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Endothelium-derived relaxing factor is an endogenous vasodilator in man

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Removal of venous endothelium in man leads to vasoconstriction and loss of dilator response to acetylcholine but not to glyceryl trinitrate. This pattern of responses can be accounted for by loss of endothelium-derived relaxing factor (EDRF), but not by loss of prostacyclin. This provides the first direct evidence for endothelium-dependent dilatation in man *in vivo*, and suggests that basal release of EDRF is a determinant of resting venous tone.

Introduction Vascular endothelium synthesises and releases a variety of vasodilator (Moncada *et al.*, 1977; Furchgott & Zawadski, 1980) and vasoconstrictor (Yanagisawa *et al.*, 1988) mediators. In animals the balance of effects favours vasodilatation since removal of endothelium results in vasconstriction (O'Neill, 1947; Lam *et al.*, 1988). In this study we have investigated whether this might also be so in human vasculature *in vivo*.

Methods Studies were performed on the large veins on the back of the hand. These vessels have no resting tone and with the subject relaxed, supine and in a warm environment, their distensibility (tone) remains constant (Nachev *et al.*, 1971). The study, which had local Ethical Committee approval, was performed on 22 healthy volunteers aged 18–32 years who gave their informed consent. Laboratory temperature was kept constant between 26–28°C. Venous diameter was recorded by measuring the linear displacement of a light-weight probe resting on the skin over the summit of the vein when the pressure in a congesting cuff placed around the upper arm was lowered from 40 mmHg to 0 mmHg (Nachev *et al.*, 1971). Drugs, or physiological saline, were infused continuously at 0.25 ml min^{-1} through a 23 SWG needle placed 10–15 mm upstream from the point of measurement.

Endothelium was removed by irrigating a 3–4 cm segment of the vein with distilled water (Bolton *et al.*, 1984). The segment was temporarily isolated by means of occluding wedges. An 'infusion' needle was placed at one end of the segment, a 'withdrawal' needle at the other, and the lumen irrigated with distilled water given at 5 ml min^{-1} for 30–40 min. After irrigation the wedges were removed and vein diameter measured. Venous responses to local infusions of noradrenaline (NA), acetylcholine (ACh) and glyceryl trinitrate (GTN) were measured before, and at 2 and 14 days after, irrigation. Subjects took soluble aspirin (600 mg) 30 min before the first control measurements were made and then daily for the next 7 days. Aspirin was again taken 30 min before recordings in those subjects restudied at 14 days.

Results are presented as mean ± s.e. mean and compared by use of Student's *t* test for paired data when $P < 0.05$ was considered significant. Drug-induced constriction is expressed as a percentage of vein diameter recorded during infusion of saline; dilatation is expressed as percentage increase in diameter from that of the NA-constricted vein.

Results In 16 subjects veins were irrigated with distilled water. Two of the irrigated segments thrombosed and could not be studied further. Both subjects had failed to comply with the study protocol by not taking aspirin.

In one set of experiments, involving 9 subjects, vein diameter was measured before, and 30 min after, irrigation. In each subject irrigation decreased resting vein diameter; pre-irrigation diameter was 1.31 ± 0.11 mm, post-irrigation diameter 0.60 ± 0.11 mm ($P < 0.01$). In 6 subjects measurements were repeated at 48 h, and in each the vein diameter remained reduced (0.69 ± 0.19 mm, $P < 0.01$; Figure 1). In 2 subjects, measurements were repeated at 14 days, and vein diameter had returned to the pre-irrigation size (Figure 1).

Evidence of smooth muscle integrity was sought by measuring venous responses to infusions of NA before, and 48 h after, irrigation in 6 subjects. NA (60 ± 13.9 pmol min^{-1}) caused a 51.6 ± 9.4% reduction in vein diameter before irrigation; after irrigation a similar reduction in diameter (55.6 ± 4.8%) was produced by NA (38 ± 13.7 pmol min^{-1}); the doses of NA were not significantly different. On both occasions the constriction was maintained throughout the infusion and

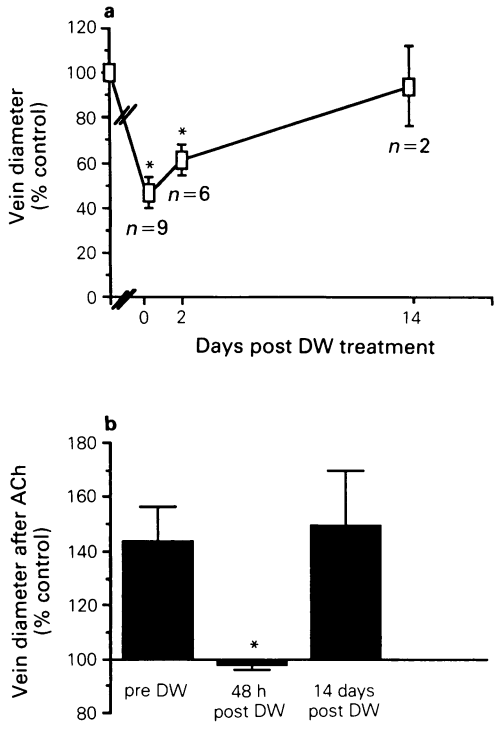

Figure 1 (a) Venoconstriction in man following removal of vascular endothelium *in vivo* with time. Vein diameter was measured at a distension pressure of 40 mmHg before (control), and at 30 min, 48 h and 14 days after, removal of endothelium with distilled water (DW) and is expressed as a percentage of the control; * denotes significantly different from control, $P < 0.01$. (b) The response of noradrenaline preconstricted dorsal hand vein to acetylcholine (1 nmol min^{-1}) before, 48 h and 14 days after removal of the endothelium by DW. Vein diameter is expressed as a percentage of the diameter recorded during infusion of NA alone; * denotes significantly different from the dilatation to ACh seen pre-DW, $P < 0.02$.

reversed at the same rate on stopping the drug. In these subjects pharmacological evidence for loss of endothelium was sought by use of ACh. In vessels preconstricted to a similar degree by NA, ACh (1 nmol min^{-1}) caused a 44 ± 13% dilatation before irrigation, but in no subject did this dose cause dilatation on the second day following irrigation ($P < 0.02$; see Figure 1). In 2 subjects studied at 14 days the dilator response to ACh was restored, and was identical to the pre-irrigation response (Figure 1).

In a second set of experiments the response of denuded vessels to GTN was examined. Five subjects were studied. In 4, denudation caused reduction of resting vein diameter (pre-irrigation control size 1.05 ± 0.27 mm; second day post-irrigation size 0.5 ± 0.08 mm). The fifth subject, however, failed to venoconstrict following denudation and showed no response to NA, even in doses of up to 800 pmol min^{-1}. It was assumed therefore, that in this subject the irrigation had impaired smooth muscle function. This subject was not studied further and was excluded from analysis. In the subjects in whom denudation caused a reduction in resting vein diameter, infusion of GTN into veins pre-constricted to a similar degree with NA, caused dose-dependent dilatation which was the same before, and after, denudation. Before denudation GTN, 3 and 6 pmol min^{-1}, caused the NA pre-constricted vein to dilate by 53.8 ± 9.9% and 77.3 ± 12.6% respectively, and two days after denudation by 53.8 ± 6.1% and 80.0 ± 10.6%. In all 4 subjects infusion of a larger dose of GTN (50 nmol min^{-1}) on the second day increased resting vein diameter from 0.50 ± 0.08 mm to 0.93 ± 0.21 mm ($P < 0.05$), returning it almost to the pre-irrigation control size (1.05 ± 0.27 mm).

In a third set of experiments, aspirin was infused into endothelium-intact veins of 6 subjects for 30 min, to give a calculated plasma concentration within the vein segment of 2 mM. Aspirin neither changed basal vessel tone (mean diameter before aspirin 1.2 ± 0.05 mm, after aspirin 1.23 ± 0.06 mm), nor altered the constrictor response to NA. The reduction in vein diameter produced by NA (80 pmol min^{-1}) was 49.6 ± 6.5% before aspirin, and 53.1 ± 5.4% after aspirin.

Discussion The results of this study provide the first direct evidence of endothelium-dependent dilatation in man *in vivo*, and demonstrate that the endothelium exerts a continuous dilator influence. Evidence for the presence of EDRF is based upon the observations that in NA pre-constricted dorsal hand veins with intact endothelium ACh and GTN dilate the vessels, whereas after irrigation with distilled water, only GTN is effective.

In addition we present evidence for basal EDRF release independent of ACh. Removal of the endothelium led to venoconstriction which gradually reversed over two weeks, a time by which the endothelial lining would have regenerated (Hayashi *et al.*, 1988) and the dilator response to acetylcholine had returned.

Basal release of prostacyclin (PGI_2) is unlikely to account for our findings as local infusion of aspirin altered neither resting vessel tone nor the constrictor response to NA.

Platelet-derived constrictor substances are also unlikely to contribute to the maintained constriction; first, the subjects took aspirin to inhibit

thromboxane synthesis; second, the constriction following denudation was the same at 30 min as at 48 h, yet platelet adhesion and activation vary with time, and would be greatest immediately after endothelial removal.

In conclusion, we suggest that EDRF plays a physiological role in the control of venous tone in man. Confirmation, however, must await a specific inhibitor of EDRF for use *in vivo*.

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Platelet-activating factor (Paf) antagonist, WEB 2086, protects against Paf-induced hypotension in *Macaca fascicularis*

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1 The actions of intravenously administered platelet-activating factor (Paf) (0.1–3.33 nmol kg^{-1}) and the effect of a recently described Paf antagonist, WEB 2086, were investigated in the anaesthetized open-chest monkey, *Macaca fascicularis*.

2 Paf dose-dependently reduced blood pressure, left ventricular pressure (LVP) and its first differential LV *dP/dt*.

3 Mean pulmonary artery pressure, recorded in three animals, was essentially unchanged by any dose of Paf.

4 WEB 2086 (0.22 μmol kg^{-1}, i.v.) attenuated the Paf-induced changes in BP, LVP and LV *dP/dt*. The dose-response curve for fall in BP was shifted to the right by one order of magnitude.

5 Histamine-induced cardiovascular changes (systemic hypotension and tachycardia) were not affected by prior administration of WEB 2086.

6 WEB 2086 should be of value in assessing the role of Paf in pathophysiological conditions.

Introduction

Interest in antagonists of platelet-activating factor (Paf, 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine) stems from the potent biological effects of Paf *in vitro* and *in vivo* (see Braquet *et al.*, 1987) and its possible involvement in hypersensitivity reactions (Koltai *et al.*, 1986), and endotoxin shock (Doebber *et al.*, 1985). The cardiovascular effects of intravenous Paf have been extensively studied in the dog (Kenzora *et al.*, 1984) and rabbit (Heffner *et al.*, 1983; Lefer *et al.*, 1984) and include systemic hypotension, pulmonary hypertension and decreased cardiac contractility. Several Paf antagonists are currently available. WEB 2086, 3-[4-(2-chlorophenyl)-9-methyl-6H-thieno-(3,2-f)(1,2,4)-triazolo-(4,3-a)(1,4)-diazepine-2-yl]-1-(4-morpholinyl)-1-propanone, has been shown to be a potent inhibitor of Paf and endotoxin-induced hypotension in the rat (Casals-Stenzel, 1987) and of anaphylactic bronchoconstriction and hypotension in the guinea-pig (Pretolani *et al.*, 1987).

The purpose of this study was two fold. Firstly, the effect of intravenous Paf was examined on the cardiovascular system of the anaesthetized macaque monkey. Secondly, the effect of one dose of WEB 2086 was examined for its ability to antagonize the effects of Paf.

Methods

Six adult male macaque monkeys (*Macaca fascicularis*, 2.4–9.2 kg) received intramuscular injections of atropine (33 μg kg^{-1}) followed by ketamine (50 mg kg^{-1}). Fentanyl (15 μg kg^{-1} i.v.) was administered and the animals were intubated via a tracheotomy incision. Ventilation was maintained with O_2/N_2O (50% O_2:50% N_2O) using an SRI Small Animal Ventilator 5056. Pentobarbitone (4 mg kg^{-1} plus 0.75 mg kg^{-1} h^{-1}) and physiological saline were administered via the left femoral vein. The animals

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were placed on a heating pad and their rectal temperature monitored (Bioscience Homeothermic Blanket System) in order to maintain body temperature. The left femoral artery was cannulated for withdrawal of samples for blood gas analysis (Corning blood gas/pH analyzer 179). Blood gas tensions and acid-base balance were maintained within normal limits. A cannula was introduced via the right femoral vein into the inferior vena cava and served for the administration of drugs. ECG was recorded continuously. The left common carotid artery was cannulated and blood pressure (BP) recorded from the aortic arch (Elcomatic transducer). The heart was exposed by performing a sternotomy and constructing a pericardial cradle. To obtain a measurement of left ventricular pressure (LVP), the cardiac apex was stabbed with a 22G Quik-cath (Vicra, Travenol Laboratories, Ireland). This was secured to the myocardium with 5-0 catgut and connected to an Elcomatic transducer when flashback occurred. Heart rate was derived from the signal obtained. In two monkeys, right ventricular pressure (RVP) was similarly obtained whilst, in three larger animals, pulmonary arterial pressure (PAP) was recorded by introduction of a 5FG Swan-Ganz catheter (S-Pace, Sheffield, S. Yorkshire) into the pulmonary artery. A 30-min stabilisation period followed completion of the surgery. Data were processed with an Epson QX10 microcomputer. The first differential of LVP, LV dP/dt, was derived. This provided an index of myocardial contractility. Hexadecyl Paf (0.1–3.33 nmol kg^{-1}) and histamine (120 and 406 nmol kg^{-1}) were injected i.v. after at least 10 min of stable cardiovascular parameters. WEB 2086 (0.22 μmol kg^{-1}) was administered and Paf (3.33–33.3 nmol kg^{-1}) and histamine (doses as before) repeated after 15 min had elapsed.

Materials

The drugs and chemicals used and their sources were: atropine sulphate (Sigma, Poole, Dorset), fentanyl (Janssen, Wantage, Oxon), histamine acid phosphate (BDH, Poole, Dorset), human serum albumin (Sigma, Poole, Dorset), ketamine (Warner Lambert, Southampton, Hants), pentobarbitone (Rhône Poulenc, Dagenham, Essex) hexadecyl platelet-activating factor (Bachem, Bubendorf, Switzerland), WEB 2086 (Boehringer Ingelheim, F.R.G.). Histamine and WEB 2086 were dissolved in physiological saline. Paf was stored dissolved in chloroform/methanol (80/20 v/v) at −20°C. For each experiment an aliquot volume was evaporated to dryness and the Paf was redissolved in saline containing 0.25% (w/v) human serum albumin. Volumes of drugs injected i.v. did not exceed 0.33 ml kg^{-1}.

Statistical analysis

Means and standard errors of the means (mean ± s.e.mean) of n observations are presented. Further analysis was performed with Student's paired t test. A P value less than 0.05 was considered significant.

Results

Platelet-activating factor dose-dependently reduced BP and peak LVP. The fall in BP was gradual, reaching a peak at 4.1 ± 1.2 min ($n = 4$) after injection of 3.33 nmol kg^{-1}. The baseline BP for this dose was 73.9 ± 4.8 mmHg. Mild and transient changes in HR occurred (less than 8%). Fall in LVP was associated with a decreased peak LV dP/dt. This was reduced from the baseline level (4490 ± 290 mmHg s^{-1} by 61.9 ± 4.5% for 3.33 nmol kg^{-1} Paf ($P < 0.01$, $n = 4$). ECG changes indicative of ischaemia, particularly ST depression and T wave flattening or inversion, accompanied responses to doses of 1.0 and 3.33 nmol kg^{-1}. Histamine also induced systemic hypotension, BP falling by 19.5 ± 6.0% ($n = 5$) and 27.7 ± 3.4% ($n = 4$) for 120 and 406 nmol kg^{-1} respectively. For each dose, the fall in BP was significant ($P < 0.05$). Heart rate increased by 12.9 ± 4.9% and 22.4 ± 3.6%. In two experiments, Paf caused peak RVP to fall slightly at the same time as BP and LVP fell; when PAP was recorded directly, essentially no change occurred with any dose of Paf. Histamine (120 nmol kg^{-1}) increased RVP by 8.0 mmHg ($n = 2$) and mean PAP by 2.7 mmHg ($n = 2$). The higher dose increased PAP by 3.3 ± 0.2 mmHg ($n = 3$).

Injection of WEB 2086 (0.22 μmol kg^{-1}) caused no

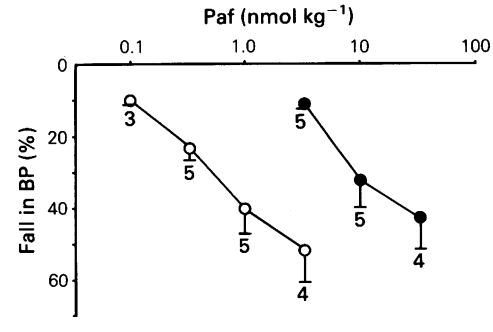

Figure 1 Accumulated data for peak fall in mean blood pressure (BP) expressed as a percentage of control (ordinate scale) following intravenous injection of platelet-activating factor (Paf) (dose in nmol kg^{-1}, abscissa scale) before (○) and 15 min after (●) WEB 2086 0.22 μmol kg^{-1} i.v. Means and standard errors of the means are shown, together with n for each point.

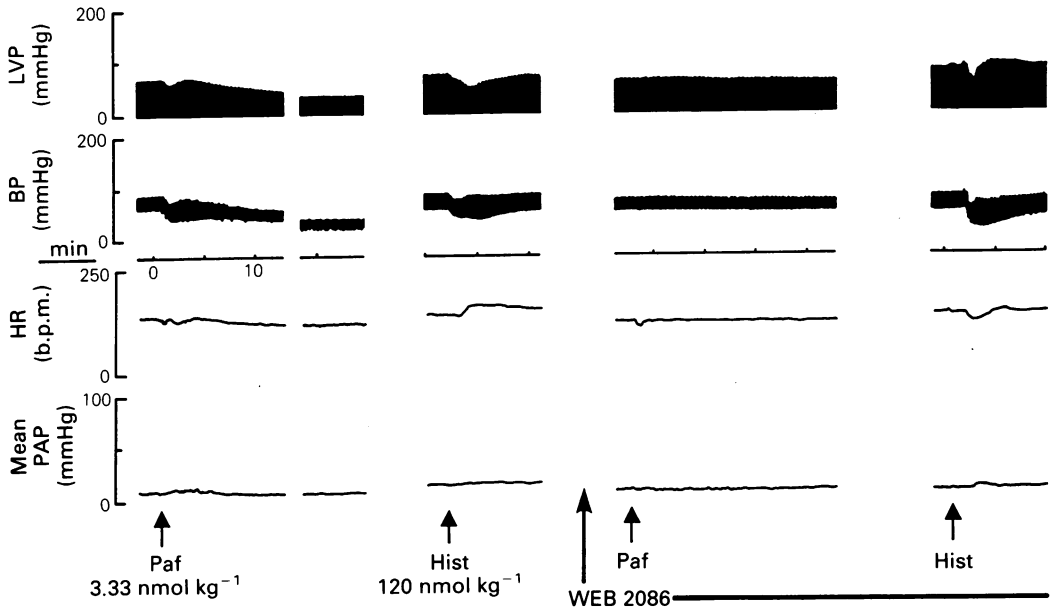

Figure 2 Typical record from an experiment in which Paf and histamine (Hist) were administered i.v. to an anaesthetized macaque before and after administration of WEB 2086 0.22 μmol kg^{-1}, i.v. The parameters shown are left ventricular pressure (LVP), systemic blood pressure (BP), heart rate (HR) and mean pulmonary arterial pressure (PAP), min = minute intervals; b.p.m. = beats per minute. Fall in LVP and BP caused by injection of Paf, but not that caused by injection of histamine, is attenuated by WEB 2086.

haemodynamic alteration in any animal. WEB 2086 markedly attenuated all cardiovascular responses induced by 3.33 nmol kg^{-1} Paf. Fall in mean BP was reduced from 51.4 $\pm$ 9.0% to 11.1 $\pm$ 1.4% (n = 5). LV dP/dt was reduced by only 16.5 $\pm$ 10.1%. For both parameters, these falls were significantly less than the pre-WEB 2086 responses ($P < 0.05$). Figure 1 shows a dose-response curve for the Paf-induced fall in BP. After the administration of WEB 2086, the curve was shifted to the right by approximately one order of magnitude. The fall in BP due to histamine was not significantly affected by WEB 2086. Figure 2 shows a typical experimental record in which the effects of Paf (3.33 nmol kg^{-1}) and histamine (120 nmol kg^{-1}) are shown. Both substances reduced LVP and BP but Paf induced longer-lasting responses. WEB 2086 markedly attenuated the Paf-induced changes. However, the histamine-induced reduction in LVP and BP persisted.

Discussion

Platelet-activating factor induced profound but reversible cardiovascular changes including fall in BP, decreased cardiac inotropic state and evidence of cardiac ischaemia. When it could be measured, Paf did not, however, produce a measurable change in PAP. In the anaesthetized dog, 0.7 nmol kg^{-1} Paf i.v. has been reported to induce a 75% rise in mean PAP (Kenzora *et al.*, 1984). This may indicate a species difference with respect to the effect of Paf on the pulmonary vasculature. The relatively slow development of peak BP reduction may be due to gradual decline in cardiac output and loss of plasma volume. Evidence for Paf-induced loss of intravascular fluid in the baboon has been presented by McManus *et al.* (1981). A recent observation by Handley *et al.* (1987) has indicated that injected [^{3}H]-Paf disappears initially fairly rapidly from the primate circulation, with a $t_{1/2}$ of 30–40 s. This seems to suggest that the continued presence of Paf in the blood is not required for the development of peak haemodynamic changes. Loss of cardiac intropy may reflect a direct effect of Paf on the heart, or a reduced cardiac workload.

Fall in BP and the other Paf-induced changes were attenuated by the recently-described antagonist WEB 2086 (Casals-Stenzel *et al.*, 1986). This compound has a $t_{1/2}$ of approximately 3 h when administered i.v. to man (Heuer, personal communication).

WEB 2086 did not affect the hypotension evoked by histamine and, in the guinea-pig (Pretolani *et al.*, 1987), the same systemic dose did not affect hypotension and bronchoconstriction induced by 5-hydroxytryptamine.

In conclusion, Paf induced profound cardiovascular changes in the primate *M. fascicularis*. Changes in the systemic circulation predominated. These effects were antagonized by WEB 2086. Further studies, aimed at establishing the relative potency of WEB 2086 and other Paf antagonists in the primate, are indicated. Antagonists of Paf such as WEB 2086 should prove to be of value in assessing the role of Paf in cardiovascular pathophysiological states in man.

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The effect of relaxants working through different transduction mechanisms on the tonic contraction produced in rat aorta by 4β-phorbol dibutyrate

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1 We have examined the effects of a range of smooth muscle relaxants on the maintained contractions produced in rat aortic rings by the protein kinase C activator, 4β-phorbol dibutyrate; these effects were compared with those on the contraction induced by the selective α_1-adrenoceptor agonist, methoxamine. The phorbol ester, at 0.3 μM, gave a sustained contraction which was, on average, of approximately the same magnitude as the maximum contraction produced by methoxamine, 10 μM.

2 The β-adrenoceptor agonist, isoprenaline (0.01–1 μM) caused a dose-related relaxation of the methoxamine-induced contraction but had no effect on the contraction induced by the phorbol ester.

3 An activator of adenylate cyclase, forskolin (0.01–1 μM) produced a dose-related relaxation of the methoxamine-induced contraction and at 0.01–10 μM caused relaxation of the contraction induced by the phorbol ester. Similar results were obtained with the potassium channel activator, cromakalim (0.001–10 μM).

4 An activator of guanylate cyclase, sodium nitroprusside (0.001–100 μM) caused a dose-related relaxation of both the methoxamine-induced and the phorbol ester-induced contraction, being more effective on the former than on the latter. Similar results were obtained with enprofylline (1–1000 μM).

5 Methoxamine (10 nM–100 μM), given cumulatively, caused a dose-related contractile response. Pretreatment with isoprenaline (1 μM), enprofylline (10 μM) and nicorandil (1 μM) resulted in partial decrease of the subsequent response to methoxamine, while nicorandil (10 μM), forskolin (1 μM), sodium nitroprusside (10 μM) and cromakalim (1 μM) totally abolished it.

6 The phorbol ester, given cumulatively, caused increasing contraction in the concentration range 30 nM–10 μM. Pretreatment with forskolin (1 μM), sodium nitroprusside (10 μM), isoprenaline (1 μM), enprofylline (10 μM), nicorandil (1 μM or 10 μM), or cromakalim (1 μM or 10 μM), resulted in partial decrease of the subsequent response to 4β-phorbol dibutyrate.

7 These results are discussed in the light of the suggestion that protein kinase C may have a role in the 'latch-bridge' phase of smooth muscle contraction, and that inappropriate activation of protein kinase C may contribute to the pathogenesis of hypertension and other conditions involving vasospasm.

Introduction

It is generally accepted that an early event in stimulus-response coupling in many cells is the breakdown of polyphosphoinositides with generation of both diacylglycerol (DAG), which activates protein kinase C (C-kinase), and inositol trisphosphate (IP_3), which mobilizes intracellular calcium (Nishizuka, 1984; 1986; Berridge & Irvine, 1984). Recent work indicates that this system of signal transduction operates in smooth muscle cells (Baron *et al.*, 1984; Somlyo, 1985). Inositol trisphosphate has been shown to release calcium from intracellular stores in skinned muscle fibres from porcine arteries (Suematsu *et al.*, 1984) and to cause tension development in permeabilised vascular smooth muscle (Somlyo *et al.*, 1985). The phorbol esters, which are C-kinase activators, have been found to cause tonic contraction in rat and rabbit vascular smooth muscle (Rasmussen *et al.*, 1984;

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Danthuluri & Deth, 1984; Forder *et al.*, 1985; Nakaki *et al.*, 1985; Miller *et al.*, 1986; Wagner *et al.*, 1987; Singer & Baker, 1987) and in guinea-pig parenchymal strips (Dale & Obianime, 1985; 1987; Obianime *et al.*, 1988). Rasmussen & Barrett (1984) have suggested that in many cell types the IP_3/calcium system is responsible for the initial transient response to an agonist and the DAG/C-kinase system for the later sustained response.

A sustained contraction of vascular smooth muscle is involved in several pathological conditions. Local vasospasm occurs in various peripheral vascular disorders, while a generalized increase in vascular tone is seen in hypertension. The molecular basis of this pathological tonic contraction is not clearly understood. As regards hypertension, it is generally considered that proliferation of resistance vessel smooth muscle occurs (Mulvany, 1987) and that an increased Na^+ load in the vascular smooth muscle cells may be important in the pathogenesis (Daniel, 1981; Blaustein, 1977). An abnormality in calcium metabolism may also be significant and may be secondary to the changes in Na^+ concentration (Blaustein 1977; Abbott, 1988). In this context, it may be significant that there is now evidence that proliferation of several cell types involves C-kinase activity which then activates the Na^+/H^+ antiport resulting in an increase in intracellular Na^+ (reviewed by Berridge & Irvine, 1984), and that both an increased Na^+/H^+ exchange in the neutrophils (Feig *et al.*, 1986) and an increase in the content of DAG in the red blood cells (Kato & Takenawa, 1987) is found to occur in spontaneously hypertensive rats. The possible relevance of the phosphoinositide signalling system in hypertension has recently been emphasised (Buhler *et al.*, 1986; Heagerty & Ollerenshaw, 1987).

Since it could well be that inappropriate activation of the C-kinase is implicated in the pathogenesis of hypertension and other conditions involving tonic contraction of vascular smooth muscle, we considered that it would be of interest to study the effect of relaxants on vascular tissue contracted by activating C-kinase. We have carried out experiments to answer two questions. Firstly, what effect do the relaxants have if they are administered to an aortic strip precontracted with 4β-phorbol dibutyrate (4β-PDBu), compared to their effect on a strip precontracted with an α_1-adrenoceptor agonist, methoxamine? Secondly, what effect will the drugs have if they are used to pretreat the aortic strip before a cumulative response to 4β-PDBu or methoxamine is evoked?

Methods

Male Sprague-Dawley rats (280–350 g) were killed by cervical dislocation, exsanguinated and the aortae removed. Six rings were cut from each aorta, mounted in 5 ml organ baths under 0.5 g tension, and maintained at 37°C in Krebs-Henseleit solution aerated with 95% O_2 and 5% CO_2. Contractions were recorded isometrically with Pye-Ether UFI transducers and Servoscribe Pen recorders (Lab Data Services). Tissues were incubated for 2 h, with frequent changes of the bathing solution, before use.

Aortic rings with intact endothelium were used since this represents more accurately the physiological situation *in vivo*. In every ring the relaxant effect of acetylcholine (1 μM) on a contraction elicited by noradrenaline (10 μM) was tested to confirm the presence of functional endothelium (Furchgott, 1984). As a standard protocol, methoxamine was then administered repeatedly until consistent responses were obtained, with 0.1, 1, and 10 μM concentrations given cumulatively, the latter concentration causing maximum contraction – higher concentrations having previously been shown not to have a greater effect.

Protocol for the studies on the effect of relaxants on methoxamine- and 4β-phorbol dibutyrate-induced contractile responses

In each of twenty-three experiments, six rings taken from the same animal were set up as stated above. Each of the five rings, was then exposed to one of the following relaxants during the presence of the maximal methoxamine contraction: isoprenaline, enprofylline, sodium nitroprusside, forskolin, cromakalim or nicorandil. In each case increasing concentrations of the relaxants were administered cumulatively. The sixth ring served as a time-matched control and after being exposed to methoxamine was left without washing (see Figure 1a). The tissues were then washed several times and allowed to equilibrate for 1 h, during which period the bath fluid was frequently replaced. The tissues were then exposed to 4β-PDBu, 10 nM–0.3 μM, given cumulatively, and the protocol used for the effect of the relaxants on the methoxamine-induced contraction was repeated on this 4β-PDBu-induced contractile response (see Figure 1). In a further three experiments, the protocol for the effect of isoprenaline on tissue precontracted with 4β-PDBu was slightly modified. The effect of isoprenaline was tested concurrently on rings precontracted with (i) 4β-PDBu (0.01–0.3 μM) alone, (ii) 4β-PDBu (0.01–0.3 μM) after previous exposure to the methoxamine regime which had been followed by thorough washing with Krebs solution, and (iii) 4β-PDBu (0.01–0.3 μM), after previous exposure to the methoxamine regime which had been followed by isoprenaline (0.001–1 μM) and then thorough washing with Krebs solution.

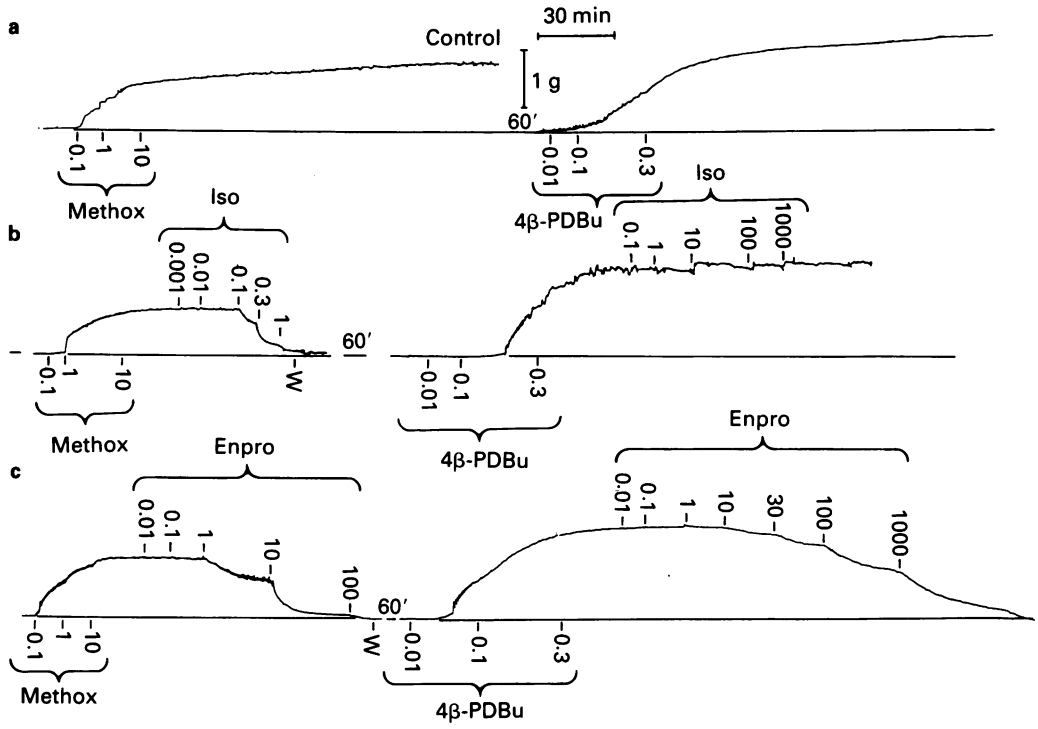

Figure 1 The responses of three rat aortic rings tested concomitantly, showing the effect of two smooth muscle relaxants given cumulatively, on both the methoxamine (Methox) and 4β-phorbol dibutyrate (4β-PDBu) contractile responses. (a) Control responses of methoxamine and of 4β-PDBu, given cumulatively. (The tissue was washed after the methoxamine contraction). (b) The relaxant effect of isoprenaline (Iso). (c) The relaxant effect with enprofylline (Enpro). All concentrations are in μM. W = wash.

The percentage waning, if any, of the time-matched control of the methoxamine- or 4β-PDBu-induced contractile response was subtracted in assessing the magnitude of drug-induced relaxation used for graphical representation.

Protocol for the studies on the effect, on both the methoxamine- and phorbol ester-induced contractile responses, of pretreatment of aortic rings with relaxants

In each of nineteen experiments, six rings were taken from the same animal and maximal contractile responses to methoxamine were established, as explained above. Each of the five rings was then exposed to one of the following relaxants: isoprenaline, 1 μM; sodium nitroprusside, 10 μM; enprofylline, 10 μM; cromakalim, 1 and 10 μM; nicorandil, 1 and 10 μM; forskolin, 1 μM. Each relaxant was left in the bath for 30 min, after which a cumulative concentration-response curve to either 4β-PDBu (1 nM–10 μM), or methoxamine (1 nM–100 μM) was obtained in the continued presence of the relaxant. One ring was not exposed to a relaxant before the response to spasmogen was elicited, thus serving as a control.

Drugs

The drugs used were noradrenaline hydrochloride, isoprenaline sulphate, 4β-phorbol 12,13-dibutyrate, dimethyl sulphoxide (all from Sigma), forskolin (Calbiochem), enprofylline (Draco), and cromakalim (Beechams Pharmaceuticals). Nicorandil was a gift from Professor T. Bolton of St. George's Hospital Medical School, London. Methoxamine, isoprenaline, sodium nitroprusside and nicorandil were all dissolved in deionized distilled water (DDW). 4β-PDBu and enprofylline were initially dissolved in dimethyl sulphoxide (DMSO), from which dilutions were made in DDW. Forskolin was dissolved in 70% ethanol and dilutions made in DDW. The concentration of DMSO or ethanol in the bath did not exceed 0.1%: at this concentration neither DMSO nor ethanol had any effects on their own, nor did they affect the methoxamine-induced responses.

The composition of the Krebs-Henseleit solution was as follows (mM): NaCl 118, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.1, $CaCl_2$ 2.5 (all chemicals from BDH, U.K.).

Statistical analysis of data

Values are presented as means ± s.e.mean; where no error bars are indicated in the figures, the s.e.mean is too small to be shown. The statistical difference of the differences between responses was assessed by means of Student's *t* test. Shifts in the EC_{50} of curves were assessed by the Mann-Whitney U test, a non-parametric test being employed since it could not be assumed that the data were normally distributed.

Results

The effect of relaxants on precontracted rings

Methoxamine in the concentration range (10 nM–100 μM) caused an immediate contraction of the aortic rings, a maximum response being obtained with 10 μM and above. In 5 preliminary experiments, contractions to 0.3 μM 4β-PDBu (1.9 ± 0.12 g) were not significantly different from the contraction with 10 μM methoxamine (1.7 ± 0.14 g). These two concentrations were then used in the subsequent experiments to compare the effect of relaxants on the contractions produced by the two spasmogens. In control rings the contractions with both methoxamine and 4β-PDBu were maintained for more than 60 min (Figure 1a). Isoprenaline (0.01 μM–1 μM) caused a dose-related decrease of methoxamine-induced contraction and complete reversal at 10 μM, but had no effect on the 4β-PDBu-induced contraction (Figure 1b and 2a). This lack of effect of isoprenaline on the 4β-PDBu contraction was observed whether the rings had been exposed to the methoxamine/isoprenaline regime ($n = 8$) or to methoxamine alone ($n = 3$), or had not been exposed to any agent at all before 4β-PDBu ($n = 3$). Forskolin (0.01–1 μM) caused a dose-related decrease with complete reversal of the methoxamine-induced contraction at 3 μM and virtually complete reversal of the 4β-PDBu-induced contraction at 10 μM (Figure 2b). It appeared to be more active on the methoxamine-induced response (EC_{50} 120 nM) than the 4β-PDBu-induced response (EC_{50} 300 nM) but, based on *t* tests, the difference between the two curves was not significant (Figure 2b). Enprofylline (1–1000 μM) and sodium nitroprusside (0.001–100 μM) also caused complete reversal of both the methoxamine and the 4β-PDBu response but were each more potent in relaxing the former than the latter contraction (Figures 1c, 2c, 2d). Cromakalim (0.001–10 μM)

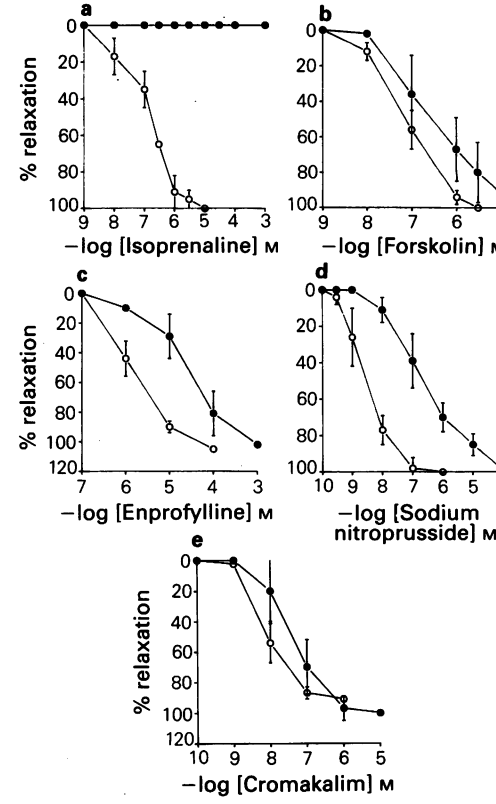

Figure 2 The effect of the five different smooth muscle relaxants given cumulatively on contractions of the rat aortic rings caused by methoxamine, (10 μM, ○) and 4β-phorbol dibutyrate (4β-PDBu, 0.3 μM, ●). (a) isoprenaline, ($n = 5$) (b) forskolin, ($n = 4$) (c) enprofylline, ($n = 5$) (d) sodium nitroprusside, ($n = 5$) (e) cromakalim, ($n = 5$).

caused a dose-related reversal of the contractions with both spasmogens (Figure 2e). With sodium nitroprusside the EC_{50} for relaxation of the methoxamine-induced contraction was 3 nM, whereas for relaxation of the 4β-PDBu-induced contraction it was 200 nM. With enprofylline the EC_{50} concentrations were 1.4 μM for the methoxamine-induced contraction and 20 μM for the 4β-PDBu-induced contraction, and with cromakalim they were 3.3 nM and 40 nM respectively.

The effect of pretreating rings with relaxants before administering methoxamine or 4β-phorbol dibutyrate

When administered before methoxamine, the relaxants, isoprenaline (1 μM) and enprofylline (10 μM) caused a dextral shift of the subsequent cumulative-response to methoxamine obtained over the concentration-range 10 nM–100 μM (Figure 3a). The

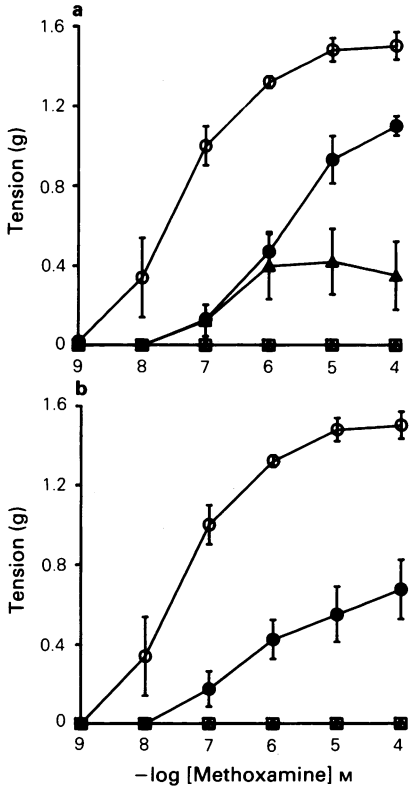

Figure 3 Cumulative concentration-response curves for methoxamine alone (○) and in the presence of six different relaxants as follows: (a) isoprenaline, 1 μM (●); forskolin, 1 μM (△); enprofylline, 10 μM (▲); sodium nitroprusside, 10 μM (□): (b) cromakalim, 1 μM (△); nicorandil, 1 μM (●) and 10 μM (□); ($n = 3$). Note that the control curves in (a) and (b) are the same, since in each set of experiments all the relaxants were tested concurrently, using tissues from the same animal.

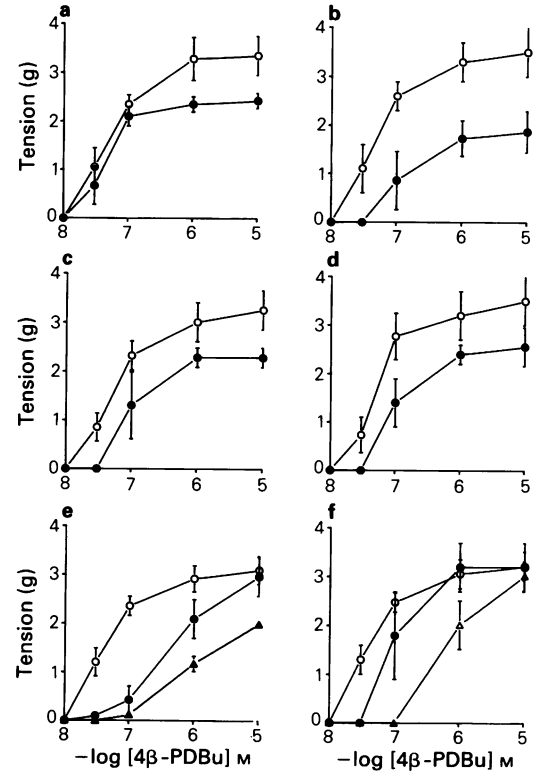

Figure 4 Cumulative concentration-response curves for 4β-phorbol dibutyrate (4β-PDBu) alone (○) and in the presence (●) of six different relaxants as follows: (a) isoprenaline (1 μM, $n = 4$); (b) forskolin (1 μM, $n = 3$); (c) enprofylline (10 μM, $n = 3$); (d) sodium nitroprusside (10 μM, $n = 4$); (e) cromakalim, 1 μM (●) and 10 μM (▲) ($n = 5$); (f) nicorandil, 1 μM (●) and 10 μM (△), ($n = 5$).

shift of the methoxamine concentration-effect curve measured at the EC_{50} in the presence of isoprenaline (1 μM) was 1.8 log units and the maximal response obtained in the presence of enprofylline (10 μM) was below the EC_{50} value of the control curve (Figure 3a). Forskolin (1 μM) and sodium nitroprusside (10 μM) totally abolished the response of aortic rings to methoxamine (Figure 3a). Cromakalim (1 μM) also abolished the response to methoxamine (Figure 3b). Nicorandil (1 μM and 10 μM) caused a dose-related inhibitory effect to the methoxamine dose-response curve (Figure 3b), the latter dose totally abolishing the methoxamine response.

When administered before 4β-PDBu, the relaxants isoprenaline (1 μM), forskolin (1 μM), enprofylline (10 μM) and sodium nitroprusside (10 μM), all produced a decrease in the subsequent cumulative response to 4β-PDBu obtained over the concentration range 0.03–10 μM, in each case causing a reduction in the maximal response (Figure 4a,b,c,d). In all 3 experiments with forskolin, (in contrast to the other relaxants) the 4β-PDBu response eventually waned after reaching the maximum. Cromakalim (1 μM and 10 μM) caused a dose-related rightward-shift in the cumulative 4β-PDBu response, the latter concentration resulting in a decreased maximal response (Figures 5b and 4e). Nicorandil (1 μM and 10 μM) also caused a dose-related rightwards shift in the 4β-PDBu dose-response curve, but with no change in maximum response (Figures 5c and 4f). Note that these concentrations of nicorandil correspond to the low therapeutic range. On a Mann-Whitney U test the shift of the EC_{50} of the control 4β-PDBu curve by cromakalim (1 μM and 10 μM) was significant at $P = 0.014$, and the shift by nicorandil (10 μM) was

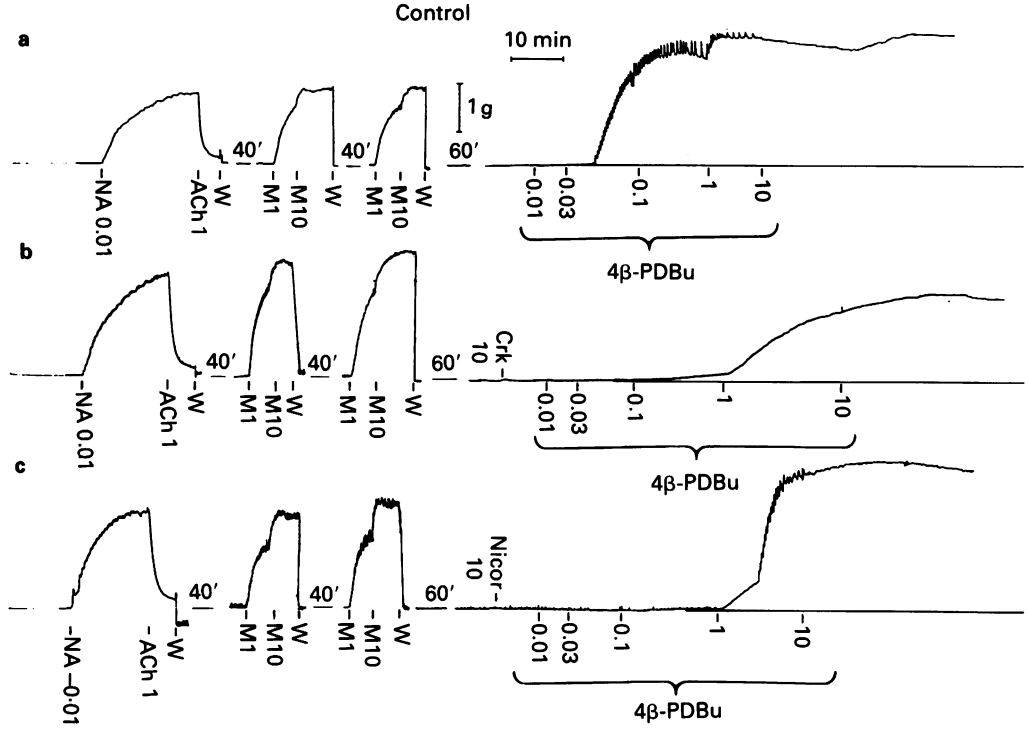

Figure 5 The effect of pretreatment with hyperpolarizing agents on 4β-phorbol dibutyrate (4β-PDBu)-induced contractile responses of rat aortic rings, tested concomitantly. The response to cumulative concentrations of 4β-PDBu: (a) in normal Krebs-Henseleit solution, (b) in the presence of cromakalim (Crk), (c) in the presence of nicorandil (Nicor). All concentrations are in μM. M = methoxamine, W = wash.

significant at $P = 0.008$. In addition to impairing the 4β-PDBu-induced contractile responses, all these agents reduced also the rate of the phorbol ester-induced contractile responses, the reduction being much more pronounced with the hyperpolarizing agent, cromakalim (Figure 5).

Discussion

It has been reported that agents which stimulate vascular smooth muscle, such as angiotensin and phenylephrine, cause during the contraction a 2-stage change in the intracellular calcium concentration, consisting of an initial large calcium transient succeeded by a smaller sustained calcium response (Morgan & Morgan, 1982). Studies by Dillon *et al.* (1981) and Askoy *et al.* (1983) have given rise to the proposal that two calcium-dependent processes are implicated in contraction, occurring sequentially, an initial phosphorylation of myosin which initiates rapid cycling of cross-bridges and tension development, succeeded by a second process of force maintenance. This second phase, reported to have a lower requirement for calcium (Askoy *et al.*, 1983), is associated with a decreased cytosolic calcium (Morgan & Morgan, 1982) and a reduced level of myosin phosphorylation (reviewed by Sommerville & Hartshorne, 1986). Tension maintenance in this second phase has been explained in terms of slowly-cycling cross-bridges, termed 'latch bridges' (Dillon *et al.*, 1981). It is generally considered that an additional regulatory mechanism must be involved in this stress maintenance phase. Hai & Murphy (1988) have recently put forward a theoretical model in which calcium activation of myosin light chain kinase alone would be both necessary and sufficient to explain cross-bridge cycling and latch; however, Bruschi *et al.* (1988) have produced experimental evidence which accords with the idea that an additional regulatory mechanism is necessary. Since this additional mechanism must function at low cytosolic calcium concentrations, it

presupposes a calcium target with a high affinity for calcium. Rasmussen *et al.* (1984) have proposed that this second regulatory mechanism involves the C-kinase pathway. In this regard, it has been shown that in the presence of DAG, C-kinase can be activated at the resting physiological intracellular calcium concentration (for review see Nishizuka, 1986). Evidence that is consistent with Rasmussen's hypothesis has been put forward by Griendling *et al.* (1986) who found that in cultured rat aortic smooth muscle cells, angiotensin II induced not only an initial transient breakdown of phosphatidyl inositol bisphosphate with generation of IP_3 and DAG, but also a later sustained increase in DAG, the endogenous C-kinase activator. This second phase was not associated with calcium mobilization.

If C-kinase activity is involved in force maintenance, the mechanism by which it produces its effects is not obvious. However, as specified above, it is clear that direct activation of the C-kinase by phorbol esters can produce a sustained contraction of smooth muscle from several tissues. Previous results in guinea-pig lung parenchyma indicate that the phorbol-induced contraction is not due to the release of endogenous spasmogens and also that there is synergism between C-kinase activators and agents which increase cytosolic calcium; threshold concentrations of C-kinase activators shift the concentration-response curve of the calcium-increasing agents to the left and *vice versa* (Dale & Obianime, 1987; Obianime *et al.*, 1988). These latter results imply that activation of the C-kinase may increase the sensitivity of the calcium-mediated contractile mechanisms as suggested by Rodger (1986).

In this context, and bearing in mind the possibility that inappropriate activation of protein kinase C could be implicated in pathological vascular changes, we considered that it would be of interest to examine the effect of smooth muscle relaxants, including some agents used in the treatment of hypertension, on the tonic vascular contraction produced by protein kinase C activation. We have compared these effects with the effects on methoxamine-induced contractions. We have examined agents which act on 4 by 5 different mechanisms, some involving activation of receptors, some involving post-receptor transduction pathways.

The β-adrenoceptor agonist, isoprenaline, at $10\,\mu M$, a concentration that caused maximal relaxation of the methoxamine-induced contraction, had no effect at all on rings precontracted with 4β-PDBu. However, pretreatment of the rings with isoprenaline before exposure to 4β-PDBu resulted in depression of the maximum response, albeit without a shift in the concentration-effect curve. In contrast, pretreatment with isoprenaline before exposure to methoxamine, caused not only a depression of the maximum methoxamine response but also a significant shift of the methoxamine concentration-effect curve. β-Adrenoceptor-mediated vasodilatation is generally considered to be due to activation of adenylate cyclase and increase in cyclic AMP (Kukovetz *et al.*, 1981; Hardman, 1984). Evidence has been put forward that the cyclic AMP-dependent kinase phosphorylates myosin light chain kinase apoenzyme and decreases its sensitivity to activation by Ca^{2+}/calmodulin (Conti & Adelstein, 1981) although other mechanisms involving alterations of membrane ion fluxes or decreased sarcoplasmic calcium concentration have been proposed (Jones *et al.*, 1984; Kamm & Stull, 1985). A possible explanation for the lack of effect of isoprenaline on tissue precontracted with 4β-PDBu is that the phorbol ester caused phosphorylation (and thus desensitization) of the β-adrenoceptor (Kelleher *et al.*, 1984; Sibley *et al.*, 1984). If this is so, it is not entirely surprising that *pretreatment* with isoprenaline before phorbol ester administration caused some reduction of the subsequent 4β-PDBu contraction, since the stimulation of adenylate cyclase would have begun before the 4β-PDBu-activated C-kinase could interfere with β-receptor-mediated transduction mechanisms. The relaxant efficacy, on tissue precontracted with 4β-PDBu, of agents which by-pass the receptor – forskolin, an adenylate cyclase activator, and enprofylline which increases cyclic AMP by inhibiting phosphodiesterase – appears to support this. It is of interest that forskolin has more potent relaxant effects on the C-kinase contraction of vascular muscle than on airway muscle, inasmuch as $1\,\mu M$ forskolin caused 97.8% reversal of the contraction evoked in rat aortic strips by phorbol myristate acetate, $10\,\mu M$ (Obianime & Dale, unpublished observations), but only 21% reversal in the guinea-pig parenchymal strip (Dale & Obianime, 1985). Previous studies have shown that activation of C-kinase impairs α-adrenoceptor responses (Danthuluri & Deth, 1984).

Sodium nitroprusside is an effective relaxant of smooth muscle used in some cases of hypertension. It is reported to act by virtue of directly stimulating guanylate cyclase (Rapoport & Murad, 1983; Ignarro & Kadowitz, 1985) and thus increasing cyclic GMP and activating the cyclic GMP-dependent kinase. This kinase can phosphorylate myosin light chain kinase resulting in decreased myosin phosphorylation (Nishikawa *et al.*, 1984); the kinase also accelerates Ca^{2+} extrusion (Sumimoto *et al.*, 1987). Increased cyclic GMP also results in decreased phosphoinositide hydrolysis (Rapoport, 1986). In the present study, sodium nitroprusside, at $1\,\mu M$ caused total relaxation of the methoxamine-induced contraction. On the 4β-PDBu-induced contraction this concentration was not totally effective,

but 100 μM caused complete relaxation. When used for pretreatment, sodium nitroprusside, 10 μM, while totally obliterating the methoxamine response, marginally shifted the 4β-PDBu concentration-response curve dextrally. The full physiological role of cyclic GMP and its interrelationship with cyclic AMP in the relaxation of vascular smooth muscle is not yet entirely clear (Kukovetz *et al.*, 1981). There is clearly a difference in the mechanisms involved in relaxation in different types of smooth muscle. Sodium nitroprusside actually increased the contraction of the guinea-pig lung parenchymal strip precontracted under another C-kinase activator, phorbol myristate acetate (Dale & Obianime, 1985).

Cromakalim is a novel smooth muscle relaxant with antihypertensive properties (Ashwood *et al.*, 1984), which acts by increasing K^+ permeability, thus hyperpolarizing the tissue (Hamilton *et al.*, 1986). It reduces the contractile response of vascular tissue to noradrenaline (Weir & Weston, 1986). In the present study cromakalim was able to reverse both the methoxamine-induced and the 4β-PDBu-induced contractions, the respective ED_{50} values being 3.3 nM and 40 mM. If used for pretreatment at 1 μM and 10 μM, it decreased the subsequent 4β-PDBu contraction and totally abolished the methoxamine response. In this context it is of interest that 4β-PDBu has been found to inhibit the cromakalim-induced ^{86}Rb efflux with an IC_{50} of 70 nM (Coldwell, 1988).

Nicorandil is an anti-anginal agent reported to cause vasodilatation by opening potassium channels in smooth muscle and causing hyperpolarization (Itoh *et al.*, 1981; Inoue *et al.*, 1983). However, nicorandil-induced relaxation is not always associated with hyperpolarization (Karashima *et al.*, 1982) or the opening of ^{86}Rb-permeable K^+ channels (Weston, 1987). This agent may produce its effects in part by its action of increasing cyclic GMP (Sumimoto *et al.*, 1987). In the present study, pretreatment with nicorandil had a rather different effect on the subsequent cumulative dose-response curve to 4β-PDBu from that of cromakalim in that, unlike cromakalim, it caused a dextral shift in the dose-response curve with no decrease in maximum. It also caused a dose-related dextral shift of the methoxamine concentration-effect curve and, at 10 μM, totally abolished the methoxamine response.

In general, when used in pretreatment, all the drugs, including those used as, or proposed as antihypertensive agents (and being used in this study at therapeutically relevant concentrations) were effective in decreasing the subsequent cumulative response to 4β-PDBu, with forskolin, cromakalim and nicorandil being the most effective. When the effect of pretreatment on the 4β-PDBu-induced contraction was compared with the effect of pretreatment on the methoxamine-induced contraction, all the relaxants proved to be markedly more effective on the methoxamine-induced response although the order of effectiveness was similar. On tissues precontracted by phorbol ester, only isoprenaline was totally ineffective, all the other agents causing relaxation, significant effects being produced by therapeutically relevant concentrations of the agents used as antihypertensives. These data imply that, if the maintained contraction induced by phorbol ester represents an *in vitro* model of pathological vasospasm, then strategies for developing new drugs to combat this phenomenon are more likely to succeed if they are aimed at modifying post-receptor transduction pathways than at receptor-mediated events.

We wish to thank Robert Muid and Joyce Mancini for technical assistance, and Vivien Grant and Dionne Menday for secretarial assistance. We would also like to express our appreciation for the gift of cromakalim from Dr M.J. Soulal of Beecham's Research Laboratories (UK) and of nicorandil from Professor T. Bolton of St George's Hospital Medical School (London).

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Functional and ligand binding studies suggest heterogeneity of platelet prostacyclin receptors

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1 This study describes attempts to compare prostacyclin (IP-) receptors in human, pig, horse, rabbit and rat platelets and in circular muscle of human, rabbit and dog mesenteric and pig gastro-epiploic arteries. Three stable prostacyclin analogues, iloprost, cicaprost and 6a-carba-prostacyclin (6a-carba-PGI_2) and a prostaglandin endoperoxide analogue EP 157 (previously shown to mimic prostacyclin on human platelets) were used.

2 Our main conclusion is that prostacyclin receptors on human, pig and horse platelets are similar in nature, but distinct from those on rabbit and rat platelets. Functional studies (inhibition of aggregation) showed that iloprost and cicaprost always had similar potencies whereas 6a-carba PGI_2 was much more potent than EP 157 on rabbit and rat platelets (300 and 1000 fold on a molar basis) compared with human, pig and horse platelets (2, 7 and 7 fold respectively). Measurement of initial rates of cyclic AMP production confirmed these orders of potency.

3 Although pig platelets were quite sensitive to inhibition by EP 157 (threshold = 10 nM in some experiments), maximal inhibition of aggregation was not always achieved (20 μM). EP 157 also produced only small elevations of cyclic AMP and inhibited rises in cyclic AMP induced by iloprost. It is possible that EP 157 has a lower efficacy than iloprost at the IP-receptor and on pig platelets it can sometimes act as a partial agonist.

4 Human, pig and horse platelet membranes bound [^{3}H]-iloprost at 30°C and this binding was inhibited by the four prostanoids. On human and pig membranes the order of potency was cicaprost = iloprost > 6a-carba PGI_2 > EP 157. The order of potency may be similar on horse platelet membranes, but the analysis is complicated by the presence of a second component of [^{3}H]-iloprost binding that is inhibited by iloprost and 6a-carba PGI_2 but not by cicaprost. This binding may be due to the presence of an EP_1-receptor, since iloprost and 6a-carba PGI_2 but not cicaprost are known to have potent EP_1-receptor agonist actions on smooth muscle preparations. IC_{50} values for cicaprost inhibition on human, pig and horse membranes were 110, 90 and 165 nM respectively. The need for IP-receptor radioligands of greater specificity is apparent from these studies.

5 Minimal binding of [^{3}H]-iloprost to rabbit and rat platelet membranes was obtained at 30°C. Lowering the incubation temperature to 4°C and ensuring that the temperature did not rise during the filtration process increased binding and allowed inhibition curves to be obtained. The results suggest a lower binding affinity for [^{3}H]-iloprost, associated with a higher dissociation rate for the radioligand-receptor complex. IC_{50} values for cicaprost were 900 nM for rabbit and 640 nM for rat platelets. In a similar manner to horse platelet membranes, the presence of a second binding site for [^{3}H]-iloprost was detected on rabbit platelet membranes.

6 Sensitivity to the relaxant action of iloprost on the arterial smooth muscle preparations decreased in the order: human mesenteric, dog mesenteric, rabbit mesenteric, pig gastro-epiploic. Cicaprost was always slightly more potent than iloprost (1.2–2.8 fold). On the pig vessel preparation 6a-carba PGI_2 did not produce complete relaxation. The possibility that this is due to an opposing contractile action mediated via EP_1 or EP_3 receptors is discussed.

7 EP 157 relaxed the human, pig and rabbit arterial preparations at concentrations 100–200 times those of iloprost. This correlates well with its IP-receptor agonist potency on human, pig and horse platelets. The results obtained with EP 157 further demonstrate the potential difficulties in separating platelet inhibitory and vasodilator properties of prostacyclin mimetics in man.

Introduction

Over the past ten years tremendous chemical effort has been invested in the development of analogues of PGI_2 (prostacyclin) that are both chemically and metabolically stable (see Aristoff, 1985). A major pharmacological objective has been to maintain platelet inhibitory activity whilst reducing vasodilator activity, the latter being responsible for marked falls in systemic blood pressure and unpleasant vascular headaches in the clinical situation (see Whittle & Moncada, 1984).

Using a 'selectivity ratio', Whittle and Moncada (1984) investigated the selectivity of a number of PGI_2 analogues and concluded that there appear to be inherent differences between prostacyclin or IP (Kennedy *et al.*, 1983) receptors since platelet activity is more readily lost than vascular activity. However, as these authors have emphasised, not only did this exercise involve comparison between *in vivo* and *in vitro* data, the depressor activity *in vivo* was measured in the rat and inhibition of aggregation *in vitro* was measured on the human platelet. Furthermore, a number of studies have been published describing PGI_2 analogues with antagonist activity at IP-receptors in the vasculature but with agonist activity in the platelet (Fassina *et al.*, 1985; Corsini *et al.*, 1987). Unfortunately, the significance of the marked prostaglandin E (PGE)-like stimulant (EP_1-receptor agonist) activity of a number of PGI_2 analogues, for example iloprost (Dong & Jones, 1982; Dong *et al.*, 1986; Sheldrick *et al.*, 1988), is only slowly being realised. This low agonist specificity may be particularly important when measuring vasodilatation, since physiological antagonism of the IP-receptor-mediated action will occur if the tissue contains a PGE-sensitive constrictor system.

In this study we have examined, under *in vitro* conditions, the prostacyclin-sensitive systems in washed platelets from man, pig, horse, rabbit and rat and in visceral arterial vessels from man, pig, rabbit and dog. Our aim was two-fold—first to compare IP-receptor agonist potencies on platelets and vessels from the same species, particularly man, and secondly to probe further our preliminary finding that relative agonist potencies on human and horse platelets differ from those found with rabbit and rat platelets (Wilson *et al.*, 1987). Since iloprost (Figure 1) appeared to be the most comprehensively studied of the stable prostacyclin analogues described in the literature (see Gryglewski & Stock, 1987), it was used as the standard agonist. It is important to be aware, however, that it is a mixture (approx. 50:50) of two diastereoisomers epimeric at C16 (Skubulla & Vorbrüggen, 1983). The related compound cicaprost (Figure 1) (Stürzebecher *et al.*, 1986), on the other hand, is a single chemical entity (16β-methyl) and

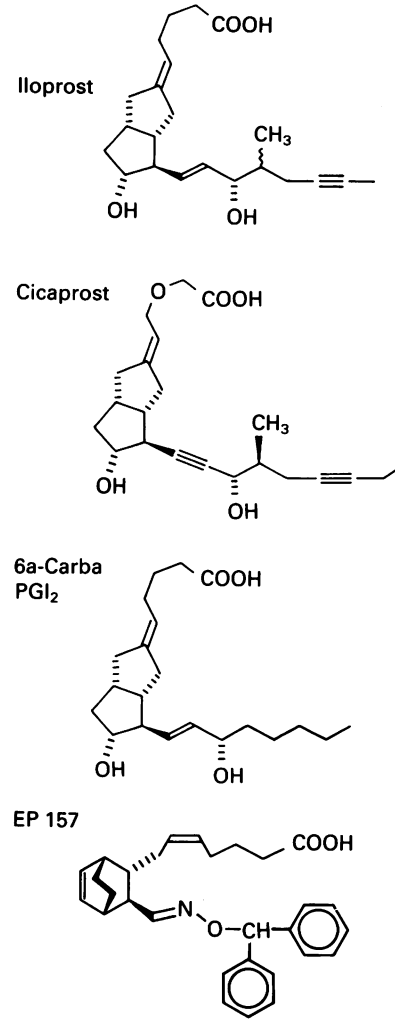


Figure 1 Structures of the four prostacyclin mimetics, iloprost, cicaprost, 6a-carba prostacyclin (6a-carba PGI_2) and EP 157, under study.

moreover it only has very weak EP_1-receptor agonist activity (Dong *et al.*, 1986). It is of particular interest since its 13,14-acetylenic bond adds further restriction to sidechain movement over that conferred by the rigid C4–C13 structure.

We were also keen to explore further the prostaglandin-like activity of the prostaglandin endoperoxide analogue EP 157 (Figure 1). This compound bears little structural resemblance to PGI_2 yet in the human platelet it inhibits adenosine-5'-diphosphate (ADP)-, platelet-activating factor (Paf)- and thromboxane-induced aggregation, maximally activates membrane-bound adenylate cyclase and

displaces [^{3}H]-iloprost binding (Armstrong *et al.*, 1986). On prostacyclin-insensitive smooth muscle preparations (rabbit aorta, guinea-pig trachea) it specifically blocks thromboxane (TP-) receptors. We were intrigued to know whether it would also mimic the relaxant action of PGI_2 on vascular smooth muscle.

Methods

Preparation of washed platelet suspensions

Human Blood was obtained from the antecubital vein of healthy human volunteers and collected into polypropylene tubes containing ACD solution (disodium hydrogen citrate 2 g, glucose 3 g, water to 120 ml; 1 ml per 5 ml blood). Centrifugation at 180 *g* for 20 min afforded platelet-rich plasma (PRP) which was treated with 20 nM PGI_2 and centrifuged at 450 *g* for 20 min. The supernatant was discarded and the pellet carefully suspended in Ca^{2+}-free Krebs solution. The washed platelet suspension was left at 37°C for 1 h to ensure complete decay of the PGI_2.

Rat Rats were anaesthetised by inhalation of ether, the abdominal aorta cannulated and blood collected into ACD. Washed platelet suspensions were prepared as above.

Rabbit New Zealand White rabbits (2–4 kg, males) were anaesthetised with pentobarbitone sodium (30 mg kg^{-1}) infused into a marginal ear vein. Both carotid arteries were cannulated, the blood collected into ACD and washed platelet suspensions prepared as above.

Pig Pigs (30–35 kg) were sedated with Stresnil (20 mg kg^{-1} intramuscularly) and then anaesthetised by infusion of pentobarbitone sodium (20 mg kg^{-1}) into an ear vein. Both carotid arteries were cannulated and following heparinisation (300 u kg^{-1} i.a.) blood was collected into ACD and washed platelet suspensions prepared as above.

Horse Blood was withdrawn from the jugular vein and collected into ACD containing heparin (10 u ml^{-1}). Washed platelet suspensions were prepared as above.

Platelet aggregation studies

Photometric measurements of platelet aggregation were made using a modified Cary 118C spectrophotometer. Washed platelet suspension (1 ml), Krebs solution (1 ml) and 0.9% NaCl solution (0.4 ml) were placed in a disposable polystyrene cuvette and allowed 2 min to equilibrate in the cell block at 37°C. Stirring was achieved by a stainless steel rod revolving at 1000 r.p.m. The aggregating agent was then added in 0.1 ml 0.9% NaCl solution. Inhibitors were added 2 min before the aggregating agent replacing all or part of the 0.4 ml of saline.

Platelet cyclic AMP determinations

One ml aliquots of washed platelet suspensions were incubated with selected concentrations of prostaglandin or saline control for 30 s at 37°C, and the reaction quenched by the addition of ethanol. A 30 s incubation time provides an estimate of the initial rate of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation and avoids the problem of reversal of stimulation of cyclic AMP seen with some prostanoids (e.g. PGE_1) over longer time courses (Gorman *et al.*, 1977). The samples were extracted and the cyclic AMP levels measured by a protein-binding assay as described previously (Armstrong *et al.*, 1986). Cyclic AMP levels were expressed as multiples of the basal level.

Displacement of [^{3}H]-iloprost binding

Platelet pellets were prepared from PRP as described above, suspended in ice-cold 5 mM Tris-HCl buffer, pH 7.4 and disrupted by sonication. After centrifugation at 100,000*g* for 15 min at 4°C, the supernatant was discarded and the platelet membranes resuspended in 50 mM Tris-HCl buffer, pH 7.4 by sonication on ice. Protein concentration was determined by the method of Lowry *et al.* (1951). The binding of [^{3}H]-iloprost (specific activity 440 GBq mmol^{-1}, Amersham) to platelet membranes from each species was studied. Each assay tube contained 2 pmol [^{3}H]-iloprost, 1 μmol $MgSO_4$, 5 μM Tris-HCl (pH 7.4) and cell membranes in a total volume of 100 μl. Prostaglandins were dissolved in part of the Tris buffer with the aid of ethanol (final conc. in assay tube = 1%). After incubation for 20 min at 30°C, bound radioligand was separated by rapid filtration through Whatman GF/B glass filter discs using a Brandel cell harvester. Each filter was washed three times with 2 ml ice-cold 50 mM Tris-HCl buffer, pH 7.4, dried and then suspended in OptiPhase 'Safe' (LKB) for scintillation counting.

This procedure had to be modified for rat and rabbit platelet membranes to 40 nM [^{3}H]-iloprost and an incubation period of 30 min at 4°C.

Isolated smooth muscle preparations

Rabbit superior mesenteric artery and pig gastroepiploic artery were removed after exsanguination of animals and placed immediately in Krebs solution.

Dog mesenteric artery was obtained from dogs under pentobarbitone anaesthesia being used for other purposes. Human mesenteric artery was obtained from patients undergoing gastro-intestinal surgery for a number of conditions. In all cases connective tissue and fat were removed from apparently healthy sections of artery and 4 rings (2 mm wide) obtained by transecting the artery. The rings were set up in conventional organ baths (10 ml) for isometric tension recording by Grass FT03C force displacement transducers coupled to a Grass polygraph. Arterial rings were stretched to an optimal resting tension of 1.5 g and readjusted to this level throughout the equilibration period (1 h). All rings were initially contracted with 100 μM phenylephrine (PE) to establish the tissue maximum. After washout, 1 μM PE was added and if after 10 min the contraction had not reached 60% of maximum, the PE concentration was increased to 2 or 3 μM. Stable tone levels between 60 and 80% of maximal were obtained in all preparations. Responses of the dog mesenteric artery rings to PE were more variable and KCl, 10 or 15 mM, was used instead giving stable tone levels of 40–60% of maximum. On each preparation two cumulative concentration-response relationships to iloprost were obtained followed by one for the test compound. Preliminary experiments showed no change in responsiveness to iloprost between second and third exposures and consequently equi-effective molar ratios (EMR) were calculated from IC_{50} values for iloprost (second exposure) and the test compound. EMR derived from single preparations obtained from 5 different animals were averaged and s.e.mean calculated.

Compounds

PGI_2, cicaprost and iloprost were gifts from Schering AG, Berlin and 6a-carba PGI_2 was a gift from Wellcome, U.K. The stable prostacyclin analogues were dissolved in saline as their sodium salts with the aid of solid $NaHCO_3$. PGI_2 sodium salt was dissolved in 50 mM Tris-HCl buffer, pH 9.0 to give a 50 $\mu g\,ml^{-1}$ solution and this was diluted with saline immediately before use and kept on ice. EP 157 (*rac* 5-endo-(6'-carboxyhex-2'Z-enyl)-6-exo-diphenylmeth oxyiminomethyl-bicyclo[2.2.2]oct-2-ene) was prepared in our laboratory. The crystalline material was dissolved in ethanol at 10 $mg\,ml^{-1}$ and this solution was diluted with saline containing 0.5 $mg\,ml^{-1}$ Na_2CO_3 to give a 200 μM stock solution of the sodium salt. SQ 22536 (9-(tetrahydro-2-furyl)-adenine) was synthesised by us and had properties identical to an original sample provided by Squibb, U.S.A., 16,16-Dimethyl PGE_2 was purchased from Upjohn Diagnostics, U.S.A. and AH 6809 (6-isopropoxy-9-oxoxanthen-2-carboxylic acid) and SC 19220 (10-(acetylhydrazinocarbonyl)-8-chloro-10, 11-dihydrodibenz(b,f)(1,4)oxazepine) were gifts from Glaxo, U.K. and Searle, U.S.A. respectively.

Results

Inhibition of platelet aggregation

Aggregation in washed platelet suspensions from man, pig, horse and rabbit was induced by Paf: typical concentrations just sufficient for irreversible aggregation were 250, 150, 2 and 1 nM respectively. Rat platelets were completely insensitive to Paf, and ADP (4 μM) was used instead. The sensitivities of the platelet suspensions from the five species to inhibition of aggregation by the standard agonist iloprost varied considerably as shown by the IC_{50} values in Table 1. Human platelets were by far the most sensitive, with measurable inhibition always present at an iloprost concentration of 0.1 nM. Iloprost and the two other prostacyclin analogues studied, cicaprost and 6a-carba PGI_2 had parallel log concentration-response curves and with the exception of pig platelets, always completely inhibited aggregation at concentrations 3–5 times their respective IC_{50} values. Equi-effective molar ratios (EMR) calculated from appropriate IC_{50} values are given in Table 1. Cicaprost was of similar potency to iloprost on all five platelet suspensions, whereas 6a-carba PGI_2 was relatively more active on rabbit and rat platelets compared with human, pig and horse platelets.

On human and horse platelets log concentration-response curves for EP 157 were parallel to those of iloprost and EP 157 was about 50 and 100 times less active respectively than iloprost (Table 1). When individual preparations of pig platelets were compared, it was found that threshold inhibitory concentrations of iloprost were similar (0.4–1.0 nM), but the slopes of the log concentration-inhibition curves varied considerably and the concentrations required for maximal inhibition as a multiple of the corresponding IC_{50} values varied between 3 and 30. On two preparations with steep iloprost slopes the log concentration-response curve for EP 157 was slightly shallower than that of iloprost but EP 157 produced maximum inhibition (Figure 2). On another four preparations with shallower iloprost slopes, the EP 157 inhibition curve was again shallower than the iloprost curve and in these cases EP 157 at concentrations up to 20 μM gave only 75–95% inhibition of aggregation (Figure 2). The mean EMR for EP 157 on pig platelets are similar to those found for human and horse platelets (Table 1). On rabbit and rat platelets EP 157 was a weak inhibitor (no effect seen below 5 μM) and complete inhibition was only seen with the maximum concentration tested (20 μM) on

Table 1 A comparison of the abilities of prostacyclin mimetics to inhibit aggregation and to raise cyclic AMP levels (values in parentheses) in platelets from different species

	Equi-effective molar ratio				
Prostanoid	*Human*	*Pig*	*Horse*	*Rabbit*	*Rat*
Iloprost*	1.0	1.0	1.0	1.0	1.0
Aggregation IC_{50} =	0.15 ± 0.04 nM	2.3 ± 0.8 nM	23 nM	17.1 ± 2.6 nM	4.7 ± 1.5 nM
Cicaprost	0.88 ± 0.06 (1.0)	1.1 ± 0.26 (1.20)	0.4 (1.0)	1.3 ± 0.21 (0.91)	0.6 ± 0.09 (0.96)
6a-Carba PGI_2	31 ± 6 (23)	13.5 ± 6.7 (27)	15 (5.8)	3.3 ± 0.4 (2.9)	3.5 ± 0.5 (2.7)
EP 157	54 ± 9 (72)	101 ± 23† (>1100)	98 (57)	990 ± 210* (>890)	3500 ± 1500† (>1300)

* Standard agonist for both inhibition of aggregation and elevation of cyclic AMP.
In each aggregation experiment, an equi-effective molar ratio was calculated from appropriate IC_{50} values; means ± s.e.mean of six experiments, except for horse = 3, are given.
† Complete inhibition of aggregation not always produced by 20 μM EP 157.
In the cyclic AMP experiments, mean (6 experiments) log concentration-response curves were plotted and the concentration of each agonist required to increase the cyclic AMP level to 5 times basal was determined. An equi-effective molar ratio (iloprost = 1.0) was then calculated.

the more sensitive preparations: similar inhibition was seen in the presence of 1 μM indomethacin.

Elevation of platelet cyclic AMP levels

Basal cyclic AMP levels in the washed platelet suspensions were as follows: human 10.5 ± 1.1, pig 17.2 ± 2.4, horse 18.5 ± 1.6, rabbit 12.2 ± 1.0 and rat 25.6 ± 3.1 pM (mean ± s.e.mean, $n = 6$). Concentration-response relationships for elevation of cyclic AMP in platelets from man, horse, pig and rat are shown in Figure 3. Data obtained with rabbits platelets were similar to those for rat platelets except that the 6a-carba PGI_2 curve did not plateau at the 300 nM concentration level. With horse, rabbit and rat platelets concentrations of the three prostacyclin analogues which produce submaximal or just maximal inhibition of aggregation also produce large and easily measureable elevations (>2 fold) of cyclic AMP. EMR for cyclic AMP elevation measured at the 5 fold value correspond well with EMR for inhibition of aggregation (Table 1). In the horse platelet EP 157 also markedly elevates cyclic AMP and the two EMR also correlate well. In rabbit and rat platelets EP 157 produces only small rises in cyclic AMP and this agrees with its very weak anti-aggregatory potency.

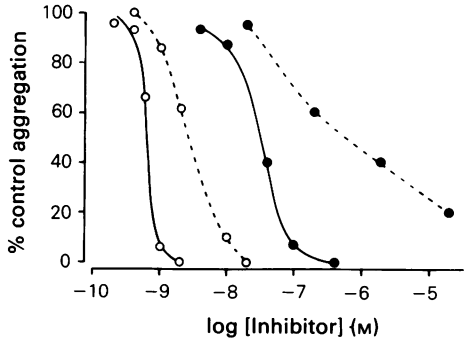


Figure 2 Concentration-response relationships for inhibition of Paf-induced aggregation in washed pig platelets by iloprost (○) and EP 157 (●). In the first experiment (continuous lines), the inhibition curve to iloprost was steep and EP 157 produced complete inhibition of aggregation. In the second experiment (broken lines), the iloprost curve was shallower and EP 157 failed to produce maximal inhibition at the highest concentration tested (20 μM). Each point represents a single measurement.

EMR for 5 fold elevation of cyclic AMP are also given for human and pig platelets in Table 1. With the exception of EP 157 on pig platelets, there is a good correlation between the cyclic AMP EMR and the anti-aggregation EMR. However, this is not a satisfactory method of making the comparison, since the concentrations of each IP-receptor agonist which raise cyclic AMP to accurately measureable levels are far in excess of those sufficient for submaximal inhibition of aggregation. This is particularly important when the activity of EP 157 on pig platelets is considered. In the experiments shown in Figure 3, the rises in cyclic AMP induced by 0.1, 1.0 and 10 μM EP 157 were 0.99 ± 0.13, 1.20 ± 0.07 and 1.26 ± 0.05 fold (mean ± s.e.mean, $n = 6$) respectively. By comparison with the corresponding portion of the iloprost curve, these small cyclic AMP elevations could certainly be responsible for inhibition of aggregation. The very small increment in cyclic AMP on raising

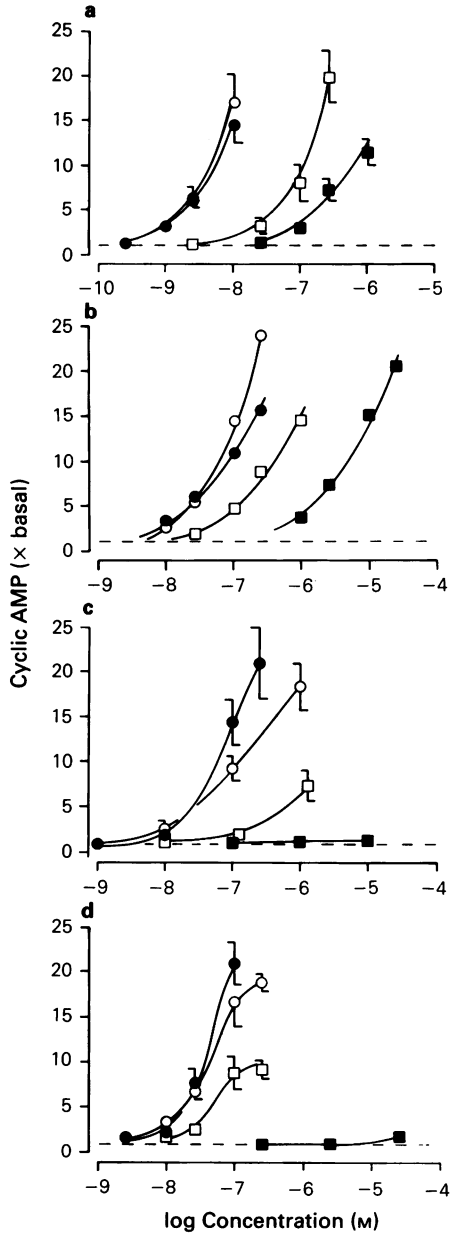


Figure 3 Elevation of cyclic AMP levels in washed platelet suspensions from (a) man, (b) horse, (c) pig and (d) rat induced by cicaprost (○), iloprost (●), 6a-carba PGI_2 (□) and EP 157 (■). Each point is the mean of 6 separate determinations, except in the horse where $n = 3$. Vertical lines indicate s.e.mean. Note the different concentration scale for the human platelet.

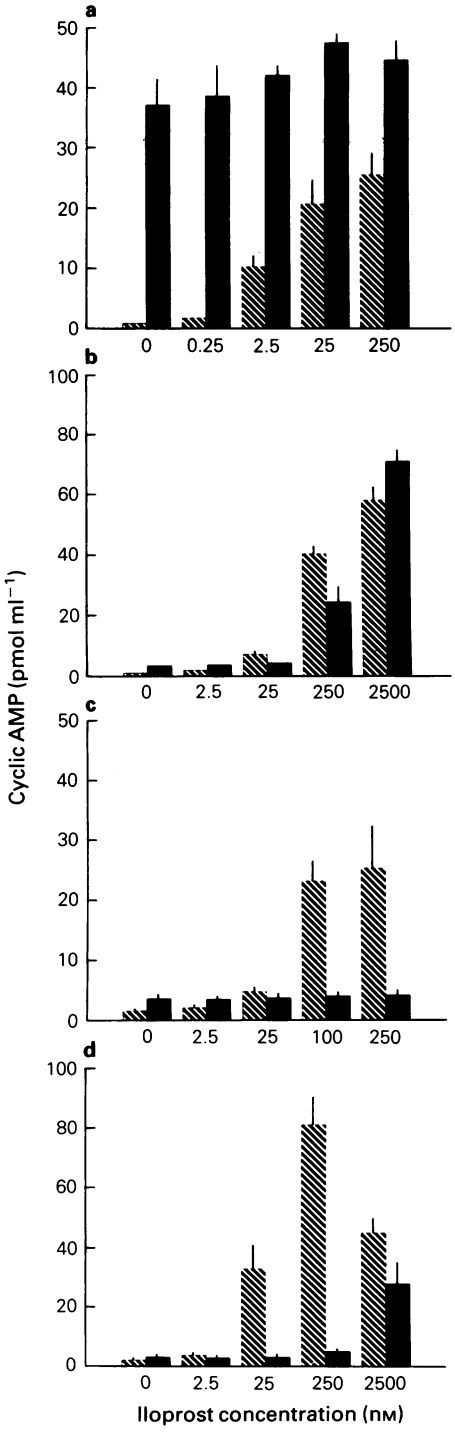
the concentration of EP 157 from 1.0 to 10 μM could also account for the difficulty in obtaining complete inhibition of aggregation in some experiments.

In order to test the EP 157/cyclic AMP link in the pig platelet, the effect of the adenylate cyclase inhibitor SQ 22536 (Harris *et al.*, 1979) was investigated. In each of four experiments matching responses to iloprost and EP 157 corresponding to 40–80% inhibition of Paf-induced aggregation were obtained. SQ 22536 (100 μM) added 2 min before prostanoid addition always reduced the iloprost and EP 157 responses to similar extents. For example, in one experiment SQ 22536 reduced the inhibition due to 3 nM iloprost from 43 to 22% and that due to 500 nM EP 157 from 43 to 25%; it is difficult to abolish completely prostacyclin-like anti-aggregatory activity with this agent.

Rises in cyclic GMP levels can also mediate inhibition of aggregation in the human platelet (see Armstrong *et al.*, 1985 for relevance to prostanoid inhibitory activity) and we thought it wise to investigate this aspect in the pig platelet, in view of the rather feeble activation of adenylate cyclase by EP 157. Neither iloprost (1 μM) nor EP 157 (10 μM) increased cyclic GMP levels in pig platelets (as measured by Amersham kit). Also N-methylhydroxylamine (0.4–4 μM), an inhibitor of guanylate cyclase (Deguchi *et al.*, 1978), had no effect on the inhibitory actions of EP 157 on pig platelets. Indeed, in the pig platelet the guanylate cyclase activator sodium nitroprusside (400 nM) is not an inhibitor of aggregation but an aggregating agent, and this effect is blocked by N-methylhydroxylamine.

Antagonism of iloprost action by EP 157

In view of the small rises in cyclic AMP produced by EP 157 in pig platelets and its inability to inhibit aggregation completely, it was decided to investigate whether this analogue would oppose the action of iloprost in raising cyclic AMP levels. Concentration-response relationships for iloprost (30 s incubation) were obtained in the absence and presence of 20 μM EP 157 (added 2 min before iloprost) on human, pig, rabbit and rat platelets (Figure 4). With human platelets the rise in cyclic AMP produced by EP 157 alone was so large (about 50 times basal) that it was difficult to determine the nature of its interaction with iloprost. In contrast, on rabbit platelets 20 μM EP 157 raised cyclic AMP by only 2.1 ± 0.3 fold (mean ± s.e.mean, $n = 6$) and there was some antagonism of the effect of iloprost. In rat and pig platelets where EP 157 raised cyclic AMP by 1.86 ± 0.11 ($n = 7$) and 1.63 ± 0.04 ($n = 6$) fold respectively, a more marked inhibition of iloprost action was seen.



Displacement of [^3H]-iloprost binding to platelet membranes

The abilities of the four prostanoids to displace [^{3}H]-iloprost binding to membrane preparations from human, horse, pig, rabbit and rat platelets were studied. We found that our previously published assay conditions for human platelet membranes (30°C for 20 min with 20 nM radioligand; Armstrong *et al.*, 1986) also gave acceptable levels of displaceable binding in pig and horse membranes. On both human and pig membranes displacement curves for iloprost and cicaprost were virtually superimposable; at 10 μM of each agent (about 100 times the IC_{50}) the displaceable binding was 75.5 $\pm$ 3.6 and 75.7 $\pm$ 1.3% of total binding (mean $\pm$ s.e.mean, $n = 5$) for human and 78.6 $\pm$ 0.7 and 77.5 $\pm$ 2.2 ($n = 5$) for pig membranes. IC_{50} values (10 μM cicaprost displacement = 100%) are given in Table 2. However, on the horse membranes the iloprost displacement curve was situated to the left of the cicaprost curve (Figure 5) and at 10 μM the displacement for iloprost was 65.4 $\pm$ 3.5% of total binding and for cicaprost 51.1 $\pm$ 4.3% ($n = 5$); these values are significantly different ($P < 0.05$, Student's t test). The displacements for 6a-carba PGI_2 and its isomer $\Delta^{6,6a}$-6a-carba PGI_1 at 10 μM were 62.3 $\pm$ 2.0 and 62.0 $\pm$ 3.8% and these values were not significantly different from the 10 μM iloprost displacement ($P > 0.05$). These results would suggest that [^{3}H]-iloprost binds to two sites on horse platelet membranes and that iloprost, $\Delta^{6,6a}$-6a-carba PGI_1 and 6a-carba PGI_2, but not cicaprost, can displace the radioligand from both sites. If we assume that cicaprost binds specifically to the IP-receptor and that iloprost binds to the second site with a considerably higher affinity than it binds to the IP-receptor, then the portion of the iloprost curve in Figure 5 between 85% (10 nM) and 35% (10 μM) of control binding can be used to estimate an IC_{50} value for displacement of IP-receptor binding by iloprost. This value is 210 nM, similar to the cicaprost IC_{50} of 165 nM (Table 2). This procedure cannot be applied to the other two prostacyclin analogues since they appear to displace from both sites over similar concentration ranges.

With rabbit and rat platelet membranes the original assay conditions gave minimal binding. By raising the radioligand concentration to 40 nM, lowering the incubation temperature to 4°C (30 min)

Figure 4 Elevation of cyclic AMP induced by iloprost alone (hatched columns) and in the presence of 20 μM EP 157 (solid columns) in (a) human, (b) rabbit, (c) pig and (d) rat platelets. Mean of 4 experiments with human, pig and rat platelets and 5 experiments with rabbit platelets are shown. Vertical lines show s.e.mean.

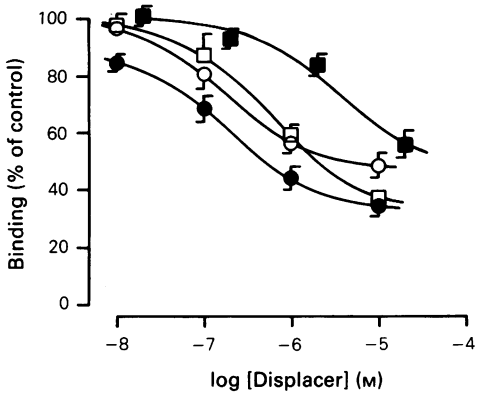


Figure 5 Displacement of [^{3}H]-iloprost binding to horse platelet membranes by cicaprost (○), iloprost (●), 6a-carba PGI_2 (□) and EP 157 (■). Each value is the mean of 5 separate determinations and vertical lines show s.e.mean.

and making sure that the temperature of the membranes did not rise above 4°C at any time during the wash cycle, total binding was increased and about 50% displaceable binding was obtained with 10 μM iloprost. With rabbit membranes, the overall picture was similar to the horse membranes. The iloprost displacement curve was situated to the left of the cicaprost curve and displacement at 10 μM was 49.9 $\pm$ 5.4% for iloprost and 33.3 $\pm$ 2.4% ($n = 5$) for cicaprost (statistically significant difference, $P < 0.05$). This trend was less marked in the rat membranes and the displacement at 10 μM iloprost (48.4 $\pm$ 4.1%) was not significantly different ($P > 0.05$) from that due to cicaprost (41.3 $\pm$ 6.0, $n = 4$). IC_{50} values are shown in Table 2. the higher IC_{50} values for cicaprost on both rabbit and rat membranes indicate a lower affinity prostacyclin binding site compared to the human, pig and horse membranes.

Relaxation of arterial smooth muscle

The relaxant action of the four prostanoids was determined on ring preparations of human mesenteric artery, rabbit mesenteric artery and pig gastro-epiploic artery. Vascular tissue from the horse was not obtainable and several rat preparations did not show high sensitivity to iloprost as a relaxant agent. Although platelets were not available from the dog, mesenteric artery was and this was also studied. Log concentration-response curves for reduction of tone produced by phenylephrine (usually 1 μM) in human, rabbit and pig vessels and by KCl in the dog vessel are shown in Figure 6 and EMR are given in Table 3. Sensitivity to iloprost decreased in the order human, dog, rabbit, pig. Cicaprost was slightly more active than iloprost on each of the four preparations. EP 157 showed threshold relaxation at 20 nM in the human vessel and at 200 nM on the dog, rabbit and pig vessels and at the highest concentration tested gave relaxations 70–95% of the iloprost maximum. The log concentration-response curve to 6a-carba PGI_2 was shallow on the pig gastro-epiploic artery and concentrations above 100 nM often gave discrete contractile responses. We surmised that 6a-carba PGI_2 might be acting on either TP- or EP_1-receptors to produce an opposing contractile action. An action on TP-receptors seems unlikely since the TP-receptor antagonist EP 092 (Armstrong *et al.*, 1985) at 1 μM did not affect the 6a-carba PGI_2 relaxation curve; the contractile action of the TP-receptor agonist U-46619 was markedly antagonised by EP 092 (dose-ratio = 20). Analysis of PGE-like activity on the pig vessel is difficult since both PGE-

Table 2 A comparison of the abilities of prostacyclin mimetics to displace [^{3}H]-iloprost binding from platelet membranes of different species

	IC_{50} values: 10 μM cicaprost displacement = 100% (nM)					
Prostanoid	*Human*	*Pig*		*Horse*	*Rabbit*	*Rat*
Assay temperature =	30°C	30°C	4°C	30°C	4°C	4°C
Iloprost	110	90	90	55* (210)	100* (700)	320
Cicaprost	110	90	95	165	900	640
$\Delta^{6,6a}$-6a-Carba PGI_1	140	170	—	75*	310	540
6a-Carba PGI_2	870	700	680	325*	1000	980
EP 157	2030	8200	7400	4700	7500	7000

IC_{50} values were calculated from mean log concentration-displacement curves derived from 5 separate experiments.
* Displacement by 10 μM prostanoid was significantly greater than that of 10 μM cicaprost ($P < 0.05$, one-tailed t test).
IC_{50} values in parentheses for iloprost on horse and rabbit membranes were obtained by assuming that displacement of binding to IP-receptors was negligible at 10^{-8} iloprost and near maximal at 10^{-5} M iloprost (i.e. the postulated higher affinity binding site for the radioligand has been eliminated from the estimation).

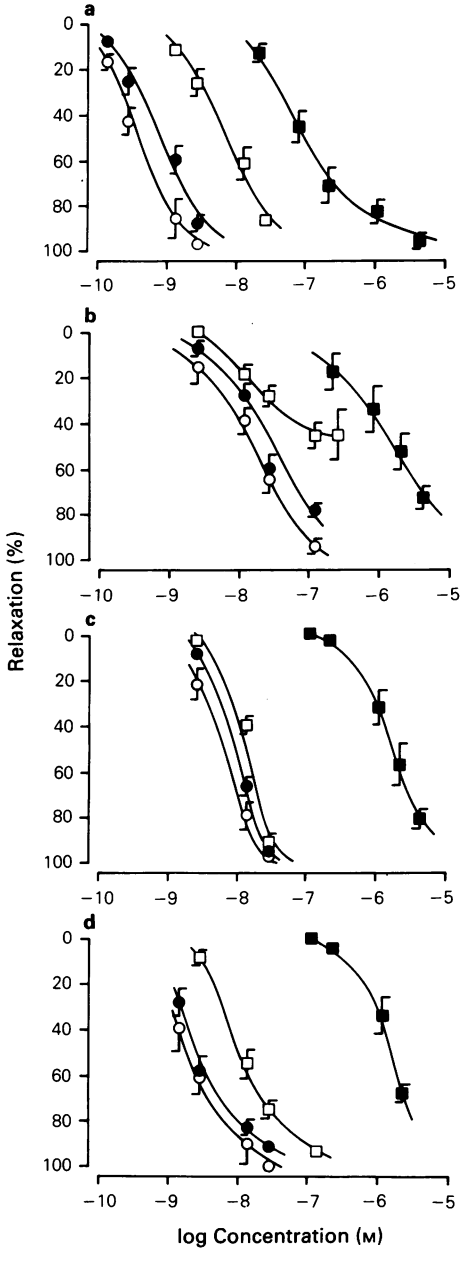


Figure 6 Log concentration-responses curves for relaxation of (a) human mesenteric artery, (b) pig gastro-epiploic artery, (c) rabbit mesenteric artery (means of 6–9 experiments) and (d) dog mesenteric artery (4–6 experiments) by cicaprost (○), iloprost (●), 6a-carba PGI_2 (□) and EP 157 (■). Tone was initially induced by phenylephrine (usually 1 μM) in the human, pig and rabbit preparations and by KCl (10 or 15 mM) in the dog prepration. Vertical lines show s.e.mean.

sensitive relaxant and contractile systems are present. Thus PGE_2 at low concentrations (0.3–10 nM) produced relaxation which was reversed above 20 nM and discrete contractile responses were seen as the concentration was increased. Our previous studies have shown 16,16-dimethyl PGE_2 to have greater activity than PGE_2 as a contractile agent and less activity as a relaxant agent (Dong *et al.*, 1986). On the pig vessel 16,16-dimethyl PGE_2 was a potent contractile agent in the presence or absence of phenylephrine. Without phenylephrine its EC_{50} was about 10 nM. By analogy with our studies of 6a-carba PGI_2 action on PGE-sensitive contractile systems, we would expect 6a-carba PGI_2 to show significant contractile activity at 200 nM and this agrees with the flattening of the relaxation curves in Figure 6. We attempted to block the proposed contractile activity of 6a-carba PGI_2 using both SC 19220 (Sanner *et al.*, 1973; Kennedy *et al.*, 1983) and AH 6809 (Coleman *et al.*, 1987); these compounds are known to block EP_1-receptors in other smooth muscle preparations. However, neither 30 μM SC 19220 nor 5 μM AH 6809 altered the nature of the 6a-carba PGI_2 relaxation curve, nor did they block the contractile action of 16,16-dimethyl PGE_2(dose-ratio < 2).

Discussion

Taken as a whole, the aggregation, cyclic AMP and ligand binding studies strongly suggest that IP-receptors on human, pig and horse platelets are similar in nature. Of particular note is the good agreement between IC_{50} values for displacement of [^{3}H]-iloprost binding to platelet membranes by cicaprost (man 110, pig 90, horse 165 nM). There are, however, marked differences between the three systems in their sensitivity to inhibition of aggregation by iloprost or cicaprost (human > pig > horse) and in the magnitude of cyclic AMP elevation at concentrations of iloprost or cicaprost which produce submaximal inhibition of aggregation (horse > man = pig). We wish to consider possible reasons for these differences and also whether the different profile of action of EP 157 on the pig platelets can be reasonably explained in terms of a low efficacy on the prostacyclin-sensitive system.

Two factors would appear to be important—the overall catalytic efficiency of the IP-receptor-adenylate cyclase complex and the sensitivity of the inhibitory mechanism (kinase activation/sequestration of internal Ca^{2+}/etc.?) to generated cyclic AMP. If both processes are 'highly tuned' in human platelets, then this would explain how an agonist such as iloprost at a concentration as low as 1/1000th of its

Table 3 Relaxant activities of prostacyclin mimetics on visceral arterial vessels

Prostanoid	*Equi-effective molar ratio* Human mesenteric artery	Pig gastro-epiploic artery	Rabbit mesenteric artery	Dog mesenteric artery
Iloprost	1.0	1.0	1.0	1.0
(IC_{50} =	1.2 ± 0.3 nM)	(24 ± 3 nM)	(10 ± 1 nM)	(2.4 ± 0.6 nM)
Cicaprost	0.36 ± 0.08	0.87 ± 0.17	0.53 ± 0.07	0.46 ± 0.03
6a-Carba PGI_2	9.6 ± 1.5	(see text)	2.1 ± 0.2	3.6 ± 0.04
EP 157	220 ± 46	81 ± 26	230 ± 32	898 ± 134

Tone was induced with phenylephrine (usually 1 μM) on the human, pig and rabbit preparations and by KCl (10 or 15 mM) on the dog preparations.
Each value is the mean ± s.e.mean of 6–9 observations in the human, pig and rabbit vessels and 4–6 observations in the dog vessel.

binding K_d raises cyclic AMP to a level sufficient to activate the inhibitory process. In addition, EP 157 with a lower affinity (Table 2) and a (postulated) lower efficacy than iloprost would still be a potent full agonist (IC_{50} = 8 nM). In horse platelets the low sensitivity to iloprost appears to be mainly due to an insensitivity of the inhibitory process to cyclic AMP: EP 157 could still produce sufficient biological stimulus to inhibit aggregation completely through efficient coupling of receptor and cyclase. In pig platelets the lower responsiveness to iloprost could be due to a poorly coupled or suppressed adenylate cyclase system. In this case an IP-receptor agonist with a reduced efficacy (e.g. EP 157) will probably raise cyclic AMP levels only marginally and, even though the inhibitory mechanism is highly sensitive to cyclic AMP, complete suppression of platelet aggregation will be difficult to achieve. The ability of EP 157 to suppress iloprost-induced elevation of cyclic AMP in pig platelets lends support to the idea that EP 157 can behave as a partial agonist at the prostacyclin-sensitive inhibitory system of the pig platelet. It is known that Ca^{2+} can inhibit adenylate cyclase activity (Rodan & Feinstein, 1976) and it is possible in the pig platelet that continuous release of Ca^{2+} from internal stores suppresses the enzyme. In preliminary experiments on pig platelets we have shown that TMB-8 (100 μM) markedly increases cyclic AMP production in response to either iloprost or EP 157, whereas diltiazem (10 μM) and verapamil (10 μM) cause minimal potentiation. TMB-8 is known to block Ca^{2+} release from internal stores, unlike diltiazem and verapamil which inhibit Ca^{2+} entry from outside the platelet (Rodan & Feinstein, 1976). We are investigating this phenomenon further.

Our binding experiments with [^{3}H]-iloprost on rabbit and rat platelet membranes were less satisfactory than those carried out on human, pig and horse membranes for three reasons: (1) the poor binding of [^{3}H]-iloprost at 30°C and the higher IC_{50} values for displacement by iloprost and cicaprost in the 4°C experiments point to a lower binding affinity for the two most potent prostacyclin mimetics. Indeed it is questionable whether a filtration-type separation method was at all suitable for these measurements. (ii) Non-specific binding of [^{3}H]-iloprost is higher in the rabbit and rat membranes. (iii) There is a possibility that [^{3}H]-iloprost was binding to another site besides the IP-receptor (this also appears to be the case in the horse platelet membranes). The nature of this other site is not clear. We have shown that iloprost, $\Delta^{6,6a}$-6a-carba PGI_1 (Dong *et al.*, 1986) and 6a-carba PGI_2 (Jones & Lawrence, unpublished observations) all have potent EP_1-receptor agonist activity whereas cicaprost does not. The presence of EP_1-receptors seems the most likely possibility at this time.

Although [^{3}H]-iloprost has been used by several groups, including our own, to detect and characterize prostacyclin binding sites (Town *et al.*, 1982; Rucker & Schror, 1983; Hall & Strange, 1984; Armstrong *et al.*, 1986), it is obvious from the present studies that it is not an ideal radioligand in all situations. Radiolabelled cicaprost may be more satisfactory owing to its very weak potency at EP_1-receptors. However, its affinity for rabbit and rat platelet IP-receptors is quite low and it may be that we shall have to wait for the development of specific IP-receptor antagonists before a more suitable high affinity radioligand becomes available. However, despite our reservations, the ligand binding data in conjunction with the corresponding aggregation and cyclic AMP studies do suggest that IP-receptors on rabbit and rat platelets are subtly different from those on human, pig and horse platelets. Of particular note is the very much greater activity of 6a-carba PGI_2 over EP 157 on rabbit/rat platelets compared with human/pig/horse platelets.

To date, little has been published on the actions of stable, prostacyclin analogues on human blood

vessels *in vitro*. Hadhazy (1986) showed that PGI_2 relaxes human mesenteric artery with an IC_{50} of about 7 nM and that the pulmonary and femoral arterial vessels are less sensitive. He also showed that the sensitivity of mesenteric arterial vessels to the relaxant effect of PGI_2 decreases in the order man, dog, rabbit, pig. Our results with iloprost and cicaprost parallel these findings. The IC_{50} for relaxation of our most sensitive preparation, the human mesenteric artery, by iloprost is about 1 nM. We found the mesenteric artery and several other arterial vessels from the pig to be insensitive to iloprost; however, the gastro-epiploic artery was reasonably sensitive ($IC_{50} \sim 25$ nM).

The true potency of the prostanoids studied here on the prostacyclin-sensitive relaxant systems may be obscured by actions at other prostanoid receptors. Although each vessel relaxes to low concentrations of PGE_2, we suspect that agonist activity at EP_2-receptors is negligible, since none of the compounds (iloprost, cicaprost or 6a-carba PGI_2) shows this type of activity on the guinea-pig or cat trachea; $\Delta^{6,6a}$-6a-carba PGI_1 was excluded from these studies because it does have significant EP_2-receptor agonist activity (Dong *et al.*, 1986). Opposing contractile activity through activation of EP-receptors is a more likely possibility and may explain the reversal of the 6a-carba PGI_2 relaxation on the pig gastro-epiploic artery. Although 6a-carba PGI_2 has EP_1-receptor agonist activity, its action on the pig vessel was unaffected by the EP_1-receptor antagonists SC 19220 and AH 6809. We have recently shown that 6a-carba PGI_2 also inhibits contractions of the guinea-pig vas deferens elicited by field stimulation (Lawrence & Jones, 1989). This type of activity has been linked to a third PGE receptor subtype (EP_3) by Coleman and colleagues (1987). Although this activity of 6a-carba PGI_2 is fairly weak (one-hundredth of PGE_2) the relatively low sensitivity of the pig vessel to IP-receptor agonists may allow it to become apparent. Rucker & Schror (1983) investigated [^{3}H]-iloprost binding to smooth muscle cells of pig aorta and found that the Hill plot was linear with a slope of 1.9. This was explained in terms of positive cooperativity, with two agonist molecules binding to one site. An alternative explanation suggested by the authors was binding of the ligand to a component other than the IP-receptor. It is possible that this component is an EP_1- or EP_3-receptor.

On the human, pig and rabbit vessels EP 157 showed relaxant activity at concentrations some 100–200 times greater than those of iloprost. This is a similar order of activity to that found on human, pig and horse platelets. We tentatively assume that this relaxant activity of EP 157 is due to an agonist action at IP-receptors. EP 157 is about 900 times less active than iloprost on the dog mesenteric artery *in vitro*. Before commencing these studies we had performed several experiments on anaesthetised dogs, in which changes in mesenteric arterial blood-flow were recorded in response to close-intra-arterial infusion of different prostanoids. Iloprost was a highly active dilator whereas EP 157 was without effect. It was these experiments that convinced us that platelet anti-aggregatory activity might be separated from vasodepressor activity by modification of prostanoid structure. Is there still a valid case for this view in relation to human therapeutics? Our experiments on human platelets and mesenteric artery would not lead one to be optimistic. However, we have demonstrated the possibility of partial agonism at IP-receptors and we feel that there is an outside chance that an IP-receptor agonist, with lower efficacy but higher affinity than EP 157, could effectively suppress platelet aggregation whilst having minimal relaxant activity on the vasculature.

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Investigation of the vasoconstrictor action of subarachnoid haemoglobin in the pig cerebral circulation *in vivo*

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1 Angiographic techniques have been used to study the influence of intracisternally injected haemoglobin on the diameters of the main intrathecal and representative extrathecal (ascending pharyngeal and facial) cranial arteries of the anaesthetized pig.

2 Intracisternal injection of haemoglobin caused concentration-dependent decreases in the diameters of intra- but not extrathecal arteries suggesting that haemoglobin possesses local vasoconstrictor activity.

3 When infused into one ascending pharyngeal artery, acetylcholine (ACh) caused slight dilatation of the intrathecal arteries but no change in the diameters of the ascending pharyngeal and facial arteries. The dilator response induced by ACh in the intrathecal arteries was converted into frank constriction after intracisternal injection of haemoglobin (cerebrospinal fluid concentration approximately 2×10^{-5} M).

4 These findings are consistent with the hypothesis that subarachnoid haemoglobin can induce cerebral artery constriction by acting as an extraluminal 'sink' for intimally released endothelium-derived relaxing factor (EDRF) and may be relevant to the pathogenesis of vasospasm after subarachnoid haemorrhage in man.

Introduction

Haemoglobin is one of the several blood components which have been implicated in the aetiology of delayed cerebral artery spasm following subarachnoid haemorrhage (SAH) in man (Osaka, 1977; Ozaki & Mullan, 1979; Tanishima, 1980; Toda *et al.*, 1980) and the severity of the associated clinical syndrome correlates with the concentration of cerebrospinal fluid oxyhaemoglobin produced by red cell lysis (Tourtellotte *et al.*, 1964; Suzuki, 1979; Vermeulen *et al.*, 1983). Indeed, oxyhaemoglobin has been shown to constrict isolated basilar arteries in dogs (Tanishima, 1980; Wellum *et al.*, 1982; Connor & Feniuk, 1987) and cats (Osaka, 1977). The observation that low concentrations of haemoglobin inhibit endothelium-dependent relaxation *in vitro* (Martin *et al.*, 1985; Edwards *et al.*, 1986) could explain the mechanism of the vasospasm that occurs after SAH as it could potentially inhibit both basal EDRF activity (Griffith *et al.*, 1984a,b; 1987; 1988; Martin *et al.*, 1986) and EDRF activity stimulated by substances derived from blood components such as aggregating platelets (Cohen *et al.*, 1983; Houston *et al.*, 1985). To date, however, the interaction between haemoglobin and EDRF has been studied only in tissue bath experiments in which both the intimal and adventitial surface of blood vessels are simultaneously exposed to haemoglobin. We have therefore developed a model of subarachnoid haemorrhage which allows investigation of the constrictor activity of purified haemoglobin when in contact solely with the adventitial surface of intracerebral vessels *in vivo*.

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Methods

Anaesthesia was induced in pigs (18–23 kg) by intramuscular injection of ketamine hydrochloride (10 mg kg^{-1}) (Ketalar, Parke Davis and Co.). Endotracheal intubation under direct vision was performed under halothane in nitrous oxide/oxygen

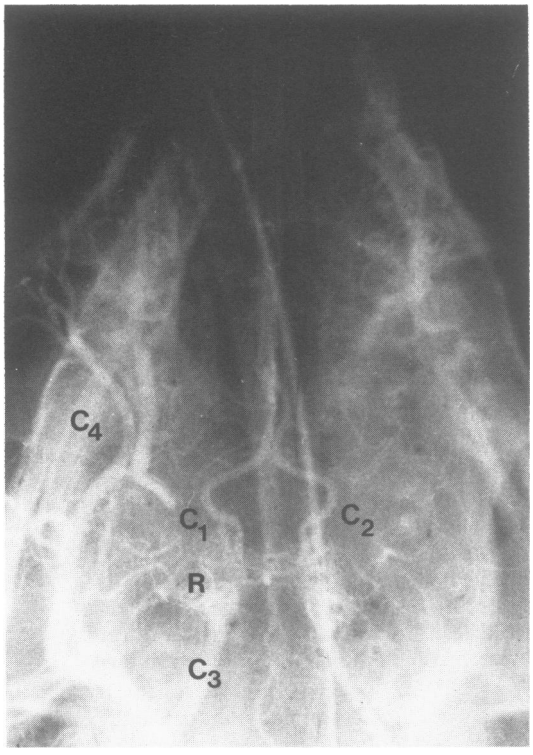

Figure 1 Representative angiogram obtained by injection of contrast medium through an ascending pharyngeal artery illustrating the topographical anatomy of the pig cerebral circulation. Intrathecal cerebral arteries (C_1, C_2) arise from the rete mirabilis (R) which is supplied by ascending pharyngeal arteries (C_3). A representative side branch of the facial artery (C_4) is also indicated. Note that unilateral injection fills both C_1 and C_2 arteries.

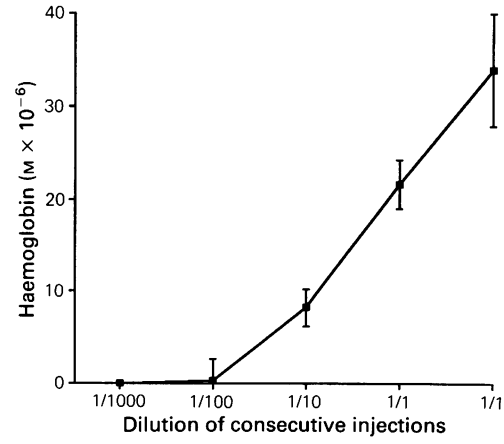

Figure 2 Standard curve plotting equilibrium concentration of CSF haemoglobin (determined spectrophotometrically) as a function of the dilution of heamoglobin solution obtained by G200 chromatography injected into the cisterna magna ($n = 6$). Two ml of the dilutions shown along the ordinate were injected cumulatively after withdrawal of 2 ml of CSF for determination of haemoglobin concentration. Haemoglobin was not detectable in CSF samples after injection of the lowest (1/1000) dilution.

anaesthesia and the animals were then mechanically ventilated with a Starling pump. Anaesthesia was maintained by intermittent bolus injections (200 mg) of pentabarbitone (Sagital, May and Baker, Ltd., Dagenham) and ventilation with nitrous oxide (66%) in oxygen to maintain a constant end tidal P_{CO_2} of 40 mmHg. The carotid and femoral arteries were separately cannulated and catheters positioned in the carotid artery with their tips just proximal to the origin of the ascending pharyngeal artery for angiography and in the abdominal aorta for continuous monitoring of systemic blood pressure. Angiography was performed by hand injections (0.3 ml kg^{-1}) of iohexol (Omnipaque, 300 mg iodine ml^{-1}, Nycomed Ltd) via the carotid catheter at a fixed film-focus distance of 70 cm. Intervals of at least 20 min were allowed between each injection to exclude any residual vasoactive effect of the contrast media. Precision milled steel balls (2 mm in diameter) were included in the exposure for calibration of radiographic magnification. A concentrated solution of purified autologous haemoglobin in Holman's physiological buffer (approximately 100 μM) was obtained from washed red cell lysate by diffusion chromatography on a Sephadex G200 column as previously described (Edwards *et al.*, 1986). Intrathecal injections (2 ml) of Holman's buffer or purified haemoglobin solution at dilutions ranging from 0 to 1/1000 were made into the cisterna magna and the animals tilted head down for 10 min in order to allow the haemoglobin time to reach the Circle of Willis. Angiography was subsequently performed 15 min after each injection.

To ensure that haemorrhage had not occurred during needle placement, cerebrospinal fluid (CSF) was sampled before and after each haemoglobin injection. The volume of CSF withdrawn was equal to that of the injection so as to minimize changes in subarachnoid pressure. The haemoglobin concentration of the CSF samples was determined spectrophotometrically by the cyanomethaemoglobin method (Drabkin & Austin, 1932). Acetylcholine, dissolved in Holman's buffer at concentrations of 10^{-6} M, 10^{-5} M and 10^{-4} M, was infused at 4 ml min^{-1} for 5 min via the carotid catheter before and after intracisternal injection of 2 ml undiluted purified haemoglobin solution. The diameters of the main intrathecal cerebral

arteries (C_1 and C_2), the ipsilateral ascending pharyngeal artery (C_3), which is extrathecal, and a side branch of the ipsilateral facial artery of similar size (C_4) (Figure 1) were measured from the radiographs with a IBAS Kontron Semi-interactive Image Analysis System (Kontron Electronics, Munich, F.R.G.) as previously described (Griffith *et al.*, 1987; 1988). The intrathecal cerebral arteries studied form the anterior part of the circle of Willis and arise from the carotid rete mirabilis which is supplied principally by the ascending pharyngeal arteries. The control diameters of C_1 and C_2 were approximately 500 μm (range 300 to 800 μm). Diameter changes in all arteries studied were expressed as % change in control diameter and these values then averaged.

Results

A calibration curve was constructed for the CSF haemoglobin concentration after successive intracisternal injections (2 ml aliquots) of increasing concentration (Figure 2). The intrathecal C_1 and C_2 arteries exhibited concentration-dependent constriction in response to haemoglobin whereas the diameters of the extrathecal C3 and C4 arteries were unchanged (Figure 3). The mean maximum constriction induced in C1 + C2 by haemoglobin (4 ml undiluted haemoglobin solution, CSF concentration approximately 3.5×10^{-5} M) was 26% of control diameter.

Infusion of acetylcholine via the ascending pharyngeal artery before intrathecal injection of haemoglobin induced a minor degree of dilatation in the intrathecal C_1 and C_2 arteries. This was significantly different from control only at the highest concentration of acetylcholine infused (10^{-4} M) (Figure 4), when a mean maximum vasodilatation of 7.2% occurred. The diameters of the extrathecal vessels did not change during the acetylcholine infusion.

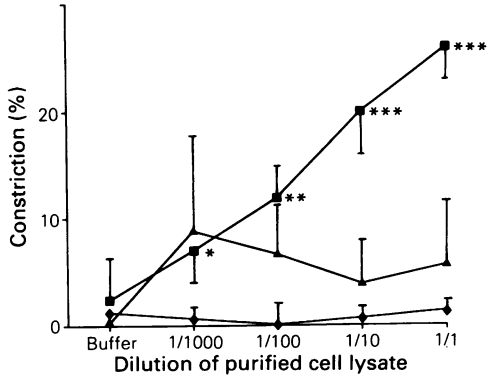

Figure 3 Graph showing % constriction (relative to control diameter) of intracerebral ($C_1 + C_2$, ■), ascending pharyngeal (C_3, ▲) and facial (C_4, ◆) arteries in response to cumulative injection of haemoglobin solution at the dilutions shown. There was a significant, concentration-dependent constriction only in intrathecal vessels (*$P < 0.05$; **$P < 0.005$; ***$P < 0.001$: $n = 6$)

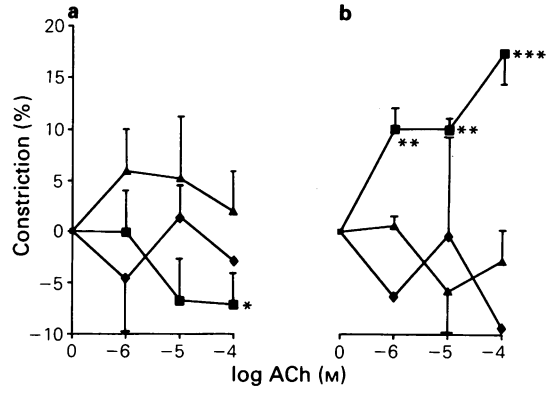

Figure 4 (a) Graph showing % changes in diameter induced in intrathecal (C1 + C2) and extrathecal (C3, C4) arteries by infusion of acetylcholine (ACh) at the molar concentrations shown ($n = 9$): C_1 and C_2 (■); C_3 (▲); C_4 (◆). There was a small but significant dilatation of the intrathecal vessels at the highest concentration of acetylcholine (*$P < 0.05$). The error bar has been omitted on the data point for the C_4 arteries at the highest concentration of ACh (10^{-4} M) because of overlap. Differences from control diameter were however insignificant. (b) Graph showing % changes from control diameter induced by injection of 2 ml of undiluted purified red cell lysate into the cerebrospinal fluid and infusion of increasing concentrations of acetylcholine ($n = 5$). CSF haemoglobin concentration was therefore approximately 2×10^{-5} M (Figure 2). Haemoglobin converted the dilatation induced by acetylcholine in the intrathecal C_1 and C_2 arteries into constriction (*$P < 0.05$; **$P < 0.005$; ***$P < 0.001$).

Responses to acetylcholine in the intrathecal arteries were altered by the presence of subarachnoid haemoglobin at a concentration of approximately 2×10^{-5} M, which corresponded to that achieved by injection of 2 ml of undiluted haemoglobin solution (Figure 2). Concentration-dependent vasoconstriction as opposed to dilatation then occurred in C_1 and C_2, the maximum mean response being 18% of control diameter when acetylcholine was infused at a concentration of 10^{-4} M (Figure 4).

Discussion

Different mechanisms may underly the pathophysiology of the acute and chronic phases of the cerebral vasospasm which occurs after

experimentally-induced subarachnoid haemorrhage, although it is possible that haemoglobin may contribute to both (Toda *et al.*, 1977; Osaki & Mullan, 1979; Duff *et al.*, 1988). As in other arteries, a number of studies have shown that haemoglobin is a potent inhibitor of EDRF-mediated relaxation in isolated cerebral arteries (Fujiwara *et al.*, 1986; Kanamaru *et al.*, 1987; Nakagomi *et al.*, 1987), presumably due to binding of EDRF which current evidence indicates may be identical to nitric oxide (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Kelm *et al.*, 1988; Furchgott 1988). *In vitro*, the constriction induced by haemoglobin in peripheral arteries is strictly dependent on the presence of an intact endothelium (Martin *et al.*, 1985; Edwards *et al.*, 1986; Tanaka *et al.*, 1987), whereas there is evidence that it also possesses direct smooth muscle constrictor activity in isolated cerebral arteries from certain species (Tanaka *et al.*, 1987; Connor & Feniuk, 1987). In the present study we have investigated angiographically the effect of subarachnoid injection of haemoglobin on intra- and extrathecal pig cerebral vessels *in vivo*.

Previous studies with isolated, intact pig arteries indicate that there is heterogeneity of arterial responsiveness in this species. Acetylcholine, for example, is able to induce endothelium-dependent relaxation in pig aorta (Gordon & Martin, 1983) but not in coronary artery (Kalsner, 1985; Graser *et al.*, 1986). A similar lack of endothelium-dependent responsiveness to acetylcholine (although not to the calcium ionophore A23187) has also been observed in canine basilar as opposed to femoral artery (Kanamaru *et al.*, 1987). In the present study, intra-arterial acetylcholine induced a small dilatation of the intrathecal vessels (although only at a high concentration) and was without effect on the extrathecal ascending pharyngeal and facial arteries. One possible explanation for these findings is that acetylcholine is a weak stimulator of EDRF release in pig cerebral vessels. It should be noted that the net response to acetylcholine will depend on the balance between direct smooth muscle constriction and stimulation of EDRF activity. Where the former predominates it may be difficult or even impossible to demonstrate EDRF release without the use of cascade bioassay techniques (Griffith *et al.*, 1984a). It is also possible that endothelium-dependent flow-mediated dilatation (Holtz *et al.*, 1983) contributed to the effect of acetylcholine in the intrathecal vessels as a secondary phenomenon.

Introduction of haemoglobin into the subarachnoid space caused concentration-dependent constriction of the intrathecal arteries. This confirms earlier reports (Osaka, 1977; Ishii & Nonaka, 1977). The observation that the ascending pharyngeal or facial arteries were unaffected suggests that the effect is mediated locally. Additionally, the small dilator response induced by acetylcholine was converted into frank constriction by subarachnoid haemoglobin. There are several possible explanations of these findings. Subarachnoid haemoglobin could act as an extraluminal 'sink' for intimally released EDRF, and could thus inhibit either basal EDRF activity (Griffith *et al.*, 1984a,b; 1987; Collins *et al.*, 1986; Martin *et al.*, 1986) or EDRF activity directly stimulated by acetylcholine itself (Furchgott, 1983). Both mechanisms would enhance the smooth muscle constrictor response to acetylcholine. We have previously shown that 1 μM haemoglobin completely abolishes endothelium-dependent relaxation stimulated by the calcium ionophore A23187 when in contact with both the intimal and adventitial surface of rabbit aorta (Edwards *et al.*, 1986). In the present study the constrictor effect of subarachnoid haemoglobin was still increasing at concentrations some 30 fold higher than this. If however the action of haemoglobin were due solely to inhibition of EDRF activity, then it would be expected to be less effective when located extraluminallyy because of the interposition of the media of the vessel wall. Concentrations greater than 1 μM would thus be necessary to obtain complete inhibition of EDRF activity. The inversion of the vasomotor response to acetylcholine could, however, also result from a primary vasoconstrictor action of haemoglobin (Tanaka *et al.*, 1987; Connor & Feniuk, 1987), release of vasoconstrictor agents such as the peptide endothelin from endothelial cells (Yanagisawa *et al.*, 1988), or release of vasoconstrictor transmitters from adventitial nerves, although there is evidence that the latter phenomenon is not important *in vitro* (Tanishima, 1980). Whilst the observations of this *in vivo* study are therefore consistent with the idea that EDRF activity can be influenced by haemoglobin located in the subarachnoid space, further studies are required to assess the contribution of these other mechanisms.

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Cholecystokinin-octapeptide constricts guinea-pig and human airways

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1 Cholecystokinin-octapeptide (CCK-OP, 10^{-10}–3×10^{-6} M) produced a concentration-dependent contractile response in guinea-pig trachea which was enhanced by both the mechanical removal of the epithelium and by indomethacin (10^{-5} M), with an EC_{50} of $6.18 \pm 0.10 \times 10^{-8}$ M.

2 Sub-threshold concentrations of CCK-OP, which did not alter the resting tone of the smooth muscle, did not alter responses produced to electrical field stimulation (EFS) or to vagal nerve stimulation in an intact tracheal tube preparation. Atropine (2×10^{-6} M) did not alter the concentration-response curve to CCK-OP, indicating that CCK-OP contraction is not mediated by cholinergic mechanisms.

3 The inhibition of neutral endopeptidase (endopeptidase-24.11) by phosphoramidon (10^{-5} M) gave a leftward shift in the CCK-OP concentration-response curve in tissues with intact epithelium obtained from normal animals, but had no effect in tissues denuded of epithelium or in tissues obtained from animals which had been actively sensitized and challenged with ovalbumin (OA).

4 CCK-OP-induced contractile responses were antagonized by the CCK-receptor antagonists dibutyryl cyclic guanosine monophosphate ($pA_2 = 4.3$) and L-364,718 ($pA_2 = 9.6$).

5 CCK-OP induced bronchoconstriction in large, but not small, human airways and was antagonized by the CCK-receptor antagonist L-364,718. CCK-OP had no effect on cholinergic neural responses elicited by EFS in human airways.

Introduction

Cholecystokinin (CCK) is a peptide hormone consisting of 33 amino acid residues which is classically associated with the gastrointestinal tract, where it is involved in a number of modulatory functions. CCK has also been localized to certain central pathways where it may function as a neurotransmitter or neuromodulator (Chiodi & Bunney, 1983; Krieger, 1983). The C-terminal octapeptide fragment (CCK-OP) has the same efficacy and potency as the complete 33 amino acid peptide (Villanueva *et al.*, 1982), and is probably the predominant form of CCK in both central and peripheral nerves (Larsson & Rehfeld, 1979).

In a number of *in vitro* models, CCK-OP has been demonstrated to have a number of modulatory functions. CCK-OP has been shown to modulate the electrically-induced release of acetylcholine (ACh) via a presynaptic effect in the frog sartorius muscle (Akasu *et al.*, 1986) and has also been shown to evoke the release of [^{3}H]-ACh from guinea-pig gallbladder via an action at the level of the postganglionic nerve terminal (Yamamura *et al.*, 1986). CCK-OP also stimulates gastrointestinal motility in the guinea-pig via a direct effect on ileal smooth muscle (Vizi *et al.*, 1973). In most smooth muscle preparations investigated, CCK-OP appears to have a higher affinity but lower efficacy than ACh. Atropine has also been observed to have a variable effect on CCK-OP-induced responses in certain smooth muscle preparations (Rakovska *et al.*, 1986), suggesting that CCK-OP may act either directly on smooth muscle or by an interaction with cholinergic mechanisms.

A number of different CCK receptors have been postulated both centrally (Moran *et al.*, 1986) and peripherally (Behar & Biancani, 1987). In peripheral systems such as the cat lower oesophageal sphincter, CCK receptor heterogeneity has been characterized to neural and muscular receptors, which are inhibitory and excitatory respectively (Rattan and Goyal, 1983). In guinea-pig gallbladder, the excitatory effect on smooth muscle is sensitive to the antagonist dibutyryl cyclic guanosine monophosphate (db cyclic GMP) whereas the inhibitory neural response is not,

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which further supports the heterogeneity of CCK-receptors (Takahashi *et al.*, 1987).

CCK-like immunoreactivity (CCK-LI) has been detected within the respiratory tract of several species (Polak & Bloom, 1982), although its precise location and function are unknown. Occasionally, CCK-LI can be visualised in certain axons that are also immunoreactive to substance P (Gibbins *et al.*, 1987).

The aim of this study was to investigate whether CCK-OP had an effect on bronchial tone, either via an interaction with cholinergic mechanisms or via a direct effect on smooth muscle.

Methods

Tissue preparation

Tracheal slices

Male Dunkin-Hartley guinea-pigs (250–500 g) were killed by cervical dislocation and the tracheae were rapidly removed. The trachea was stripped of connective tissue and opened longitudinally by cutting through the cartilage. Each trachea was cut into eight transverse pieces, each containing 3–4 cartilaginous strips. Where applicable, the removal of the epithelial layer was achieved by gentle rubbing of the inside surface of the trachea with a cotton wool applicator.

Each tracheal segment was then suspended in a 10 ml organ bath containing Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25.5, and glucose 5.05. The solution was maintained at 37°C and was gassed continuously with a mixture of 95% CO_2/5% O_2, at pH 7.4. The tissues were allowed to equilibrate for 1 h, with frequent washing, under a resting tension of 1 g, which was found to be optimal for determining changes in tension. Isometric contractile responses were measured with Grass FT.03 force-displacement transducers and recorded on a polygraph (Grass Model 7D, Grass Instruments Co., Quincy, Mass., U.S.A.).

Tracheal tube

Male Dunkin-Hartley guinea-pigs (250–500 g) were anaesthetized by an intraperitoneal injection of urethane (400 mg kg^{-1}) and the trachea, together with the intact right vagus nerve, was dissected free as previously described (Blackman & McCaig, 1983). The trachea was suspended vertically in a 10 ml organ bath containing Krebs solution and connected to a physiological pressure transducer (Gould, Holland). Preganglionic nerve stimulation was achieved via the right vagus nerve, and postganglionic nerve stimulation via transmural parallel field electrodes.

Human bronchi

The effect of CCK-OP was studied in human airways with tissues obtained from patients having undergone lung resection for bronchial carcinoma. Bronchi from the level of distal lobar to subsegmental were dissected and mounted as rings in organ baths as for guinea-pig tracheal strips.

Electrical field stimulation

The effect of CCK-OP on responses elicited to electrical field stimulation (EFS) in guinea-pig tracheal slices and human bronchi were studied by suspending the tissues between parallel platinum wire electrodes (approximately 1.5 cm apart). Biphasic square wave pulses were delivered for 15 s periods from a Grass S88 stimulator, using a supramaximal voltage of 40 V at source and a pulse duration of 0.5 ms. Stimuli were delivered every 4 min at a frequency of 8 Hz.

In the intact tracheal tube preparation, preganglionic nerve stimulation via the vagus nerve was achieved with a voltage at source of 20 V and a pulse duration of 0.2 ms. Trains of stimuli were delivered for 5 s every 55 s. Postganglionic stimulation via transmural field electrodes was achieved with a voltage of 40 V and a pulse duration of 0.5 ms. Impulse delivery was similar to that for preganglionic stimulation.

Sensitization studies

Male guinea-pigs (200–250 g) were actively sensitized to ovalbumin (OA, Grade 5), three weeks before the experiments, by a single intraperitoneal injection of 0.5 ml of saline containing 20 μg OA and 100 mg aluminium hydroxide (Andersson, 1980). The animals were challenged 24 h before use via an aerosol solution containing 250 μg^{-1} ml OA in saline. Control animals received a single intraperitoneal injection of aluminium hydroxide, 100 mg in 0.5 ml saline, and were challenged by aerosol with saline alone 24 h before use.

Drugs

Drugs were obtained from the following sources: cholecystokinin-octapeptide, dibutyryl cyclic guanosine monophosphate, indomethacin, atropine sulphate, phosporamidon, ovalbumin urethane and

bovine serum albumin (BSA) (Sigma); and L-364,718 (3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1-H-1,4-benzodiapine-3-yl)-H-indole-2-carboxamide, kindly donated by Merck, Sharp and Dohme, U.S.A.). CCK-OP stock solutions were made up in distilled water in the presence of BSA (200 μg BSA mg^{-1} peptide). Aliquots of CCK-OP were freeze dried and stored at −20°C. Indomethacin was made up in alkaline phosphate buffer (pH 7.8) of the following composition (mM): KH_2PO_4 20, Na_2HPO_4 120. High potassium Krebs-Henseleit solution was of the following composition (mM): KCl 123.9, NaCl 4.0, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.3, $NaHCO_3$ 25.5 and glucose 5.05. Stock solutions of L-364,718 were dissolved in dimethylsulphoxide (DMSO) and then diluted in distilled water to the appropriate concentration. Fresh drug solutions were made up daily. Drug additions did not exceed 1% of the bath volume. All concentrations refer to the final bath concentrations.

Analysis of results

Contractile responses to exogenously applied CCK-OP were expressed as absolute changes in tension, and then transformed to a percentage of the maximal response for each tissue which was obtained in the presence of a high potassium Krebs-Henseleit solution. The effects of exogenous drug additions, sensitization and epithelium removal on concentration-response relationships constructed to CCK-OP were assessed by Student's t test for either paired or unpaired data. Probability values $P < 0.05$ were considered significant.

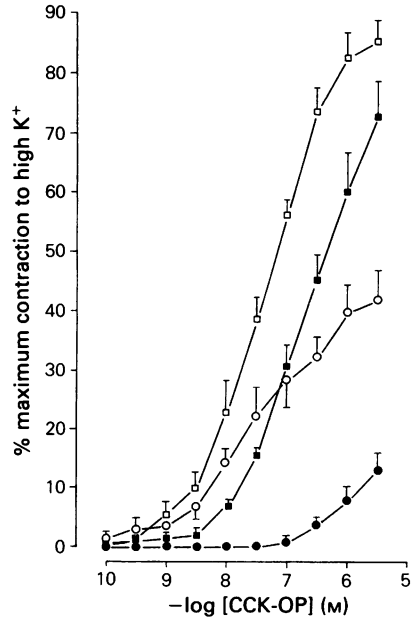

Figure 1 Effect of indomethacin (10^{-5} M) and epithelium removal on contractile responses to cholecystokinin-octapeptide (CCK-OP) in guinea-pig trachea: (●) tissues with intact epithelium (+E) in the absence of indomethacin (Indo); (○) tissues denuded of epithelium (−E) in the absence of Indo; (■) +E in the presence of Indo and (□) −E in the presence of Indo ($EC_{50} = 6.18 \pm 0.10 \times 10^{-8}$ M). All points are means, $n = 6$; vertical bars represent s.e.mean. These abbreviations are used in subsequent legends.

Results

Effect of cholecystokinin-octapeptide

In the guinea-pig trachea, CCK-OP produced a concentration-dependent contractile effect. In the presence of indomethacin, tissues denuded of epithelium produced a maximal response to high [K^+] of 85.11% ± 4.0% and an EC_{50} of $6.18 \pm 0.10 \times 10^{-8}$ M ($n = 6$) (Figure 1).

Effect of indomethacin

In the presence of indomethacin (10^{-5} M), the contractile responses induced by the exogenous application of CCK-OP were significantly enhanced, such that the concentration-response relationship was shifted in a leftward fashion ($EC_{50} = 6.24 \pm 0.15 \times 10^{-7}$ M), with a significant increase in the maximum response (72.62 ± 6.1% of response to high [K^+]), (Figure 1).

Effect of epithelium removal

Epithelium removal caused a similar leftward shift in the concentration-response curve ($EC_{50} = 6.18 \pm 0.11 \times 10^{-8}$ M) together with an increase in the maximal response (to 85.11 ± 4.0% of response to high [K^+]) obtained to the peptide (Figure 1). In the presence of indomethacin, epithelium removal produced an eight fold leftward shift in the concentration-response curve when compared with similar relationships obtained with tissues having intact epithelium. Maximal contractile responses to CCK-OP were thus obtained in tissues denuded of epithelium and in the presence of indomethacin (Figure 1).

Interaction with cholinergic nerves

Atropine (2×10^{-6} M), pre-incubated with the tissue for 30 min, had no significant effect on the concentration-response relationship to CCK-OP.

Both sub-threshold concentrations of CCK-OP which did not alter the resting tone and higher concentrations of CCK-OP which caused an increase in the resting tone of the airways had no effect on the contractile responses to EFS (40 V, 0.5 ms, 8 Hz) in the guinea-pig trachea. In the guinea-pig tracheal tube preparation CCK-OP did not enhance either preganglionic stimulation via the vagus nerve, or postganglionic stimulation via EFS ($n = 7$), indicating that CCK-OP does not have an effect on ganglionic transmission.

Effect of phosphoramidon

Pre-incubation of the tissues for 30 min with phosphoramidon (10^{-5} M) caused a 6 fold, leftward shift in the concentration-response relationship to CCK-OP, constructed using tissues with intact epithelium ($EC_{50} = 8.33 \pm 0.14 \times 10^{-8}$ M, $P < 0.05$) (Figure 2), but failed to potentiate the similar concentration-response curve constructed in tissues denuded of epithelium.

Effect of sensitization

The contractile response to CCK-OP in intact airways from guinea-pigs which were actively sensitized with ovalbumin was significantly more sensitive ($EC_{50} = 7.54 \pm 0.17 \times 10^{-8}$ M, $n = 6$), than the response in airways from control (sham-sensitized) animals ($EC_{50} = 5.26 \pm 0.12 \times 10^{-7}$ M, $P < 0.05$). The response in sensitised did not differ from that of airways from control animals denuded of epithelium ($EC_{50} = 6.84 \pm 0.21 \times 10^{-8}$ M, Figure 3).

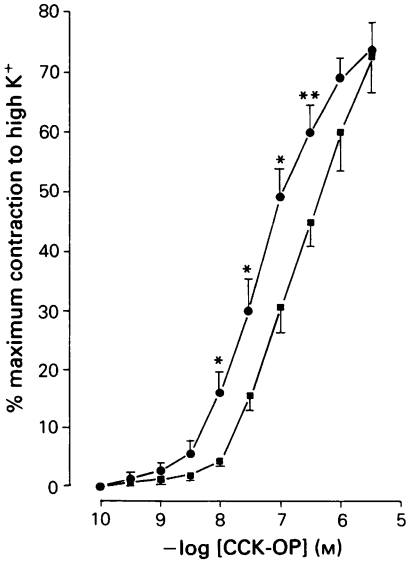

Figure 2 Responses of guinea-pig trachea to cholecystokinin-octapeptide (CCK-OP) (−E, +Indo) in the absence (■) and in the presence (●) of phosphoramidon (10^{-5} M). Points represent means, $n = 6$; vertical bars represent s.e.mean. $P < 0.05$ and $P < 0.01$ as compared to control values, by use of the Student's t test for paired data.

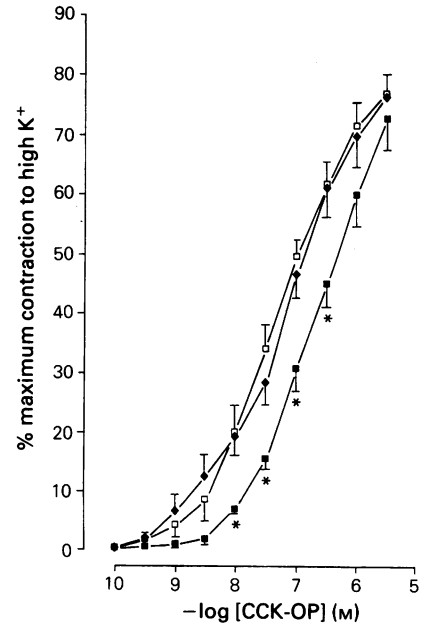

Figure 3 Effect of active sensitization to ovalbumin (OA) on cholecystokinin-octapeptide (CCK-OP)-induced responses in guinea-pig trachea: (■) +E +Indo; (□) −E +Indo from control (sham-sensitized) animals and (◆) +E +Indo from OA-treated animals. All points represent means, $n = 7$; vertical bars are s.e.mean. $P < 0.05$ as compared to control tissues (■), by use of the Student's t test for unpaired data.

Effect of cholecystokinin antagonists

db Cyclic GMP (10^{-4}–10^{-3} M) gave a parallel, rightward displacement in the concentration-response relationship to CCK-OP which was sufficient to allow an estimation of a pA_2 value from the Schild equation of approximately 4.3, (Figure 4). db Cyclic GMP did not alter the contractile response to exogenously applied acetylcholine.

The peripheral CCK-receptor antagonist, L-364, 718 (10^{-10}–10^{-8} M), produced a similar displacement to that produced by db cyclic GMP, but it was not possible to determine its full potency as a maximal contractile effect to CCK-OP in the presence of the antagonist was not achieved. The Schild equation

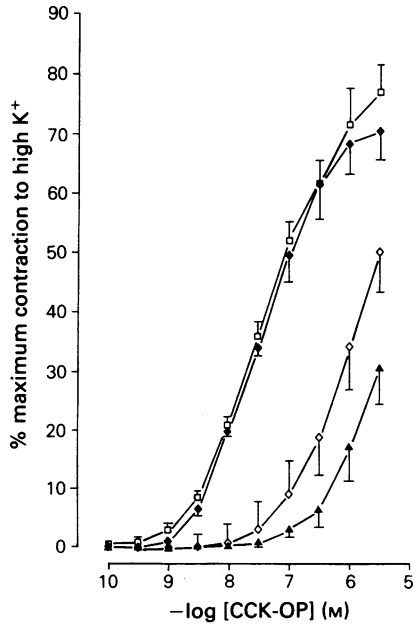

Figure 4 Responses of guinea-pig trachea to cholecystokinin-octapeptide (CCK-OP) (−E, +Indo) in the absence of (□) and in the presence of (◆) 10^{-4} M, (◇) 3×10^{-4} M and (▲) 10^{-3} M db cyclic GMP. Points represent means, $n = 6$; vertical bars represent s.e.mean.

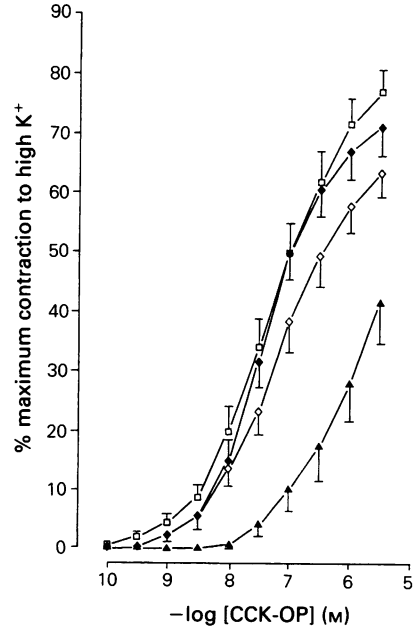

Figure 5 Responses of guinea-pig trachea to cholecystokinin-octapeptide (CCK-OP) (−E, +Indo) in the absence of (□) and in the presence of (◆) 10^{-10} M, (◇) 10^{-9} M and (▲) 10^{-8} M L-364,718. Points represent means, $n = 6$; vertical bars are s.e.mean.

gave an estimation of the pA_2 value of approximately 9.6. Control experiments for L-364,718 carried out in the presence of DMSO at a concentration corresponding to that used for 10^{-8} M L-364,718 were not significantly different from the corresponding concentration-response relationships to CCK-OP as compared with corresponding relationships constructed in the absence of DMSO (Figure 5). L-364,718 also antagonised the response to CCK-OP obtained in distal lobar bronchus, but it was not possible to calculate a value for the pA_2.

Effect of cholecystokinin-octapeptide on human airways

CCK-OP (10^{-7}–10^{-4} M) produced a concentration-dependent contractile effect in both distal lobar and large segmental bronchi ($n = 7$) (Figure 6). The contractile responses were greater in magnitude in the larger airways, but the effect of CCK-OP was successfully and significantly antagonized by the peripheral, non-peptide CCK-receptor antagonist L-364, 718, with an estimated pA_2 value of 8.3.

Discussion

Classically, cholecystokinin has been associated with the gastrointestinal tract, where a family of gastrin- and CCK-like polypeptides have been shown to contribute to the regulation of gastrointestinal function and motility (Vizi *et al.*, 1973). The overriding influence of CCK on gut motility appears to be exerted by a direct action on the smooth muscle and also by an interaction with cholinergic mechanisms, such that CCK is able to evoke atropine-sensitive and -insensitive effects (Rakovska *et al.*, 1986; Takahashi *et al.*, 1986; Yamamura *et al.*, 1986). More recently, evidence obtained from bovine gallbladder muscularis has led to the suggestion of a functional excitatory CCK receptor (Schjoldager *et al.*, 1988).

In a number of systems, such as the cat lower oesophageal sphincter (Rattan & Goyal, 1983) and the guinea-pig gallbladder (Takahashi *et al.*, 1987), two different types of CCK-OP receptors have been defined as muscular (excitatory), db cyclic GMP-sensitive and neural (inhibitory), db cyclic GMP-insensitive receptors. Within the central nervous system, CCK receptor localization by autoradiography has revealed two distinct binding patterns,

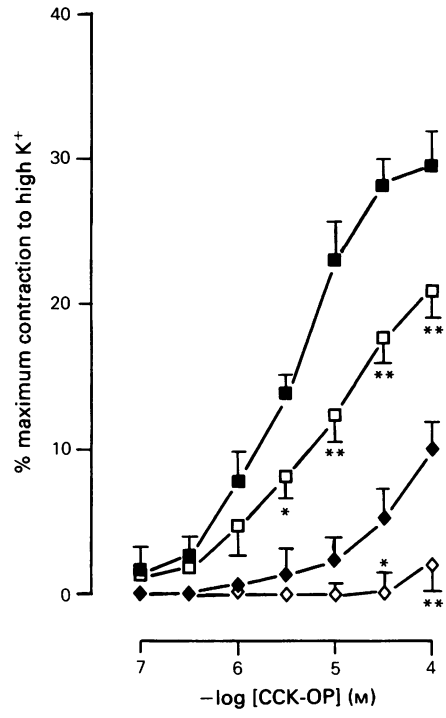

Figure 6 Contractile responses induced by cholecystokinin-octapeptide (CCK-OP) in distal lobar (■) and large segmental (◆) human bronchi and the effect of L-364,718 on these responses (□ and ◇, respectively). All points are means of airways from 7 patients; vertical bars represent s.e.mean. * $P < 0.05$ and ** $P < 0.01$ as compared to control values by use of Student's t test for unpaired data.

suggesting at least two different receptor populations, one of which exhibits binding characteristics similar to a peripheral CCK receptor (Moran *et al.*, 1986). In the guinea-pig trachea, CCK-OP gave a direct concentration-dependent contractile response which was antagonized by db cyclic GMP.

The localization of CCK within the respiratory tract has been extremely difficult to demonstrate, although CCK-LI has been detected in low concentrations in several species (Polak & Bloom, 1982). When present, the distribution of CCK-LI is greater in the trachea than in peripheral lung tissue. Within guinea-pig airways there are no detectable CCK-LI axons, except for occasional non-varicose axons in large nerve bundles running outside the wall of the trachea and bronchi. When such axons are present, however, they are also immunoreactive for substance P (Gibbins *et al.*, 1987).

In guinea-pig trachea, the CCK-OP-induced contraction was potentiated by the presence of indomethacin, suggesting that the effect of CCK may be influenced by inhibitory cyclo-oxygenase products such as prostaglandin E_2 (Orehek *et al.*, 1975; Gardiner & Collier, 1980). As with other contractile agents, the mechanical removal of the epithelial layer produced an enhancement of the contractile response of the guinea-pig trachea to CCK-OP (Barnes *et al.*, 1985; Flavahan *et al.*, 1985; Holroyde, 1986).

At sub-threshold concentrations of CCK-OP, which did not alter the resting tone of the tracheal smooth muscle, CCK-OP failed to alter the responses of the trachea to electrical field stimulation. Similarly, in the intact tracheal tube preparation, CCK-OP failed to alter the responses to vagal nerve stimulation. The presence of atropine also failed to alter significantly the concentration-response relationship to CCK-OP. These observations would suggest that, in the guinea-pig trachea, CCK-OP does not appear to interact with ganglionic transmission or with postganglionic cholinergic mechanisms.

Phosphoramidon produced a statistically significant, 6 fold leftward shift in the concentration-response relationship to CCK-OP in tissues with intact epithelium. However, in tissues denuded of epithelium, phosphoramidon had no significant effect. This suggests that neutral endopeptidase has the capacity to cleave CCK-OP and that this enzyme is located predominantly in the epithelium (Droschodt-Lanckman & Strosberg, 1983; Matsas *et al.*, 1984). The results with CCK-OP are similar to those reported with tachykinins, which are also cleaved by this enzyme (Lundblad & Persson, 1988). The responses obtained to CCK-OP in airways from sensitized and challenged animals with intact epithelium were significantly shifted in a leftward direction, suggesting that allergen exposure may result in a reduction in the functional integrity of the epithelium and/or the enzymes responsible for the degradation of the peptide.

The peripheral CCK receptor antagonist db cyclic GMP, which has allowed the differentiation of muscular (excitatory) and neural (inhibitory) CCK receptors (Rattan & Goyal, 1983; Takahashi *et al.*, 1987), produced a parallel, rightward displacement of the CCK-OP concentration-response relationship, providing an estimation of a pA_2 value of approximately 4. This is consistent with findings in gastrointestinal smooth muscle (Rakovska *et al.*, 1986). The non-peptide CCK receptor antagonist L-364,718 (Evans *et al.*, 1986; Lotti *et al.*, 1986; Hewson *et al.*, 1988) produced a similar parallel rightward shift in the CCK-OP-induced concentration-response relationship with a pA_2 of approximately 9. This is consistent with findings in guinea-pig ileum and colon (Chang & Lotti, 1986).

This non-peptide peripheral CCK receptor antagonist has a very high affinity and selectivity.

CCK-OP also produced a direct contractile response in human lobar bronchus and, to a lesser extent, in large segmental bronchi but failed to contract smaller airways. The contractile responses induced by CCK-OP in lobar and large segmental bronchi were also antagonized by L-364,718, with a similar pA_2 value to that found in guinea-pig trachea.

We conclude that in guinea-pig trachea, CCK-OP induces a direct contractile effect on airway smooth muscle. This effect is modulated by cyclo-oxygenase products and also by the functional integrity of the epithelial layer, possibly related to the presence of neutral endopeptidase in epithelial cells. In contrast to its actions in the gut, CCK-OP does not interact with cholinergic mechanisms. The effect of CCK-OP is completely antagonized by the peripheral CCK receptor antagonists db cyclic GMP and L-364,718, consistent with the presence of excitatory CCK receptors on the airway smooth muscle. CCK may, therefore, contribute to the regulation of bronchial tone, both in guinea-pig and, perhaps, in human airways. Although there may be only a few CCK containing neurones, these could be activated selectively and thus allow fine control of airway calibre via very specific pathways.

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Differential control and calcium-dependence of production of endothelium-derived relaxing factor and prostacyclin by pig aortic endothelial cells

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1 Production of endothelium-derived relaxing factor (EDRF) by primary cultures of pig aortic endothelial cells was assessed indirectly by measuring endothelial cyclic GMP content, and prostacyclin production was measured by radioimmunoassay of 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$).

2 The resting level of cyclic GMP fell significantly following removal of extracellular calcium (1 mM EGTA present), but elevations of cyclic GMP content induced by sodium azide (10 μM) or atriopeptin II (10 nM) were similar in the absence and presence of extracellular calcium.

3 Haemoglobin (10 μM) reduced the resting level of cyclic GMP in the presence, but not the absence of extracellular calcium. M&B 22,948 (100 μM), superoxide dismutase (30 u ml^{-1}), bradykinin (0.1 μM), ATP (10 μM) and ionophore A23187 (0.1 μM) each induced an increase in endothelial cyclic GMP content that was reduced in the absence of extracellular calcium.

4 In cascade bioassay experiments using endothelial cells on microcarrier beads and perfused in columns, continuous infusion of bradykinin (0.1 μM) induced release of EDRF, assayed on rabbit aortic rings, that was maximal after 2 min and still detectable up to about 16 min.

5 In the presence of extracellular calcium, the time course of bradykinin (0.1 μM)-stimulated production of EDRF, assessed as endothelial cyclic GMP content was maximal within 1 min, declined thereafter, but was still significant after 30 min. Production of 6-keto $PGF_{1\alpha}$, measured simultaneously, rose rapidly but was complete within 3 min.

6 In the absence of extracellular calcium the resting endothelial content of cyclic GMP fell, but resting production of 6-keto $PGF_{1\alpha}$ was unaffected. The rise in cyclic GMP content stimulated by bradykinin (0.1 μM) was lower than when calcium was present and was complete within 3 min, but the magnitude and time course of bradykinin (0.1 μM)-stimulated production of 6-keto $PGF_{1\alpha}$ was unaffected.

7 In the presence of TMB-8 (100 μM) resting endothelial content of cyclic GMP rose slightly, but production of 6-keto $PGF_{1\alpha}$ fell. The bradykinin (0.1 μM)-stimulated increase in cyclic GMP content was augmented, but the stimulation of 6-keto $PGF_{1\alpha}$ production was blocked. Results from cascade bioassay experiments confirmed that TMB-8 (100 μM) did not inhibit bradykinin-induced production of EDRF.

8 The data suggest that resting production of EDRF but not prostacyclin is dependent upon the presence of extracellular calcium. Bradykinin-stimulated production of EDRF is sustained and requires the presence of extracellular calcium, but stimulated production of prostacyclin is transient and may result from discharge of an intracellular pool of calcium.

9 The vascular endothelial cell appears therefore to control differentially production of EDRF and prostacyclin.

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Introduction

Endothelium-derived relaxing factor (EDRF), recently identified as nitric oxide (Palmer *et al.*, 1987; Ignarro *et al.*, 1987; Furchgott, 1988), and prostacyclin (Moncada *et al.*, 1976), are two powerful vasodilator and anti-aggregatory agents released by the vascular endothelium.

Release of EDRF and prostacyclin occurs spontaneously (Gordon & Martin, 1983; Griffith *et al.*, 1984; Martin *et al.*, 1985), or in response to chemical (Gimbrone & Alexander, 1975; Furchgott & Zawadzki, 1980) or physical stimuli (Grabowski *et al.*, 1985; Holtz *et al.*, 1984). It appears that calcium is involved in the release of both EDRF (Singer & Peach, 1982; Long & Stone, 1985; Griffith *et al.*, 1986) and prostacyclin (Weksler *et al.*, 1978; Seid *et al.*, 1983). Calcium is required for activation of phospholipase A_2 (Hong & Deykin, 1982) which liberates arachidonate from membrane phospholipid, but the calcium-dependent step in EDRF release has not been identified.

Differences in the mechanisms for production of EDRF and prostacyclin have been identified. For example, endothelium-dependent vasodilatation, resulting from agonist-induced stimulation, is well sustained (Furchgott & Zawadzki, 1980), but agonist-induced production of prostacyclin is transient, being complete within 1–3 min (Gordon & Martin, 1983; Pearson *et al.*, 1983). In addition, basal and stimulated production of EDRF both require the continuous presence of extracellular calcium (Long & Stone, 1985; Griffith *et al.*, 1986), while prostacyclin release can be elicited in its absence (Hallam & Needham, 1987). Studies with TMB-8, an inhibitor of intracellular calcium release suggest that prostacyclin production arises mainly as a consequence of mobilisation of intracellular calcium (Seid *et al.*, 1983).

We now present results of experiments in which the extracellular requirement for calcium and sensitivity to TMB-8 of EDRF release and prostacyclin production are compared.

Methods

Endothelial cell culture

Pig aortic endothelial cells were isolated and grown in culture as previously described (Martin *et al.*, 1988).

For monolayer studies the cells were seeded into Linbro plates each containing 6 wells (9.6 cm^2) and grown to confluence in an incubator at 37°C under an atmosphere of 5% CO_2 in air.

For microcarrier experiments cells were seeded onto 3 ml of Biosilon microcarrier beads (Nunc, 200 μm diameter) in a sterile siliconised Techne microcarrier flask, and grown at 37°C under an atmosphere of 5% CO_2 in air. The beads were stirred at 30 r.p.m. for 2.5 min every 30 min for 3–5 days, during which time the cells grew to confluence, which was confirmed by microscopic examination after staining a sample of the cells with Methyl Violet (0.1%, B.D.H.).

Measurement of cyclic GMP and prostacyclin

Before experimentation the tissue culture medium was removed by aspiration and the cells washed with 2 × 2 ml of Krebs solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11 and EDTA 0.03, and incubated in 2 ml of Krebs solution at 37°C under an atmosphere of 5% CO_2 in air for at least 90 min. After this time the Krebs solution was removed and replaced either with normal Krebs solution or Krebs in which the calcium had been omitted and to which ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetra acetic acid (EGTA, 1 mM) had been added. Drugs were added to the cells at the concentrations and times indicated in the Results. At the appropriate time the Krebs solution was removed and retained for analysis of prostacyclin, and the cells immediately extracted in 0.5 ml of ice-cold trichloroacetic acid (TCA, 6%), scraped off the multiwell plates and harvested. Cells remaining on the plates were recovered by extracting with a second 0.5 ml of TCA and this was combined with the first. The extracts were spun (13,000 r.p.m., 3 min, room temperature) and the pellet and supernatant separated. The DNA content of the pellet was measured by the fluorescence technique of Kissane & Robins (1958). The supernatant was neutralised and the guanosine 3' : 5'-cyclic monophosphate (cyclic GMP) content measured by radioimmunoassay as previously described (Martin *et al.*, 1985). Cyclic GMP content is expressed as fmol μg^{-1} DNA.

The prostacyclin content of the Krebs solution was determined by radioimmunoassay (measured as 6-keto prostaglandin $F_{1\alpha}$) using an antiserum kindly supplied by Dr A.C. Newby. The cross-reactivity of the antiserum at 50% displacement with prostaglandin E_2 (PGE_2), PGE_1, and $PGF_{2\alpha}$ was 5%, 1.3%, and 1.4%, respectively. Prostacyclin production is expressed as pg 6-keto $PGF_{1\alpha}$ μg^{-1} DNA.

Cascade bioassay

EDRF release from pig aortic endothelial cells grown on microcarrier beads and perfused in

Table 1 Effects of sodium azide and atriopeptin II on the cyclic GMP content of pig aortic endothelial cells in the presence and absence of extracellular calcium

Stimulus	*Ca^{2+} content of Krebs* (mM)	*Cyclic GMP* (fmol μg^{-1} DNA)	n
None (control)	2.5	14.2 ± 4.0	6
None	None	4.8 ± 0.8*	6
Azide	2.5	57.2 ± 13.3*	6
Azide	None	44.0 ± 8.8*	6
None (control)	2.5	15.0 ± 1.6	6
None	None	3.9 ± 0.5***	6
APII	2.5	35.0 ± 3.3***	6
APII	None	34.2 ± 4.3**	6

The cyclic GMP content of pig aortic endothelial cells was determined following exposure to sodium azide (10 μM) for 1.5 min or atriopeptin II (APII, 10 nM) for 3 min in the presence or absence (1 mM EGTA added) of 2.5 mM calcium. Results are expressed as the mean ± s.e.mean. *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$, denotes significant difference from control.

Table 2 Effects of haemoglobin, M&B 22,948 and superoxide dismutase on the cyclic GMP content of pig aortic endothelial cells in the presence and absence of extracellular calcium

Stimulus	*Ca^{2+} content of Krebs* (mM)	*Cyclic GMP* (fmol μg^{-1} DNA)	n
None (control)	2.5	17.8 ± 1.6	9
None	None	4.5 ± 0.4***	10
Hb	2.5	3.5 ± 0.5***	6
Hb	None	3.3 ± 0.5***	6
None (control)	2.5	15.4 ± 1.6	6
None	None	3.9 ± 0.5***	6
M&B	2.5	29.2 ± 4.7*	6
M&B	None	5.2 ± 0.08***	6
None (control)	2.5	25.1 ± 4.4	6
None	None	3.7 ± 1.0***	6
SOD	2.5	69.8 ± 14.8*	6
SOD	None	11.0 ± 1.9*	6

The cyclic GMP content of pig aortic endothelial cells was determined following exposure to haemoglobin (Hb, 10 μM) for 20 min, or to M&B 22,948 (M&B, 100 μM) or superoxide dismutase (SOD, 30 u ml^{-1}) for 3 min in the presence or absence (1 mM EGTA added) of 2.5 mM calcium. Results are expressed as the mean ± s.e.mean. *$P < 0.05$; ***$P < 0.001$, denotes significant difference from control.

columns was detected by bioassay using an endothelium-denuded ring of rabbit aorta. The preparation of endothelial cell columns was as previously described (Gordon & Martin, 1983). The perfusate, which contained flurbiprofen (10 μM, to inhibit cyclooxygenase) and superoxide dismutase (30 u ml^{-1}, to potentiate the actions of EDRF), was passed over an endothelium-denuded ring of rabbit aorta that had been suspended under 2 g resting tension and contracted sub-maximally with phenylephrine (0.1–10 μM). Tension was recorded isometrically by Ormed UF1 transducers and displayed on a Lectromed recorder.

Drugs

Adenosine triphosphate (ATP), atriopeptin II (rat synthetic), sodium azide, bradykinin triacetate, haemoglobin (bovine Type 1), ionophore A23187, phenylephrine, superoxide dismutase (bovine erythrocyte) and trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8) were obtained from Sigma. Sodium flurbiprofen was a generous gift from Dr R.V. Holland, Boots Pure Drug Co. and M&B 22,948 (2-O-propoxyphenyl-8-azapurin-6-one) was a generous gift from Dr J.E. Souness, May & Baker Ltd. All drugs were dissolved in twice-distilled water except for A23187 which was dissolved in ethanol, TMB-8 which was dissolved in 1% ethanol and M&B 22,948 which was dissolved in 10% triethanolamine.

Solutions of haemoglobin were reduced to the ferrous form with dithionite before use, as previously described (Martin *et al.*, 1985).

Statistical analysis

The resting content of cyclic GMP and basal production of prostacyclin varied in batches of cells. The validity of statistical comparisons was therefore enhanced by performing each experiment with its own internal controls. In the results, *n* represents the number of replicate dishes of cells used in experiments. Results are expressed as the mean ± s.e.mean and comparisons were made by means of Student's *t* test. A probability of 0.05 or less was considered significant.

Results

Calcium removal and cyclic GMP content

When incubated in normal Krebs solution (calcium 2.5 mM), the resting level of cyclic GMP in primary cultures of pig aortic endothelial cells was 20.9 ± 2.0 fmol μg^{-1} DNA ($n = 59$). Following 20 min incubation in nominally calcium-free Krebs

Table 3 Effects of bradykinin, ATP and ionophore A23187 on the cyclic GMP content of pig aortic endothelial cells in the presence and absence of extracellular calcium

Stimulus	*Ca^{2+} content of Krebs* (mM)	*Cyclic GMP* (fmol μg^{-1} DNA)	n
None (control)	2.5	9.1 ± 1.4	7
None	None	6.9 ± 2.3	5
Bk	2.5	86.4 ± 13.2***	8
Bk	None	24.2 ± 5.7*	6
None (control)	2.5	12.3 ± 1.9	6
None	None	9.8 ± 2.6	6
ATP	2.5	41.0 ± 6.5**	6
ATP	None	19.8 ± 2.5*	6
None (control)	2.5	31.6 ± 8.3	6
None	None	4.2 ± 0.9*	6
A23187	2.5	288.2 ± 23.2***	5
A23187	None	106.4 ± 17.8*	5

The cyclic GMP content of pig aortic endothelial cells was determined following exposure to bradykinin (Bk, 0.1 μM) for 1.5 min or to ATP (10 μM) or ionophore A23187 (0.1 μM) for 3 min in the presence or absence (1 mM EGTA added) of 2.5 mM calcium. Results are expressed as the mean ± s.e. mean. $*P < 0.05$; $**P < 0.005$; $***P < 0.001$, denotes significant difference from control.

(EGTA 1 mM present) the resting level of cyclic GMP was significantly lower ($P < 0.001$) at 7.1 ± 1.0 fmol μg^{-1} DNA ($n = 46$).

Sodium azide (10 μM, 1.5 min) and atriopeptin II (10 nM, 3 min) each elevated the cyclic GMP content to levels that were similar in the presence and absence of extracellular calcium (Table 1).

Haemoglobin, M&B 22,948 and superoxide dismutase

Treating endothelial cells for 20 min with the EDRF blocking agent, haemoglobin (10 μM), in the presence but not the absence of extracellular calcium, led to a reduction in cyclic GMP content (Table 2). The cyclic GMP content of endothelial cells obtained in the absence of calcium was not significantly different from that obtained following haemoglobin (10 μM) treatment in the presence of calcium (Table 2).

Treating endothelial cells for 3 min with either M&B 22,948 (100 μM) or superoxide dismutase (30 u ml^{-1}), two agents known to potentiate the actions of EDRF, induced rises in cyclic GMP content that were inhibited in the absence of extracellular calcium (Table 2).

Bradykinin, ATP and ionophore A23187

Three agents known to elicit EDRF production, bradykinin (0.1 μM, 1.5 min), ATP (10 μM, 3 min) and ionophore A23187 (0.1 μM, 3 min), induced rises in endothelial cyclic GMP content that were inhibited in the absence of extracellular calcium (Table 3).

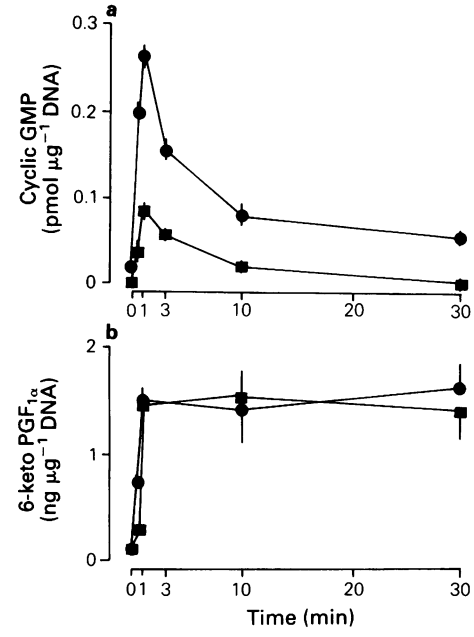

Figure 1 Time courses showing the simultaneous effects of bradykinin (0.1 μM) on endothelial cyclic GMP content (a) and 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) production (b) measured in the absence (●) and presence (■) of haemoglobin (10 μM). Results are presented as the mean of 6 observations with s.e.mean shown by vertical lines.

Effects of haemoglobin on cyclic GMP accumulation and prostacyclin production

In the presence of calcium (2.5 mM), the resting content of cyclic GMP in endothelial cells was 20·7 ± 3.8 fmol μg^{-1} DNA ($n = 6$) and the resting production of 6-keto $PGF_{1\alpha}$, measured over 40 min, was 126 ± 21 pg μg^{-1} DNA ($n = 5$). Bradykinin (0.1 μM) induced a maximum increase in cyclic GMP within 1 min, which declined thereafter but even at 10 min and 30 min was still significant (Figure 1). Production of 6-keto $PGF_{1\alpha}$, measured simultaneously, also rose rapidly but this was complete within 1 min (Figure 1).

When the EDRF-blocking agent, haemoglobin (10 μM), was present during the 40 min incubation period, the resting cyclic GMP content of the endothelial cells fell significantly, but resting 6-keto $PGF_{1\alpha}$ production was unaffected. Bradykinin (0.1 μM) induced a significantly smaller rise in cyclic GMP content in the presence of haemoglobin

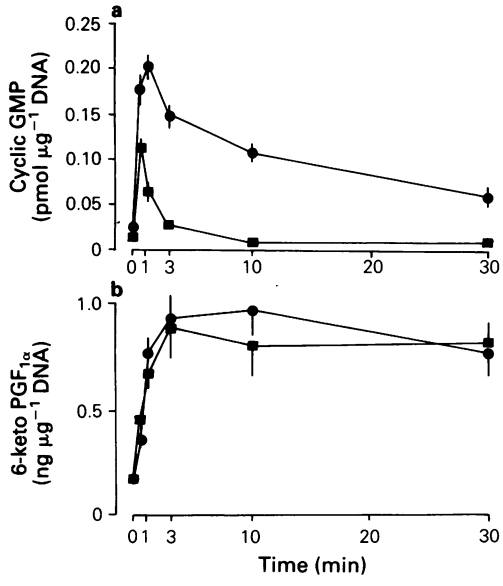

Figure 2 Time courses showing the simultaneous effects of bradykinin (0.1 μM) on endothelial cyclic GMP content (a) and 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) production (b) measured in the presence (●) and absence (1 mM EGTA added, ■) of 2.5 mM extracellular calcium. Results are presented as the mean of 12–31 observations with s.e.mean shown by vertical lines.

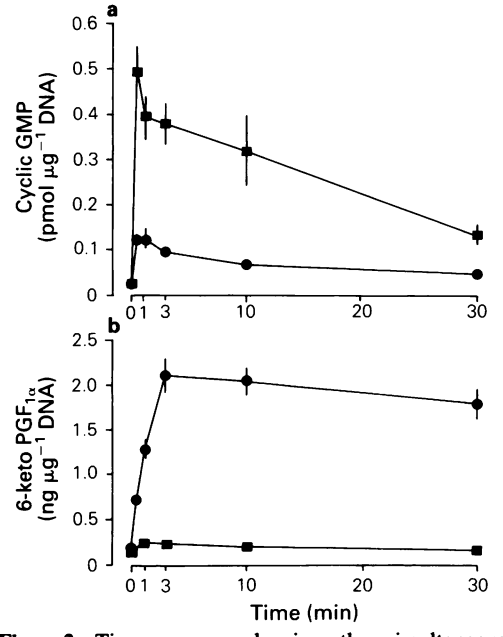

Figure 3 Time courses showing the simultaneous effects of bradykinin (0.1 μM) on endothelial cyclic GMP content (a) and 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) production (b) measured in the absence (●) and presence (■) of trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8, 100 μM). Results are presented as the mean of 17–24 observations with s.e.mean shown by vertical lines.

(10 μM), but the stimulation of 6-keto $PGF_{1\alpha}$ production was unaffected (Figure 1).

Calcium-dependence of cyclic GMP accumulation and prostacyclin production

In the absence of calcium the resting content of cyclic GMP fell significantly, but resting 6-keto $PGF_{1\alpha}$ production was unaffected (Figure 2). Bradykinin (0.1 μM) induced a rise in cyclic GMP content that was significantly smaller in magnitude than that obtained in the presence of calcium (2.5 mM) and was complete within 3 min, but stimulated production of 6-keto $PGF_{1\alpha}$ was unaffected (Figure 2).

Effects of TMB-8 on cyclic GMP accumulation and prostacyclin production

When the inhibitor of intracellular calcium release, TMB-8 (100 μM), was present during the 40 min incubation period the resting cyclic GMP content rose slightly and production of 6-keto $PGF_{1\alpha}$ fell (Figure 3). Bradykinin (0.1 μM) induced a significantly greater rise in cyclic GMP content in the presence of TMB-8 (100 μM), but the stimulation of 6-keto $PGF_{1\alpha}$ production was significantly smaller (Figure 3).

Cascade bioassay

In cascade bioassay experiments infusion of bradykinin (0.1 nM–0.1 μM) into columns of endothelial cells for 3 min periods induced concentration-dependent relaxations of rabbit aortic rings (Figure 4). In the presence of haemoglobin (10 μM) or methylene blue (20 μM) dilator responses were completely abolished (data not shown).

Continuous infusion of bradykinin (0.1 μM) induced a $71 \pm 8\%$ ($n = 5$) relaxation that was maximal after 2.0 ± 0.2 min and complete after 16.0 ± 0.3 min ($n = 5$) (Figure 5).

When TMB-8 (100 μM) was infused continuously into endothelial cell columns a $73 \pm 3\%$ ($n = 8$) relaxation of phenylephrine-induced tone was observed in rabbit aortic rings. When tone was raised to its original level by increasing the concentration of phenylephrine, infusion of bradykinin (0.1 nM–0.1 μM) induced concentration-dependent relaxations that were similar to those obtained in the absence of TMB-8 (Figure 4).

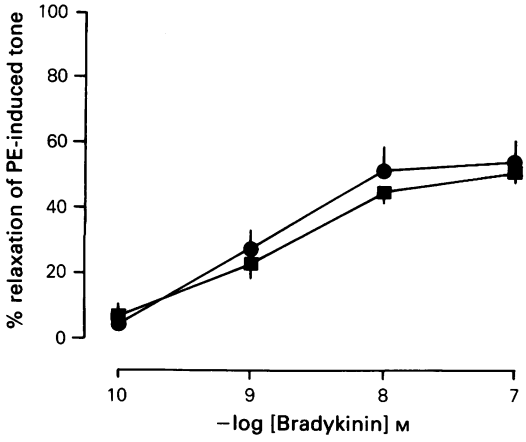

Figure 4 Log concentration-response curves obtained in cascade bioassay experiments showing the relaxation of phenylephrine (PE)-contracted, endothelium-denuded rings of rabbit aorta obtained when bradykinin (0.1 nM–0.1 μM) was infused for 3 min periods into columns of pig aortic endothelial cells on microcarrier beads in the absence (●) and presence (■) of trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8, 100 μM). Results are presented as the mean of 8 observations with s.e.mean shown by vertical lines.

Discussion

EDRF induces vasodilatation by elevating smooth muscle cyclic GMP content (Rapoport & Murad, 1983). Moreover, agents which evoke EDRF release elevate endothelial cyclic GMP content (Adams Brotherton, 1986; Martin *et al.*, 1988). These elevations are inhibited by haemoglobin or methylene blue and potentiated by M&B 22,948 or superoxide dismutase (Martin *et al.*, 1988), which indicates that they are produced by the action of EDRF on endothelial soluble guanylate cyclase. The ability of haemoglobin to lower the resting level of cyclic GMP in endothelial cells suggests that, even in the resting state basal production of EDRF is sufficient to activate endothelial soluble guanylate cyclase. Thus, the measurement of endothelial cyclic GMP content provides an indirect, but sensitive means of assessing EDRF production.

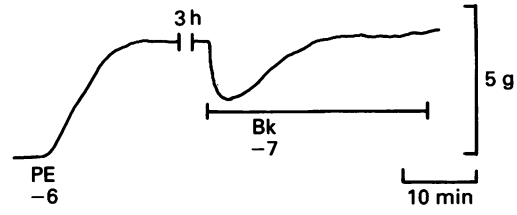

Figure 5 A typical trace obtained in a cascade bioassay experiment showing relaxation of a phenylephrine (PE)-contracted, endothelium-denuded ring of rabbit aorta obtained when bradykinin (Bk) was infused constantly for 30 min into a column of pig aortic endothelial cells on microcarrier beads. Molar concentrations are expressed in log units.

Many studies have shown that basal and agonist-stimulated production of EDRF are dependent on the presence of extracellular calcium (Singer & Peach, 1982; Long & Stone, 1985; Miller *et al.*, 1985; Griffith *et al.*, 1986; Luckhoff *et al.*, 1988). We found that calcium removal lowers resting levels of cyclic GMP in pig aortic endothelial cells to the same extent as the EDRF blocking agent, haemoglobin (Martin *et al.*, 1985). Furthermore, calcium removal prevents the rises in cyclic GMP induced by the EDRF potentiating agents, M&B 22,948 and superoxide dismutase (Martin *et al.*, 1986; Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986), as well as those induced by the EDRF releasing agents, bradykinin, ATP, and ionophore A23187. Thus, measuring endothelial cyclic GMP content has provided additional evidence to support the conclusion that basal and stimulated release of EDRF requires extracellular calcium.

It could be argued that removal of calcium did not prevent release of EDRF, but inhibited the subsequent stimulation of soluble guanylate cyclase. This is unlikely since sodium azide, a stimulant of soluble guanylate cyclase, and atriopeptin II, a receptor-mediated stimulant of particulate guanylate cyclase, induced rises in endothelial cyclic GMP content that were similar in the absence and presence of extracellular calcium.

In cascade bioassay experiments with endothelial cells on microcarrier beads, continuous infusion of bradykinin induced release of EDRF that was maximal at about 2 min and detectable for up to 16 min. EDRF production, assessed by measuring endothelial cyclic GMP content followed a similar time course: stimulation of production was maximal at 1 min and was still detectable after 30 min. Bradykinin-stimulated production of prostacyclin, measured simultaneously, also rose rapidly but, in contrast to the prolonged production of EDRF, was complete within 1–3 min, as demonstrated previously (Gordon & Martin, 1983; Pearson *et al.*, 1983). Removal of extracellular calcium differentially affected production of EDRF and prostacyclin: resting levels of cyclic GMP fell consistent with inhibition of basal EDRF production, but resting production of prostacyclin was unaffected. Furthermore, following treatment with bradykinin, stimulation of EDRF production, assessed as endothelial cyclic GMP content, still increased rapidly but reached a lower maximum and was less well sustained than that obtained in the presence of calcium, being complete within 3 min. The lower sensitivity of cascade

bioassay techniques probably explains why this transient production of EDRF in the absence of extracellular calcium has not been previously observed (Long & Stone, 1985; Griffith *et al.*, 1986; Luckhoff *et al.*, 1988). The magnitude and time course of bradykinin-stimulated production of prostacyclin was, however, similar to that obtained in the presence of calcium (2.5 mM). Thus, basal production of EDRF but not prostacyclin requires the presence of extracellular calcium. Also, bradykinin-stimulated production of EDRF is well sustained and requires the presence of extracellular calcium, but stimulated production of prostacyclin is transient and independent of the presence of extracellular calcium.

The inability of extracellular calcium removal to inhibit bradykinin-stimulated production of prostacyclin is consistent with the proposal that production is dependent mainly on release of an intracellular calcium pool (Seid *et al.*, 1983; Hallam & Needham, 1987). Direct measurement of intracellular calcium levels in endothelial cells using fura 2 or quin 2 supports this proposal (Hallam & Needham, 1987; Colden-Stanfield *et al.*, 1987). The transient rise in endothelial cyclic GMP levels induced by bradykinin in the absence of extracellular calcium may, likewise, reflect EDRF production triggered after the discharge of the intracellular pool of calcium.

If agonist-induced production of prostacyclin is controlled mainly by intracellular calcium release and production of EDRF is controlled by calcium influx, then prostacyclin production but not sustained EDRF production should be blocked by inhibitors of intracellular calcium release. Using TMB-8, we found that both basal and bradykinin-stimulated production of prostacyclin was inhibited, thus confirming previous data (Seid *et al.*, 1983). Basal and bradykinin-induced EDRF production, assessed as endothelial cyclic GMP content, were not inhibited, in fact the effect of bradykinin appeared to be potentiated. Independent confirmation that TMB-8 did not inhibit EDRF production was obtained in cascade bioassay experiments with endothelial cells on microcarrier beads. Whether TMB-8 actually increases agonist-induced production of EDRF, as suggested by experiments in which endothelial cyclic GMP was measured, warrants further investigation.

In conclusion, our data show that agonist-induced production of prostacyclin is transient but production of EDRF is more sustained. They further suggest that prostacyclin production is triggered by discharge of an intracellular store of calcium and EDRF production maintained by influx of extracellular calcium. Thus the vascular endothelial cell appears to exert differential control of production of these two powerful vasoactive agents by utilizing distinct calcium pools.

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Subclassification of α_1-adrenoceptor recognition sites by urapidil derivatives and other selective antagonists

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1 The affinities of urapidil derivatives and other antagonists for α_1-adrenoceptors labelled by [^{3}H]-prazosin were determined on membranes of six different rat tissues.

2 Urapidil and its 5-acetyl-, 5-formyl- and 5-methyl-derivative displaced [^{3}H]-prazosin from α_1-adrenoceptor binding sites in a concentration-dependent manner which varied with tissue. IC_{50} values were lower in vas deferens, hippocampus and cerebral cortex than in heart, liver and spleen. For 5-methyl-urapidil, binding to two distinct sites could be demonstrated with mean K_I values of about 0.6 and 45 nM. Saturation binding studies with [^{3}H]-prazosin in the presence of 5-methyl-urapidil indicated a competitive type of interaction between 5-methyl-urapidil and [^{3}H]-prazosin.

3 The proportion of [^{3}H]-prazosin binding sites with high affinity for 5-methyl-urapidil was 58% in vas deferens, 69% in hippocampus, 41% in cerebral cortex and 23% in myocardium. In liver and spleen virtually no high affinity sites were found. These values were in good agreement with the percentages of binding sites with high affinities for WB-4101 and phentolamine, indicating that all these antagonists bind to the same subtype of α_1-recognition sites, whereas other α-antagonists like BE 2254, yohimbine and unlabelled prazosin did not discriminate between two binding sites.

4 Preincubating membranes of the cerebral cortex with chloroethylclonidine preferentially inactivated [^{3}H]-prazosin binding sites with low affinity for 5-methyl-urapidil.

5 The antagonist potencies of 5-methyl-urapidil and WB-4101 against α_1-adrenoceptor-mediated contractile responses were higher in vas deferens than in myocardium. The α_1-mediated effects in vas deferens but not in the heart were highly susceptible to nitrendipine.

6 Using 5-methyl-urapidil, the existence of two distinct α_1-adrenoceptor recognition sites could be demonstrated which correspond to the proposed α_{1A}- and α_{1B}-subtypes. Since 5-methyl-urapidil is one of the ligands with most selectivity between these subtypes in binding studies it may serve as a valuable tool for such investigations.

Introduction

Urapidil and some of its derivatives which are substituted at the 5-position of the uracil moiety (5-acetyl-, 5-formyl-, 5-methyl-urapidil, see Figure 1) lower blood pressure by a central nervous mechanism as well as by a peripheral action (Sanders & Jurna, 1985; Kolassa *et al.*, 1986; Ramage, 1986; 1988; Fozard & Mir, 1987; Gillis *et al.*, 1987; Doods *et al.*, 1988). The high affinity for 5-hydroxytryptamine (5-HT) receptors of the 5-HT_{1A} subtype and the intrinsic activity at these receptors seems to be responsible for the central component of the antihypertensive action whereas α_1-adrenolytic properties contribute to the vasodilator effect in the periphery (van Zwieten *et al.*, 1985; Ramage, 1986; 1988; Gillis *et al.*, 1987; Gross *et al.*, 1987).

In a preliminary paper, we were able to demonstrate that 5-methyl-urapidil inhibited [^{3}H]-prazosin binding to α_1-adrenoceptors of various rat tissues with two distinct affinity constants (Gross *et al.*, 1988a). Although there is increasing evidence from functional as well as from radioligand binding studies that α_1-adrenoceptors may be divided into subtypes this issue remains highly controversial (for reviews see McGrath, 1982; Hieble *et al.*, 1986; 1987; Morrow & Creese, 1986; McGrath & Wilson, 1988). Since the decision whether α_1-adrenoceptors are a homogeneous group of receptors or not is critically dependent on the availability of subtype-selective

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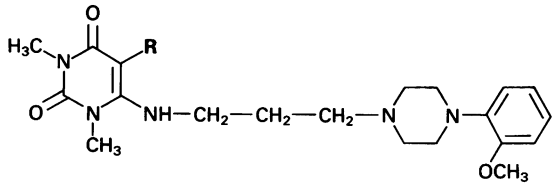

R	
—H	Urapidil
—CH_3	5-Methyl-urapidil
—C(=O)—H	5-Formyl-urapidil
—C(=O)—CH_3	5-Acetyl-urapidil

Figure 1 Chemical structures of urapidil and its derivatives substituted at the 5-position of the uracil moiety.

antagonists, we investigated whether 5-methyl-urapidil and other urapidil derivatives (Figure 1) discriminate between distinct α_1-adrenoceptor recognition sites and/or receptors and whether these sites can be reconciled with subdivisions of α_1-adrenoceptors proposed previously.

Methods

Male adult Wistar rats weighing 250 to 400 g were used for all experiments. Organs were removed immediately after the rats had been killed and used for radioligand binding and functional studies.

Radioligand binding

Hearts were perfused through the aorta with ice-cold saline and atria were cut off. After connective tissue had been removed the organs were blotted, weighed and homogenized twice for 15 s with an Ultra Turrax in 10 ml of ice-cold buffer (Tris HCl 50 mM, NaCl 100 mM, EDTA 2 mM, pH 7.4). The homogenates were filtered through 4 layers of gauze and centrifuged at 80,000 *g* for 20 min at 4°C. The pellets were resuspended in 8 ml of fresh buffer (Tris HCl 50 mM, EDTA 1 mM, pH 7.4), incubated for 10 min at 37°C, centrifuged again as described above and washed once more with 8 ml buffer.

[^{3}H]-prazosin binding was performed as described previously (Gross & Lues, 1985) with minor modifications. Membranes corresponding to 1.7 to 10 mg of initial wet weight, depending on the density of [^{3}H]-prazosin binding sites in the respective tissue, were incubated with 0.05 to 0.75 nM (saturation experiments) or 0.2 nM (competition experiments) [^{3}H]-prazosin in a final volume of 2 and 1 ml, respectively. The incubation buffer consisted of Tris HCl 50 mM, EDTA 1 mM, pH 7.4 as final concentrations. Incubations were carried out in duplicate or triplicate at 30°C and terminated after 45 min or 2 h (saturation experiments with 5-methyl-urapidil) by rapid filtration through Whatman GF/C filters using a Brandel M24R cell harvester. The filters were washed with 15 ml ice-cold buffer (Tris HCl 50 mM, pH 7.4) and subsequently dried at 100°C. Membrane-bound radioactivity retained on the filters was measured by liquid scintillation counting in a toluene/Triton x 100 mixture with an efficiency of 49%. Phentolamine (10 μM) was used to define non-specific binding which was usually 15% at 0.2 nM [^{3}H]-prazosin. The protein content of membrane suspensions was determined by the method of Lowry *et al.* (1951).

α_1-Adrenoceptor-mediated functional responses

The α_1-adrenoceptor-mediated inotropic response of the myocardium was studied as described previously (Gross *et al.*, 1988b). Strips of the right ventricle were tied to a tissue holder and mounted in an organ bath containing 20 ml of a modified Krebs-Henseleit solution saturated with 95%O_2:5%CO_2 (30°C); the solution had the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 10. Propranolol (10 μM) was used to block completely any β-adrenoceptor-mediated response, oxaprotiline (0.3 μM) and corticosterone (40 μM) were added to inhibit neuronal and extraneuronal uptake, respectively. The preparations were stimulated electrically via platinum electrodes (1 Hz, 3 ms, amplitude 20% above threshold voltage). Contractions were registered with an isometric transducer (preload 9.8 mN) and recorded with a Hellige amplifier and recorder.

Contractions of vas deferens were measured under the same conditions as described above for the positive inotropic response of heart ventricles except that the contractions were recorded without electrical stimulation; the resting tension was 4.9 mN.

Cumulative concentration-response curves in the heart and single concentration-response curves in vas deferens were constructed after 40 min of equilibration. 5-Methyl-urapidil and WB-4101 were added 30 min before the agonist.

Calculations

All values are expressed as mean ± s.e.mean of *n* independent experiments. IC_{50} values and pseudo Hill coefficients (n_H) were calculated as described by

McPherson (1983). Two-site analysis of the data was performed as described by Barlow (1983), F-test analysis was used to decide whether a one- or a two-site model was more appropriate ($P < 0.05$). IC_{50} values were transformed into K_I values by the method of Cheng & Prusoff (1973). The significance of difference between two means was assessed by use of a two-tailed unpaired Student's t test. ANOVA and Duncan's multiple range test were used for the comparison of more than two means. pA_2-values were calculated by linear regression analysis according to the method of Arunlakshana & Schild (1959).

Drugs

The following drugs were used: [^{3}H]-prazosin (specific activity 82 Ci mmol^{-1}, NEN, Boston, U.S.A.), 5-methyl-, 5-acetyl- and 5-formyl-urapidil (synthesized by Dr W. Prüsse, Konstanz, F.R.G.), (−)-adrenaline base (Hoechst, Frankfurt, F.R.G.), BE 2254 (2[β-(4-hydroxyphenyl)-ethyl-aminomethyl]tetralone hydrochloride, Beiersdorf, Hamburg, F.R.G.), chloroethylclonidine (Research Biochemicals Inc., Natick, U.S.A.), corticosterone (Sigma, Munich, F.R.G.), nitrendipine (Bayer, Leverkusen, F.R.G.), (+)-oxaprotiline hydrochloride (Ciba-Geigy, Basle, Switzerland), phentolamine hydrochloride (Ciba-Geigy, Basle, Switzerland), prazosin hydrochloride (Pfizer, Karlsruhe, F.R.G.), (±)-propranolol hydrochloride (Sigma, Munich, F.R.G.), urapidil (Byk Gulden Pharmazeutika, Konstanz, F.R.G.), WB-4101 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4 benzodioxane hydrochloride. (Research Biochemicals Inc., Natick, U.S.A.), yohimbine hydrochloride (Sigma, Munich, F.R.G.).

Results

Radioligand binding studies

[^{3}H]-prazosin in concentrations ranging from 0.01 to 0.75 nM was used to label α_1-adrenoceptors in crude particulate membrane preparations of six rat tissues. Binding of [^{3}H]-prazosin to 5-hydroxytryptamine receptors could be excluded, since high concentrations of 5-hydroxytryptamine and 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino) tetraline, up to 0.3 μM) did not displace [^{3}H]-prazosin (data not shown). Scatchard transformation of saturation binding data resulted in linear plots and pseudo Hill coefficients (n_H) not significantly different from unity ($P > 0.05$) suggesting a single population of binding sites in all tissues investigated (Table 1, see also Figure 4). However, equilibrium dissociation constants (expressed as pK_D, −log M) differed slightly between the tissues. In liver, spleen and heart

Table 1 Binding of [^{3}H]-prazosin to α_1-adrenoceptors of various rat tissues

	n	pK_D	n_H	B_{max}
Cerebral cortex	3	10.4 ± 0.03	1.09	11.4 ± 0.3
Heart	3	10.6 ± 0.03	1.05	5.9 ± 0.3
Hippocampus	4	10.2 ± 0.05	1.08	5.6 ± 0.1
Liver	5	10.7 ± 0.04	1.13	11.5 ± 0.6
Spleen	3	10.6 ± 0.12	0.96	2.7 ± 0.1
Vas deferens	3	10.2 ± 0.03	1.07	8.4 ± 0.9

B_{max} (maximal number of binding sites in fmol per mg initial wet weight) and pK_D values (−log equilibrium dissociation constants, −log M) were calculated by linear regression analysis after transformation of the data according to Scatchard (1949). n_H = Hill coefficient. Values are means ± s.e.mean of n experiments.

mean pK_D values of 10.6 and 10.7 were measured, whereas pK_D values of 10.2 were found in vas deferens and hippocampus.

As shown in Figure 2, significantly lower concentrations ($P < 0.01$, $n = 6$) of 5-methyl-urapidil are required for half-maximum inhibition of [^{3}H]-prazosin binding in vas deferens and hippocampus as compared to liver and spleen membranes. Similar results were obtained when inhibition experiments with 5-methyl-urapidil were carried out in a buffer containing NaCl 160 mM, KCl 4.6 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2.5 mM, Tris HCl 5 mM, glucose 10 mM as final

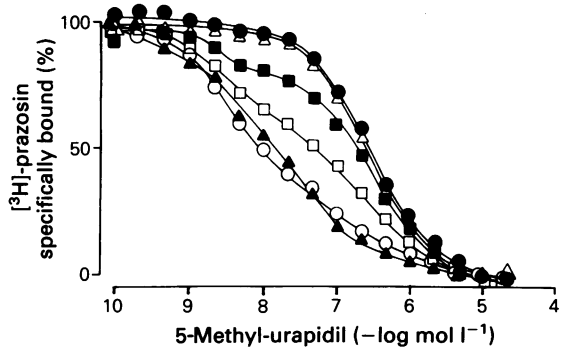

Figure 2 Inhibition of specific [^{3}H]-prazosin binding to α_1-adrenoceptors in various tissues of the rat by 5-methyl-urapidil. Crude membrane preparations of vas deferens (▲), hippocampus (○), cerebral cortex (□), heart ventricles (■), spleen (●) and liver (△) were incubated with 0.2 nM [^{3}H]-prazosin and increasing concentrations of 5-methyl-urapidil at 30°C for 45 min. Non-specific binding was determined in the presence of 10 μM phentolamine and subtracted from total binding. Values given represent means of at least 6 experiments, s.e.means were less than 4%.

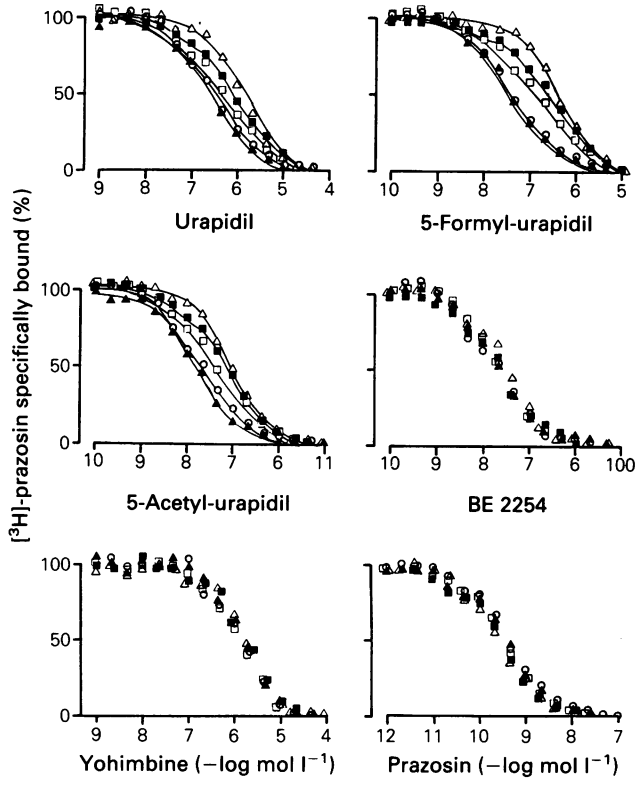

Figure 3 Inhibition of specific [^{3}H]-prazosin binding to α_1-adrenoceptors by various antagonists. Crude membrane preparations of vas deferens (▲), hippocampus (○), cerebral cortex (□), heart ventricles (■) and liver (△) were incubated with 0.2 nM [^{3}H]-prazosin and increasing concentrations of antagonists at 30°C for 45 min. Non-specific binding was determined in the presence of 10 μM phentolamine and subtracted from total binding. Values given represent means of at least 3 experiments, s.e.means were less than 6%.

concentrations, pH 7.4 (data not shown) instead of the routinely used Tris/EDTA buffer. The displacement of [^{3}H]-prazosin by 5-methyl-urapidil in cortical and myocardial membranes was not influenced by high concentrations of guanosine triphosphate (GTP, 1 mM, data not shown), whereas agonist binding (adrenaline) was clearly affected by GTP.

A tissue-specific displacement of the radioligand was also found for urapidil and its 5-acetyl- and 5-formyl-derivative (Figure 3) as well as for WB-4101 and phentolamine (Table 2). The order in which [^{3}H]-prazosin binding was inhibited in the different tissues was similar for all these compounds. The lowest IC_{50} values were always found in vas deferens and hippocampus, intermediate values in cerebral cortex and heart and the highest IC_{50} values in liver. All compounds tested inhibited [^{3}H]-prazosin binding to liver membranes with pseudo Hill coefficients (n_H) not different from unity whereas n_H values smaller than 1 were measured in the other tissues for those α_1-antagonists which discriminated most clearly between two [^{3}H]-prazosin binding sites (5-methyl-urapidil, WB-4101 and phentolamine, see Table 2).

In contrast to these compounds, unlabelled prazosin, BE 2254 and yohimbine inhibited [^{3}H]-prazosin binding to membranes of different tissues in a monophasic manner (n_H not significantly different from 1) with similar K_I values (Figure 3). Computerized analysis of the binding data revealed that 5-methyl-urapidil, WB-4101 and phentolamine bound to two distinct sites in vas deferens, hippocampus, cerebral cortex and heart but to only one site in liver and spleen (Table 2). pK_I values for the low affinity sites of tissues containing two populations of [^{3}H]-prazosin binding sites were in the same range as pK_I

Table 2 Inhibition of [^{3}H]-prazosin binding to α_1-adrenoceptors of various tissues by subtype-selective α_1-antagonists

	Tissue	n	n_H	$pK_{I\ high}$	$pK_{I\ low}$	*% high*
5-Methyl-urapidil	Vas deferens	6	0.67 ± 0.09	9.21 ± 0.21	7.55 ± 0.19	57.54 ± 7.19
	Hippocampus	6	0.53 ± 0.02	9.00 ± 0.08	7.08 ± 0.07	68.54 ± 2.44
	Cortex	20	0.57 ± 0.01	9.27 ± 0.04	7.33 ± 0.02	41.20 ± 1.52
	Heart	18	0.77 ± 0.03	9.24 ± 0.08	7.41 ± 0.02	22.82 ± 1.32
	Spleen	6	0.97 ± 0.03	—	7.53 ± 0.07	2.8 ± 2.8
	Liver	16	1.01 ± 0.02	—	7.63 ± 0.03	—
WB-4101	Vas deferens	5	0.93 ± 0.31	9.82 ± 0.05	8.89 ± 0.13	89.31 ± 9.86
	Hippocampus	6	0.65 ± 0.03	9.85 ± 0.04	8.95 ± 0.08	75.85 ± 4.10
	Cortex	6	0.66 ± 0.01	9.99 ± 0.11	8.51 ± 0.05	44.27 ± 1.30
	Heart	6	0.85 ± 0.03	10.31 ± 0.24	8.89 ± 0.05	22.28 ± 1.93
	Liver	7	1.02 ± 0.02	—	8.85 ± 0.04	—
Phentolamine	Vas deferens	3	0.75 ± 0.02	8.92 ± 0.01	7.69 ± 0.03	59.79 ± 2.51
	Hippocampus	3	0.64 ± 0.03	8.86 ± 0.49	7.19 ± 0.31	62.65 ± 8.92
	Cortex	3	0.70 ± 0.02	9.05 ± 0.02	7.65 ± 0.02	41.88 ± 1.51
	Heart	3	0.87 ± 0.02	9.26 ± 0.28	7.76 ± 0.00	17.57 ± 2.75
	Liver	3	0.93 ± 0.01	—	7.81 ± 0.02	—

$pK_I = -\log K_I$ calculated from IC_{50} values according to Cheng & Prusoff (1973). n_H = Hill coefficient. Values are means ± s.e.mean of *n* experiments.

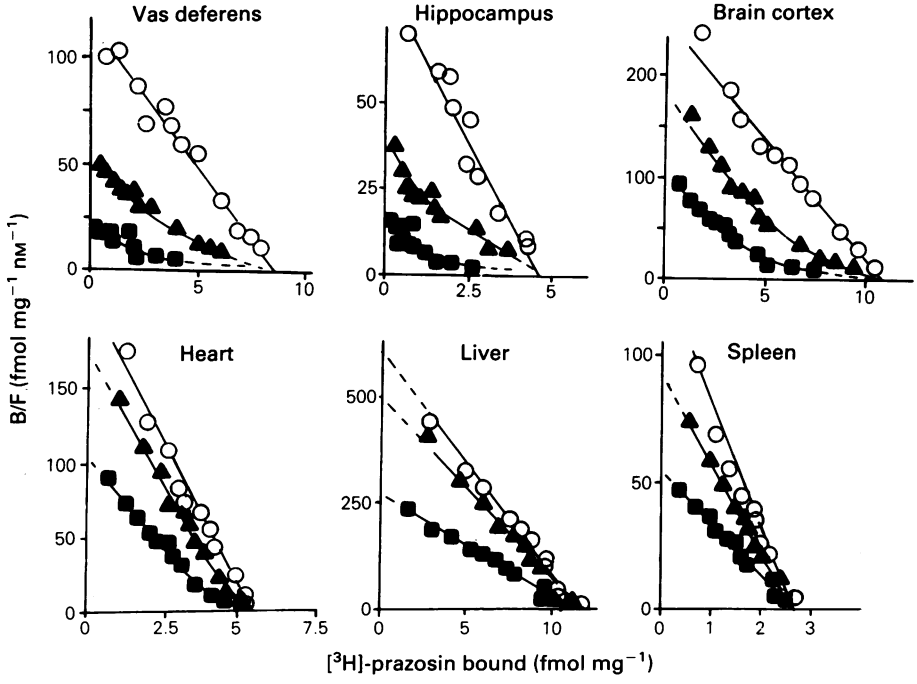

Figure 4 Binding of [^{3}H]-prazosin to α_1-adrenoceptors of various rat tissues in the absence (○) and in the presence of 5-methyl-urapidil, 3 (▲) and 30 nM (■). Membranes were incubated with increasing concentrations of [^{3}H]-prazosin ranging from 0.01 to 0.75 nM for 2 h at 30°C. Non-specific binding was determined in the presence of 10 μM phentolamine and subtracted from total binding. Results are presented as Scatchard plots. Values represent means of at least 3 experiments, s.e.means were less than 3%.

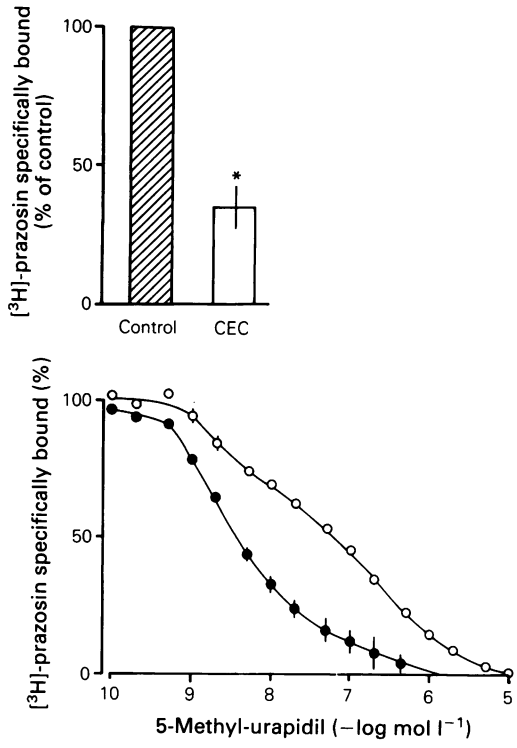

Figure 5 Effect of chloroethylclonidine (CEC) on the inhibition of [^{3}H]-prazosin binding by 5-methyl-urapidil. Membrane suspensions of the cerebral cortex were incubated without (○) and with 10 μM CEC (●) at 37°C. After 30 min the membrane suspensions were centrifuged and washed twice. CEC pretreatment caused a 63% loss of [^{3}H]-prazosin binding. Hill coefficients for the displacement of the radioligand by 5-methyl-urapidil were increased from 0.55 ± 0.01 (controls) to 0.76 ± 0.10 (CEC treatment, $P < 0.01$). Values represent means of 4 experiments with s.e.mean shown by vertical lines. * $P < 0.01$ compared to controls.

values for the respective single sites in liver and spleen. Of all compounds investigated 5-methyl-urapidil discriminated most clearly between these two populations of [^{3}H]-prazosin binding sites.

In saturation binding studies with [^{3}H]-prazosin, addition of 5-methyl-urapidil (3 and 30 nM) resulted in an apparent increase in K_D values in liver and spleen, the Scatchard plots remained linear. In those tissues, however, in which a major proportion of binding sites with high affinity for 5-methyl-urapidil had been found, the Scatchard plots became curvilinear (Figure 4).

Preincubation of cortical membranes with 10 μM chloroethylclonidine (Minneman *et al.*, 1988) for 30 min resulted in a 63% loss of specific [^{3}H]-prazosin binding ($P < 0.01$, $n = 4$). In membranes not treated with chloroethylclonidine, 5-methyl-urapidil displaced the radioligand with a shallow inhibition curve (n_H 0.55 ± 0.01, $51 \pm 2\%$ low affinity sites). After chloroethylclonidine treatment the inhibition curve became significantly steeper (n_H 0.76 ± 0.10, $P < 0.01$) and the proportion of sites with low affinity decreased ($12 \pm 1\%$, $P < 0.01$, see Figure 5).

Functional studies

Electrical stimulation of right heart ventricles produced a basal force of contraction of 10.6 ± 0.8 mN ($n = 18$). Adrenaline (0.01 to 100 μM) in the presence of 10 μM propranolol caused a positive inotropic effect of 7.3 ± 0.7 mN by stimulating α_1-adrenoceptors. The $-\log EC_{50}$ value was 6.46 ± 0.06. Preliminary experiments showed that this high concentration of propranolol was sufficient to suppress a β-adrenoceptor-mediated response even at the highest adrenaline concentration used (Gross *et al.*, 1988b). 5-Methyl-urapidil (30 to 300 nM) and WB-4101 (10 to 100 nM) caused a parallel shift to the right of the concentration-response curve. The maximum increase in tension due to α_1-adrenoceptor activation was not significantly affected by 5-methyl-urapidil in concentrations up to 0.3 μM ($n = 4$) or WB-4101 0.1 μM ($n = 6$). pA_2 values calculated from Arunlakshana & Schild plots were 7.65 (slope -1.04, $r = 1.00$) and 8.21 (slope -0.97, $r = 0.99$) for 5-methyl-urapidil and WB-4101, respectively (Figure 6).

The dihydropyridine Ca^{2+}-channel blocker, nitrendipine, at a concentration of 0.1 μM had no influence on the α_1-adrenoceptor mediated positive inotropic effect (Figure 7).

In vas deferens, α_1-adrenoceptor activation by adrenaline (0.03 to 100 μM) resulted in a maximum increase by 19.4 ± 1.7 mN ($n = 8$) with a $-\log EC_{50}$ value of 6.67 ± 0.08. The shift of concentration-response curves to the right produced by 5-methyl-urapidil (10 to 100 nM) and WB-4101 (1 to 30 nM) was significantly greater in vas deferens as compared to heart ventricles. pA_2 values of 8.47 (slope -1.04, $r = 0.98$) and 9.01 (slope -1.33, $r = 1.00$) were calculated for 5-methyl-urapidil and WB-4101, respectively (Figure 6). Both antagonists at the highest concentrations used had no significant effect ($P > 0.05$) on the maximum increase in tension.

In contrast to the α_1-mediated positive inotropic effect in myocardium, the α_1-adrenoceptor response in vas deferens was clearly affected by nitrendipine (Figure 7).

In both tissues investigated, 5-methyl-urapidil in concentrations of up to 1 μM had no intrinsic activity and can thus be considered as a pure antagonist.

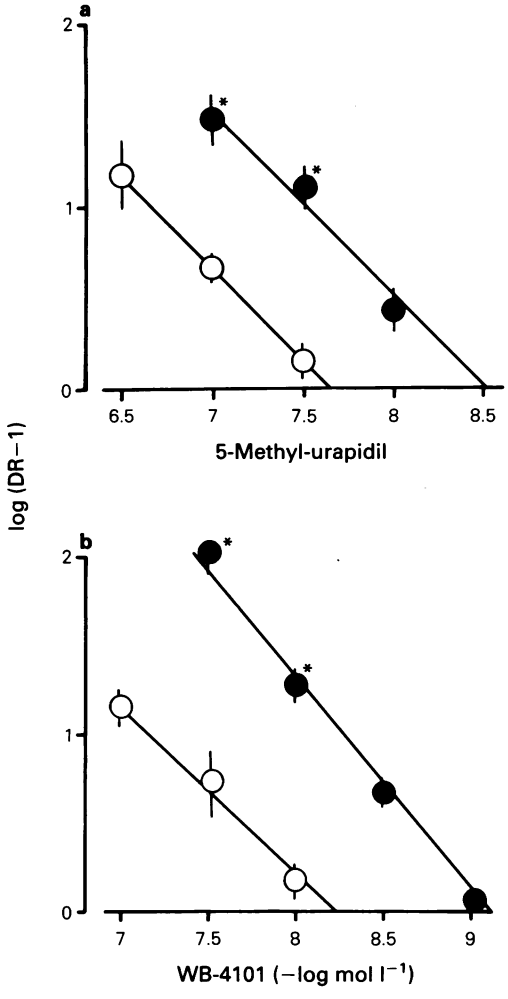

Figure 6 Concentration-dependent antagonism of the α_1-adrenoceptor-mediated positive inotropic response of right heart ventricles (○) and of the contractile response of rat vas deferens (●). Arunlakshana-Schild plots for 5-methyl-urapidil (a) and WB-4101 (b). Adrenaline (0.01 to 100 μM) in the presence of propranolol (10 μM) was used as α_1-adrenoceptor agonist. Neuronal and extraneuronal catecholamine uptake were blocked by oxaprotiline and corticosterone, respectively. Least squares fit to data reveal the following pA_2 values: pA_2 for 5-methyl-urapidil 7.65 in the heart and 8.47 in vas deferens, slopes −1.0. pA_2 values for WB-4101: 8.21 in the heart and 9.01 in vas deferens, slopes −1.0 and −1.3, respectively. Values given represent means (with s.e.mean shown by vertical lines) of at least 4 experiments for each antagonist concentration. * $P < 0.01$.

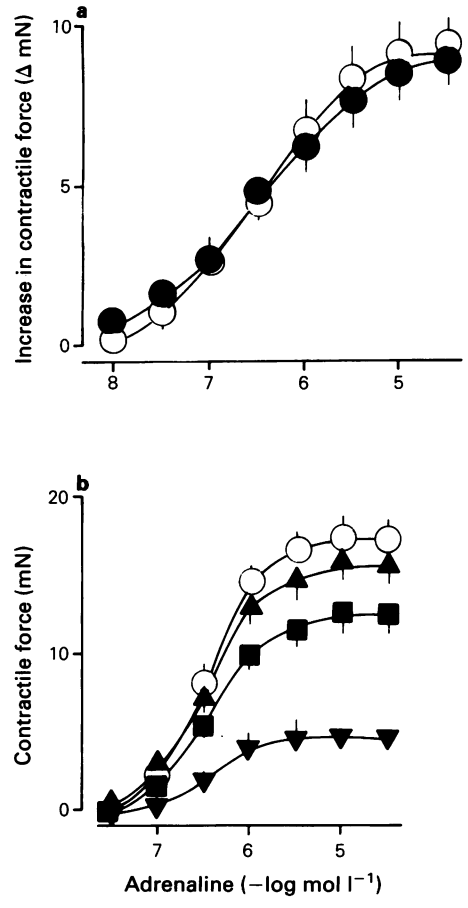

Figure 7 Effect of nitrendipine on α_1-adreno- ceptor-mediated functional responses. The positive inotropic effect in right heart ventricles (a) and the contractile response of vas deferens (b) caused by adrenaline in the presence of 10 μM propranolol was measured in the absence (○) and in the presence of nitrendipine 3 (▲), 10 (■), 30 (▼) and 100 (●) nM. Neuronal and extraneuronal catecholamine uptake were blocked by oxaprotiline and corticosterone, respectively. Values represent means of 6 experiments with s.e.means shown by vertical lines.

Discussion

In a preliminary paper (Gross *et al.*, 1988a) and in the present study we have demonstrated that 5-methyl-urapidil displaced the α_1-adrenoceptor ligand [^{3}H]-prazosin from its binding sites on membranes of six rat tissues with different affinity constants (K_I). Inhibition curves were shallow or clearly biphasic in vas deferens, hippocampus, cerebral cortex and heart

but monophasic in liver and spleen. Two-site analysis of the data strongly suggests the existence of two distinct binding sites with affinities of about 0.6 nM and 45 nM. Liver and spleen membranes seem to contain only the site with low affinity for 5-methyl-urapidil, whereas both high and low affinity sites were found in the other tissues investigated. These two binding sites in vas deferens, hippocampus, cortex and myocardium could also be detected in saturation experiments: the linear Scatchard plots of [^{3}H]-prazosin binding became curvilinear after addition of 5-methyl-urapidil, confirming the existence of two binding sites. These sites were labelled by [^{3}H]-prazosin with similar or equal affinities. In liver and spleen, addition of 5-methyl-urapidil resulted in an apparent decrease in K_D values but Scatchard plots remained linear. Unaltered B_{max} values indicated a competitive type of interaction between 5-methyl-urapidil and [^{3}H]-prazosin.

Among a series of urapidil derivatives that all inhibited [^{3}H]-prazosin binding in a tissue-dependent manner, 5-methyl-urapidil most clearly discriminated between two classes of α_1-recognition sites. The order of selectivity was: 5-methyl-urapidil > 5-formyl-urapidil ≥ 5-acetyl-urapidil > urapidil. On the other hand, several well-known α-adrenoceptor antagonists like BE 2254, yohimbine and unlabelled prazosin did not recognize distinct α_1-binding sites.

During recent years, several investigators suggested that α_1-adrenoceptors may be divided into subclasses. These attempts were based on functional data, e.g. different potencies of agonists and antagonists in various tissues and species, on differences in Ca^{2+} utilization (for reviews see Hieble *et al.*, 1986; 1987; McGrath & Wilson, 1988), on the possibility that α_1-adrenoceptors may be coupled to different second messenger pathways (García-Sainz & Hernandez-Sotomayor, 1985; Johnson & Minneman, 1986; Han *et al.*, 1987a,b) and on radioligand binding studies (Morrow *et al.*, 1985, Morrow & Creese, 1986, Han *et al.*, 1987a,b; Minneman *et al.*, 1988). However, as delineated above (see Introduction), these issues remained highly controversial and there is still no unequivocal classification. Our data presented above clearly support the findings of Morrow *et al.* (1985), Morrow & Creese (1986) and Minneman and coworkers (Han *et al.*, 1987a,b; Minneman *et al.*, 1988). Both groups found that WB-4101 and phentolamine bind to α_1-adrenoceptors in cerebral as well as in other tissues with two different affinities. The binding sites with high affinity for WB-4101 and phentolamine were designated α_{1A} (or α_{1a}), the sites with low affinity α_{1B} (or α_{1b}). Using [^{3}H]-prazosin instead of [^{125}I]BE 2254 as radioligand, we could confirm the results of these investigators. In our hands, WB-4101 as well as phentolamine proved to be selective for subtypes of α_1-recognition sites. Several lines of evidence indicate that 5-methyl-urapidil binds preferentially to α_1-sites with high affinity for WB-4101 and phentolamine and can thus be considered as a selective α_{1A}-ligand: (1) The order in which these chemically different antagonists displaced [^{3}H]-prazosin binding was similar. (2) The percentages of high- and low-affinity sites in various tissues as determined by the use of these different antagonists were in good agreement. (3) Chloroethylclonidine which has been reported to inhibit irreversibly [^{125}I]-BE 2254 binding sites with low affinity for WB-4101 (Minneman *et al.*, 1988) inactivated [^{3}H]-prazosin binding sites with low affinity for 5-methyl-urapidil as well, leaving most of the high affinity sites unaffected. Thus, our experiments with 5-methyl-urapidil support the assumption that two pharmacologically distinct α_1-binding sites exist. However, the approximately 70 fold selectivity of this new compound for α_{1A}-recognition sites clearly exceeds that of WB-4101 and phentolamine (20 to 30 fold). Therefore, 5-methyl-urapidil may serve as a valuable tool for further functional investigations of these subtypes.

Another attempt to subclassify α_1-adrenoceptors is based on the largely variable potencies of prazosin in functional experiments (Flavahan & Vanhoutte, 1986; Hieble *et al.*, 1986). In our experiments, however, K_D values for [^{3}H]-prazosin binding to various tissues differed by a ratio of about 3 or less. The fact that [^{3}H]-prazosin had the highest affinity for α_1-adrenoceptors in those organs with a high proportion of α_{1B}-sites may indicate that [^{3}H]-prazosin itself is slightly subtype-selective, but this selectivity is obviously too small to be detected by two-site analysis. However, the existence of a further α_1-recognition site with much lower affinity for [^{3}H]-prazosin cannot be excluded definitively since we used only radioligand concentrations up to 0.75 nM in our experiments.

The demonstrations of two distinct α_1-adrenoceptor recognition sites raises the question whether both sites are functionally relevant. In order to answer this question we chose two organs with a different content of α_{1A} and α_{1B}-sites in which contractile responses could be easily measured. In vas deferens at least 58% of [^{3}H]-prazosin binding sites belonged to the α_{1A}-type but in the heart only about 20%. The antagonist potencies of 5-methyl-urapidil and WB-4101 against the α_1-mediated contractile or inotropic response were about ten fold higher on vas deferens as compared to right heart ventricle, suggesting that both α_1-subtypes may contribute to functional responses. In the heart, pA_2 values of both antagonists were in good agreement with pK_I values for the α_{1B} site as determined by radioligand binding.

However, a contribution of the small amount of α_{1A}-binding sites may remain undetected by Schild analysis (Milnor, 1986). In vas deferens, on the other hand, pA_2 values intermediate between K_I values of the α_{1A} and α_{1B} sites were measured. These results may be due to a possible functional involvement of both subtypes on this tissue.

It has been suggested that α_{1A}-binding sites mediate contractions of smooth muscle which require the influx of extracellular Ca^{2+} through dihydropyridine-sensitive channels whereas activation of α_{1B}-sites stimulates inositol phosphate formation independent of extracellular Ca^{2+} (Han *et al.*, 1987a). Our experiments on heart muscle and vas deferens are in agreement with this assumption. The α_1-mediated contraction of the vas deferens, a tissue in which α_{1A}-binding sites prevail, was highly susceptible to the Ca^{2+}-channel blocker, nitrendipine. In contrast, the inotropic effect of the myocardium with 80% α_{1B}-sites was not at all inhibited by high concentrations of nitrendipine. In our opinion, however, these results are not sufficient to prove definitely that the α_{1A} and the α_{1B} subtype are linked to different second messenger pathways. Additional investigations on other tissues are clearly needed.

In conclusion, our results demonstrate that two distinct α_1-adrenoceptor binding sites can be distinguished in rat tissues by radioligand binding techniques. It remains to be established whether the same sites can be identified in other species and in man. For an unequivocal subclassification of α_1-adrenoceptors, however, further studies are needed to characterize better the biochemical and functional events mediated by these recognition sites.

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α_1-Adrenoceptor function and autoradiographic distribution in human asthmatic lung

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1 The autoradiographic distribution of α_1-adrenoceptors was investigated in non-diseased and asthmatic human lung by use of [^{3}H]-prazosin (H-PZ). To validate binding and autoradiographic methods, H-PZ binding was also measured in rat heart.

2 Significant levels of specific H-PZ binding were detected in sections of rat heart. This binding was associated with a single class of non-interacting sites of high affinity (dissociation constant, $K_d = 1.17 \pm 0.26$ nM). The maximum binding capacity (B_{max}) was 59.5 ± 4.5 fmol mg^{-1} protein.

3 In sharp contrast, very low levels of specific H-PZ binding were found in both human non-diseased and asthmatic bronchus, although a high level of binding of [^{125}I]-iodocyanopindolol (I-CYP, 50 pM) to β-adrenoceptors was detected in these airways. Furthermore, very low levels of autoradiographic grains representing specific H-PZ binding were found in all airway structures in human non-diseased or asthmatic lung parenchyma.

4 Consistent with these data, the α-adrenoceptor agonist phenylephrine failed to induce significant increases in tone in bronchi isolated from either non-diseased or asthmatic human lung. Results indicate that asthma does not involve significant increases in airway α_1-adrenoceptor function.

Introduction

It has been proposed that asthma is associated with enhanced α-adrenoceptor activity (Szentivanyi, 1980). This theory, was supported by some studies suggesting that α_1-adrenoceptor agonists such as phenylephrine and methoxamine, induced significant bronchoconstriction in asthmatics (Patel & Kerr, 1973; Snashall *et al.*, 1978; Black *et al.*, 1982; 1984), although others have produced evidence to the contrary (Thomson *et al.*, 1982). Some evidence suggests that α-adrenoceptor antagonists may be therapeutically beneficial in asthma (Griffith *et al.*, 1972; Patel & Kerr, 1975), while other studies do not support this concept (Ind & Dollery, 1983; Utting, 1979; Svedmyr, 1984; Baudouin *et al.*, 1988). Clearly, the role of α-adrenoceptors in airway smooth muscle function is controversial.

Several studies have demonstrated that α_1-adrenoceptor-mediated contractile responses in human isolated bronchial preparations are small and only evident after β-adrenoceptor blockade (Mathe *et al.*, 1971; Goldie *et al.*, 1984; 1985). However, greater α_1-adrenoceptor activity may reside at the level of bronchiolar airways (Barnes *et al.*, 1983a). Furthermore, significant α-adrenoceptor activity has been associated with increases in submucosal gland secretion (Peatfield & Richardson, 1982; Phipps *et al.*, 1982). Few studies have investigated α_2-adrenoceptor function in the lung. Barnes *et al.* (1983b) demonstrated α_2-adrenoceptor-mediated contraction of canine tracheal smooth muscle. However, this was not observed in human bronchus, although α_2-adrenoceptor-mediated attenuation of excitatory nerve transmission was documented (Grundstrom & Andersson, 1985). This is perhaps consistent with the fact that the α_2-selective agonist clonidine reduced bronchial obstruction in asthma (Lindgren *et al.*, 1986). Thus we were particularly interested in the possibility that the up-regulation of airway α_1-adrenoceptors is involved in asthma. To this end, we have examined the autoradiographic localization and distribution of specific binding sites for the α_1-selective radioligand [^{3}H]-prazosin (H-PZ) in human non-diseased and asthmatic lung parenchyma and bronchus. We have also investigated

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the functional effects of the α_1-selective agonist phenylephrine in human bronchi, to ascertain the likely physiological significance of such receptors.

Methods

Radioligand binding and autoradiography

Tissue preparation Samples of macroscopically normal lung were obtained from 3 victims of cardiovascular or automobile accidents with a mean subject age of 47.0 ± 17.4 years and a mean post-mortem age of 5.2 ± 2.0 h. Samples of asthmatic lung were also obtained from 3 severely asthmatic individuals. These subjects died rapidly before medical assistance arrived. Subject 1 was a 63 year old female (lung post-mortem age = 7 h) who used a Ventolin inhaler regularly and who died in respiratory failure. Subject 2 was a 62 year old male (lung post-mortem age = 4 h) asthmatic with chronic obstructive lung disease, for whom medication consisted of Ventolin, Moduretic, Minipress and Zyloprim. This subject died following a myocardial infarction. Subject 3 was a 60 year old male asthmatic (lung post-mortem age = 7.5 h) and who died following a coronary occlusion and who regularly used a Ventolin inhaler and oral theophylline.

Human bronchi (2–3 mm i.d.) were dissected free of parenchymal tissue and visible blood vessels and placed in aluminium foil trays containing Macrodex (6% dextran in 5% glucose). Parenchymal tissue was inflated by bronchial instillation of OCT embedding medium diluted 1:4 with 0.9% w/v NaCl solution (saline). Samples of rat ventricle were obtained from male Wistar rats (300–500 g) and placed in aluminium foil trays containing Macrodex (6% dextran in 5% glucose). All tissue samples were snap frozen in isopentane which had been quenched with liquid nitrogen. Tissue samples were then stored at −75°C until required. Serial frozen tissue sections (10 μm, autoradiographs; 16 μm, binding experiments) were cut at −30°C, mounted and thawed onto glass slides covered with gelatin. Sections were stored at −75°C for up to 2 weeks before use without loss of radioligand binding capacity.

Radioligand binding Slide-mounted sections (16 μm) of rat ventricle were incubated at 22°C for 5–60 min in Tris-HCl buffer (170 mM, pH 7.6) which contained [^{3}H]-prazosin (H-PZ; 79 Ci mmol^{-1}, 2 nM, Amersham) and the protease inhibitor phenylmethylsulphonylfluoride (PMSF, 10 μM). In another series of experiments, sections of rat ventricle were co-incubated with sections of human lung parenchyma at 22°C for 25 min in Tris-HCl buffer containing H-PZ (0.25–9 nM) and PMSF (10 μM). Specific binding of H-PZ to α_1-adrenoceptors was defined as that which was displaced by 10 μM phentolamine. Sections incubated with H-PZ were washed at 4°C in radioligand-free buffer for 1 min and again for 2 × 15 min periods, then rapidly rinsed in distilled water and wiped from slides with glass fibre filter paper (Whatman, GF/A). Tissue radioactivity was measured in a Packard liquid scintillation counter (Model B2450). The protein content of sections from each tissue block was estimated by the method of Lowry *et al.* (1951).

Autoradiography To determine the tissue distribution of α_1-adrenoceptors, slide-mounted sections (10 μm) of human non-diseased and asthmatic lung parenchyma or bronchus were incubated with H-PZ (1 nM) for 25 min in the absence or presence of 10 μM phentolamine. These preparations were washed as described for binding studies. Sections were then rapidly dried under a stream of cold dry air. Emulsion-coated (type 0) coverslips coated with NTB-3 nuclear track emulsion (Eastman Kodak Co., Rochester, N.Y.) were attached to one end of these tissue slides with cyanoacrylate adhesive and stored in desiccated light-tight X-ray cassettes at 4°C for 70 days. Autoradiographs were developed in Dektol (Kodak; diluted 1:1 with distilled water) for 3 min, rinsed in 1% acetic acid containing 2.5% Hypam hardener (Ilford) and fixed for 3 min with Hypam Rapidfix (Ilford; diluted 1:4 with distilled water) containing 2.5% Hypam hardener. Tissue sections were then lightly stained with Gill's haematoxylin for 30 s, dehydrated in graded solutions of ethanol, cleared in xylene and the coverslips re-apposed to the slides by tissue mounting in DePeX medium (BDH). Autoradiographs were viewed with a Zeiss III photomicroscope under light and dark-field illumination.

For comparative purposes, the distribution of β-adrenoceptors was also determined in slide-mounted sections of asthmatic bronchus using [^{125}I]-iodocyanopindolol (I-CYP, 50 pM) as previously described (Goldie *et al.*, 1986a).

Organ bath experiments

The mean ± s.e.mean subject age and post-mortem age of lung samples obtained from individuals with severe bronchial asthma were 29.2 ± 10.1 years and 10.5 ± 2.2 h ($n = 4$), respectively. Similarly, non-diseased lung was obtained from individuals with a mean subject age and post-mortem age of 44.4 ± 12.5 years and 8.4 ± 1.2 h ($n = 5$), respectively. There was no significant difference between non-diseased and asthmatic groups with respect to either the mean age of the subjects or the post-

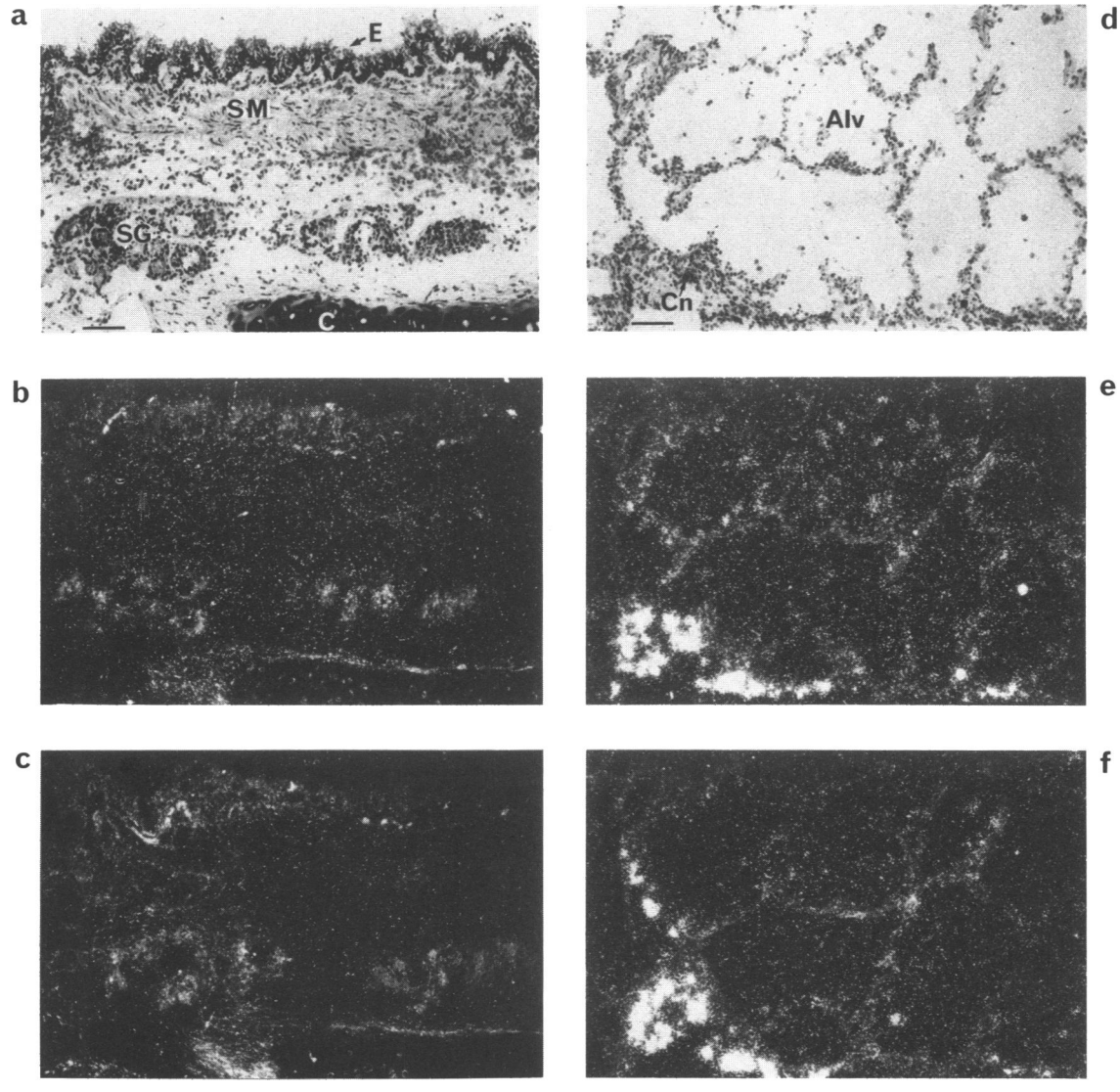

Figure 1 Photomicrographs of 10 μm thaw-mounted frozen sections of human non-diseased bronchus (a–c) and lung parenchyma (d–f). Light-field photomicrograph of (a) human bronchus showing the epithelium (E), smooth muscle (SM), submucosal glands (SG) and cartilage (C); (d) lung parenchyma showing alveoli (Alv), some of which contain carbon particles (Cn). (b and e) Dark-field photomicrographs of the above sections showing the distribution and localization of autoradiographic grains derived from [^{3}H]-prazosin (H-PZ, 1 nM) binding. (c and f) Dark-field photomicrograph showing the distribution of non-specific autoradiograph grains in respective serial sections incubated with H-PZ (1 nM) and phentolamine (10 μM). Bar = 100 μm.

mortem age of the lung samples ($P > 0.05$, non-paired t test).

Bronchial spiral preparations were dissected from both human non-diseased and asthmatic lung and suspended in organ baths as previously described (Goldie *et al.*, 1986b). All preparations were left to equilibrate for 2.5 h at 37°C in Krebs solution gassed with 5% CO_2 in oxygen before any pharmacological testing was attempted. Cumulative concentration-effect curves to carbachol were constructed in all

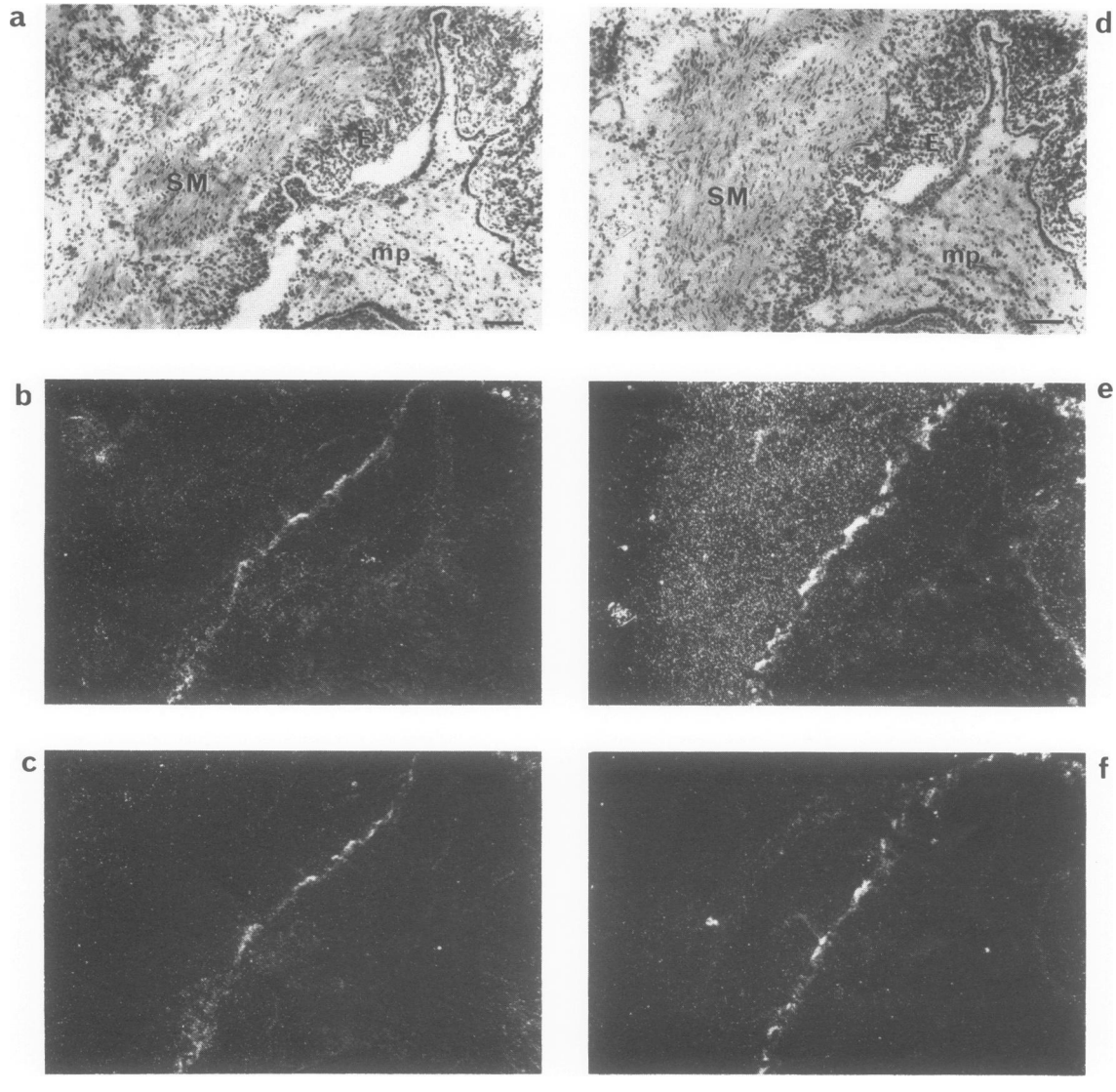

Figure 2 Photomicrographs of 10 μm thaw-mounted frozen sections of human asthmatic bronchus. (a, d) Light-field photomicrographs of 2 serial sections of human asthmatic bronchus showing smooth muscle (SM), damaged epithelium (E) and a luminal mucous plug (mp) infiltrated with cells. Dark-field photomicrographs of the above sections showing the distribution and localization of autoradiographic grains derived from (b) [^{3}H]-prazosin (H-PZ, 1 nM) and (e) [^{125}I]-iodocyanopindolol (I-CYP; 50 pM) binding. Dark-field photomicrograph showing the distribution of non-specific autoradiographic grains in respective serial sections incubated with (c) H-PZ (1nM) and phentolamine (10 μM) or (f) I-CYP (50 pM) and isoprenaline (200 μM). An artifact seen as bright areas associated with the epithelial basement membrane appears in each dark-field photomicrograph. Bar = 100 μm.

preparations used. Cumulative concentration-effect curves were also produced for (±)-phenylephrine in the absence or presence of propranolol (0.5 μM), in bronchial preparations with spontaneous or carbachol-induced tone. The concentration of carbachol used to induce tone was that concentration producing 50% (EC_{50}) of the maximum response to this agonist, as determined from the first cumulative concentration-effect curve. Preparations were washed at the end of each curve by 3 complete

changes of Krebs solution and an interval of 1 h allowed between curves.

Drugs and solutions

Carbamylcholine chloride, (±)-isoprenaline hydrochloride, (±)-phenylephrine hydrochloride, (±)-propranolol hydrochloride, theophylline (Sigma); (±)-phentolamine mesylate (Ciba); PMSF (phenylmethylsulphonylfluoride, Calbiochem). PMSF was freshly prepared in absolute ethanol. [^{3}H]-prazosin, 79 Ci mmol^{-1}; [^{125}I]-iodocyanopindolol, 2000 Ci mmol^{-1} (Amersham). The Krebs solution used throughout the study had the following composition (mM): NaCl 117.6, KCl 5.4, $NaHCO_3$ 25, KH_2PO_4 1.03, $MgSO_4$ 0.57, D-glucose 11.1 and $CaCl_2$ 2.5.

Analysis of results

All numerical results were expressed as mean ± s.e.mean. Parameters describing the concentration-dependence of H-PZ binding (dissociation constant, K_d; maximum binding capacity, B_{max}) were estimated by non-linear least squares regression analysis of data fitted to a one site binding model, by use of the computer programme MLAB (N.I.H., U.S.A.). Scatchard analysis was used to confirm these results and the nature of binding was described using Hill analysis. The probability (P) of differences between mean values was determined by use of Student's two tailed, non-paired t test and was considered significant if $P < 0.05$.

Results

Characteristics of [^{3}H]-prazosin binding

Incubation conditions appropriate for the detection of specific H-PZ binding were determined from studies using rat heart which is known to contain a significant population of α_1-adrenoceptors (Guicherey & Meyer, 1981). The specific binding of H-PZ (2 nM) in rat heart sections reached equilibrium after approximately 25 min, and was saturable and involved a single population of non-interacting, high affinity sites (Hill coefficient, nH = 1.007 ± 0.124). Non-linear regression analysis of the specific binding data using a one binding site model yielded a maximum binding capacity (B_{max}) of 59.5 ± 4.5 fmol mg^{-1} protein and a dissociation constant (K_d) of 1.17 ± 0.26 nM (where the errors were estimated directly from non-linear regression analysis of data from one heart). Specific binding accounted for 74% of total binding at a H-PZ concentration of 0.25 nM and 60% at a H-PZ concentration of 3.5 nM.

In sharp contrast, no evidence of a time-related increase in H-PZ (2 nM) binding or of binding saturability was obtained in tissue specimens from 3 samples of human non-diseased lung. Similarly, no significant levels of specific H-PZ binding were detected in tissue sections from asthmatic lung samples.

Autoradiography

The distribution of autoradiographic grains derived from H-PZ (1 nM) binding was assessed in human lung tissue in an attempt to identify locations of small populations of α_1-adrenoceptors, which might not be detected in binding studies. However, only very low levels of specific H-PZ binding were observed in specimens of human non-diseased bronchus and lung parenchyma (Figure 1) and asthmatic bronchus (Figure 2) and lung parenchyma. Some areas of peripheral lung tissue contained black deposits resembling carbon particles or tar (Figures 1d), presumably derived from atmospheric pollutants including cigarette smoke. These areas are associated with high levels of non-specific binding (Figures 1f). Specific grain densities over smooth muscle and submucosal glands in bronchi from both human non-diseased and asthmatic lung were also very low. In contrast to the low levels of specific H-PZ binding in asthmatic bronchus, this tissue contained high levels of specific I-CYP binding which represents β-adrenoceptors (Figure 2).

Organ bath experiments

Phenylephrine caused partial concentration-dependent relaxation in 10 of 11 bronchial preparations from human non-diseased lung with spontaneously developed (Figure 3a) or carbachol-induced tone (Figure 3b), while 1 preparation failed to respond to phenylephrine but contracted in response to carbachol. In contrast, in the presence of the β-adrenoceptor antagonist propranolol (0.5 μM), phenylephrine caused small concentration-dependent increases in tone in 6 of 10 bronchial preparations from 5 separate non-diseased lung samples (Figure 3c). Maximal phenylephrine-induced contractions amounted to 10, 11, 12, 13, 18 and 24% of the respective maximum contractile response to carbachol.

In one bronchial preparation from an asthmatic lung sample, phenylephrine caused an increase in basal tone equivalent to 14% of the maximal contractile response to carbachol. Conversely, concentration-dependent relaxation was obtained in 2 of 4 bronchial preparations with spontaneous tone from 3 asthmatic lungs (Figure 3d). The 2 bronchial

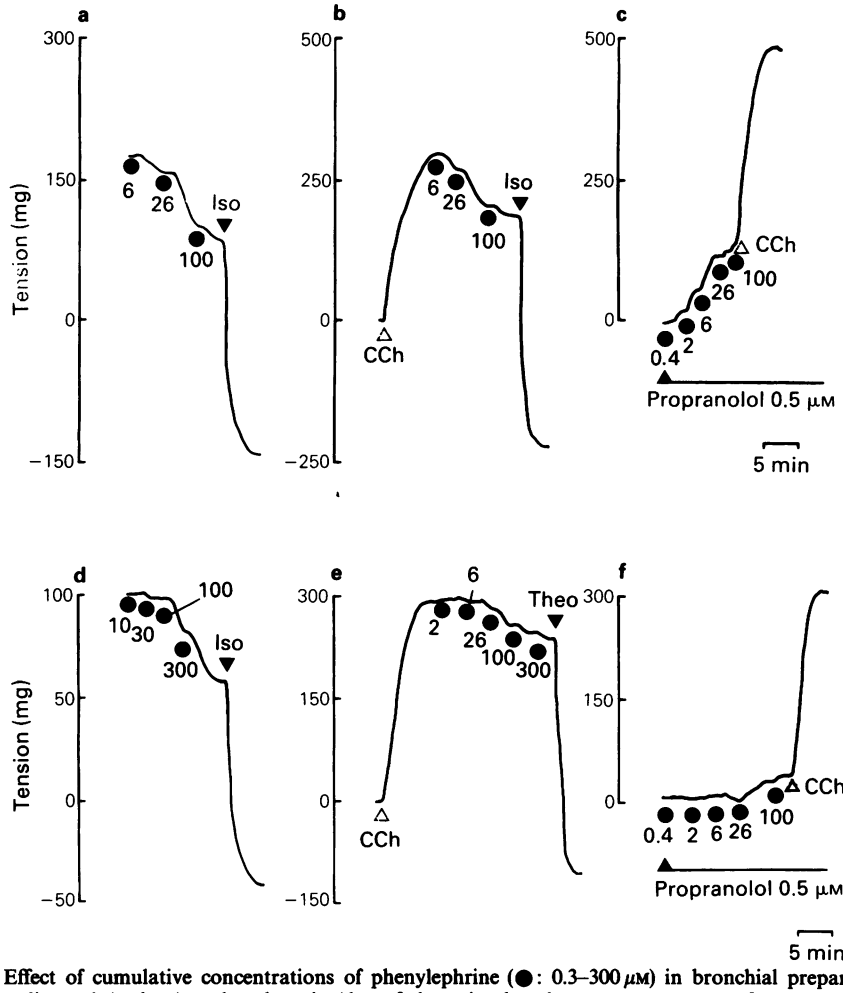

Figure 3 Effect of cumulative concentrations of phenylephrine (●: 0.3–300 μM) in bronchial preparations from human non-diseased (a, b, c) and asthmatic (d, e, f) lung in the absence or presence of propranolol (0.5 μM). CCh = carbachol (0.3–1 μM). Iso = isoprenaline (6.4 μM). Theo = theophylline (1 mM).

preparations which were unresponsive to phenylephrine did contract in response to carbachol and relaxed in response to isoprenaline. Phenylephrine also caused concentration-dependent relaxation in each of 5 bronchial preparations with carbachol-induced tone from 4 asthmatic lung samples (Figure 3e). Even in the presence of propranolol (0.5 μM), these preparations failed to contract in response to phenylephrine (Figure 3f).

Discussion

After β-adrenoceptor blockade, adrenaline, noradrenaline or phenylephrine-induced stimulation of human pulmonary α-adrenoceptors has been shown to cause vascular smooth muscle contraction (Goldie *et al.*, 1982), submucosal gland secretion (Phipps *et al.*, 1982), inhibition of excitatory neurotransmission (Grundstrom & Andersson, 1985; Andersson *et al.*, 1986) and weak contractions of bronchial smooth muscle (Goldie *et al.*, 1984; 1985). While the physiological significance of airway smooth muscle α-adrenoceptor function in healthy lung is uncertain, some studies suggest that α_1-adrenoceptor function is enhanced in asthma (Black *et al.*, 1982; 1984; Szentivanyi *et al.*, 1984), perhaps as a result of the presence of mediators including histamine (Kneussel & Richardson, 1978). However, we were unable to demonstrate such enhancement in the presence of

histamine (Goldie *et al.*, 1985). Other studies have also failed to show increased α-adrenoceptor activity in asthmatic compared to non-asthmatic bronchi (Svedmyr, 1984; Goldie *et al.*, 1988; Barnes, 1986).

Samples of post-mortem asthmatic human lung are obtained very infrequently. The need to obtain approximately age-matched samples of non-diseased and asthmatic human lung within 11 h post-mortem in order to conduct appropriate comparisons of both functional and autoradiographic data, further reduces the number of suitable tissue samples. Despite these constraints, it is clear from the present study that asthma does not involve significantly increased airway α_1-adrenoceptor function. Indeed, phenylephrine caused only small contractions of bronchi from both non-diseased and asthmatic lung. Furthermore, in all but one case, weak contractions were only elicited after propranolol-induced blockade of β-adrenoceptors. In the absence of propranolol, phenylephrine caused β-adrenoceptor-mediated partial relaxation of these bronchi, which emphasizes the relative insignificance of α_1-adrenoceptor function in human bronchial smooth muscle.

Autoradiographic experiments were conducted in order that populations of α-adrenoceptors present throughout the lung including airway smooth muscle could be examined. Consistent with previous findings (Sholtz *et al.*, 1980; Guicherey & Meyer, 1981; Muntz *et al.*, 1986), high levels of specific H-PZ binding to sections of rat heart were detected in binding analyses.

In sharp contrast, very low levels of specific H-PZ binding were detected in association wtih airway structures within human lung parenchyma and bronchi. Interestingly, bronchiolar airways in ferret lung (Barnes *et al.*, 1983a) have been shown to contain significant numbers of α_1-adrenoceptors. This suggests that great care must be taken when extending conclusions drawn from studies using animal lung to human airways. However, it is noteworthy that there was no evidence of significant numbers of bronchiolar α_1-adrenoceptors in rat lung (Xue *et al.*, 1983). Conversely, high levels of binding of I-CYP to β-adrenoceptors were detected in various structures within bronchi from both non-diseased and asthmatic lung.

While the present study shows that α_1-adrenoceptor activity in healthy and asthmatic human airway structures is low, these findings are contrary to results from some studies using homogenized membrane preparations from human non-diseased (Szentivanyi *et al.*, 1979; Barnes *et al.*, 1980b), asthmatic (Szentivanyi *et al.*, 1979) and bronchitic lung (Barnes *et al.*, 1980b) and in guinea-pig lung (Barnes *et al.*, 1979; 1980a). The detection of low but significant numbers of α_1-adrenoceptors in these investigations may reflect the much higher protein content of membrane preparation aliquots compared with that in lung tissue sections. Importantly, however, the number of α_1-adrenoceptors detected in these membrane preparations was at least 5–10 fold lower than the number of β-adrenoceptors present. While the possibility of some post-mortem fall out of α_1-adrenoceptors in airway smooth muscle cannot be excluded, we have previously demonstrated powerful α_1-adrenoceptor-mediator contraction of human pulmonary artery obtained up to 14 h after death (Goldie *et al.*, 1982). Thus it seems more likely that the number of α_1-adrenoceptors in human bronchi are too low to be readily detected by radioligand binding or light microscopic autoradiography.

The present study has clearly demonstrated the absence of significant numbers of airway α_1-adrenoceptors in human lung. These results are in line with studies reporting only weak α-agonist-induced bronchoconstriction (Thomson *et al.*, 1982) and disappointing therapeutic efficacy with α-adrenoceptor antagonists in asthma (Svedmyr, 1984).

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β-Adrenoceptor blocking effects of a selective β_2-agonist, mabuterol, on the isolated, blood-perfused right atrium of the dog

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1 Effects of (±)-1-(4-amino-3-chloro-5-trifluoromethyl-phenyl)-2-tert.-butylamino-ethanol hydrochloride (mabuterol) on pacemaker activity and atrial contractility were investigated in the isolated and blood-perfused right atrium of the dog.

2 Mabuterol, injected into the sinus node artery of the isolated atrium, dose-dependently increased atrial rate and contractile force at doses of 0.01–10 nmol but the responses to over 10 nmol of mabuterol gradually decreased and mabuterol at higher doses induced biphasic cardiac responses, i.e., negative followed by positive cardiac responses.

3 The maximal increases in atrial rate and contractile force induced by mabuterol were 41.4% and 12.9%, respectively, of the maximal chronotropic and inotropic effects of isoprenaline.

4 Positive chronotropic and inotropic responses to mabuterol were dose-dependently inhibited by a selective β_2-adrenoceptor antagonist, ICI 118,551. These responses were only slightly attenuated by atenolol.

5 Mabuterol (1–300 nmol) dose-dependently inhibited both dobutamine- and procaterol-induced positive chronotropic and inotropic responses.

6 These results indicate that mabuterol causes weak positive chronotropic and inotropic effects on the perfused canine right atrium by activating β_2-adrenoceptors, and that higher concentrations non-selectively block both β_1- and β_2-adrenoceptors.

Introduction

Mabuterol, ((±)-1-(4-amino-3-chloro-5-trifluoromethyl-phenyl)-2-tert.-butylamino-ethanol hydrochloride), is a newly synthesized orally active and long-acting β_2-adrenoceptor agonist and bronchodilator. Mabuterol exhibits marked bronchodilator activity accompanied by only slight effects on the cardiovascular system in guinea-pigs and dogs (Murai *et al.*, 1984). Osada *et al.* (1984) found that i.v. administration of mabuterol dose-dependently decreased blood pressure and increased heart rate, but at higher doses, mabuterol decreased heart rate in rats, cats and dogs. They also reported that a high dose of mabuterol possessed β_1-adrenoceptor blocking activity in guinea-pig isolated atrium.

Lands *et al.* (1967a,b) first subclassified β-adrenoceptors into β_1- and β_2 subtypes with absolute organ-specific distribution such as β_1-receptors in the heart and β_2-receptors in the lung. Recently, it has been shown that both receptor subtypes exert a physiological role in cat, dog and human hearts by use of pharmacological (O'Donnell & Wanstall, 1980; Zerkowski *et al.*, 1986; Friedman *et al.*, 1987), biochemical (Brodde *et al.*, 1984; Gille *et al.*, 1985; Kaumann & Lemoine, 1987) and receptor-binding studies (Hedberg *et al.*, 1980; Manalan *et al.*, 1981; Stiles *et al.*, 1983). However, only β_1-receptors mediate positive chronotropic and inotropic responses in rat and guinea-pig hearts (Wilson & Lincoln, 1984; Molenaar & Summers, 1987). Recently, we have reported the existence of positive chronotropic and inotropic responses mediated by β_2-adrenoceptors in addition to β_1-adrenoceptors in the isolated, blood-perfused right atrial and left ventricular preparations of the dog (Akahane *et al.*,

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1988; 1989). Therefore, this study was carried out to examine the selectivity of mabuterol for β-receptor subtypes. We also studied whether mabuterol selectively blocked β_1-adrenoceptors in the isolated, blood-perfused right atrium of the dog.

Methods

Preparation of the isolated, blood-perfused atrium of the dog

Thirty six donor dogs, weighing 8–25 kg, were anaesthetized with sodium pentobarbitone, 30 mg kg^{-1} i.v. Sodium heparin (500 USP units kg^{-1}, i.v.) was administered at the beginning of the perfusion of the isolated atrial preparation and 200 USP units kg^{-1} were given each hour thereafter. Isolated right atrial preparations were obtained from another 36 mongrel dogs weighing 7–15 kg. Each dog was anaesthetized with sodium pentobarbitone 30 mg kg^{-1} i.v. Sodium heparin (200 USP units kg^{-1}, i.v.) was given, and the right atrium was then excised and immersed in cold Tyrode solution. The wet weight of the atrial preparations varied between 8–14 g. The sinus node artery was cannulated via the right coronary artery and perfused with blood conducted from the carotid artery of the support dog by the aid of a peristaltic pump (Harvard Apparatus Model 1210). A pneumatic resistance was placed in parallel with the perfusion system so that the perfusion pressure could be maintained constant at 100 mmHg. The rate of blood flow to the isolated atrium was 3–9 ml min^{-1}.

The ventricular margin of the atrium was anchored to a stainless steel bar and was placed in a cup-shaped glass container which was kept at a constant temperature of 37°C. The superior part of the atrium was connected to a force-displacement transducer (Nihon Kohden AP 620G) by a silk thread. The atrium was usually stretched to a resting tension of 2 g. The isometric tension was recorded on a thermo-writing rectigraph (Nihon Kohden WT 685T). A pair of electrodes, each with an inter-electrode distance of 1.5 mm, was brought into contact with the epicardial surface of the isolated atrium in order to record the atrial electrogram. The atrial rate was derived from the atrial electrogram with a cardiotachometer (Nihon Kohden AT 600G). The details of this preparation have been described previously (Chiba *et al.*, 1975a,b).

Experimental protocols

Chronotropic and inotropic responses to mabuterol ($n = 8$) and isoprenaline ($n = 6$) were first determined in the isolated, blood-perfused right atrial preparation. Positive chronotropic and inotropic responses to either mabuterol, to a selective β_1-agonist, dobutamine (Williams & Bishop, 1981) or to a selective β_2-agonist, procaterol (Yabuuchi *et al.*, 1977) were obtained and the effects of a selective β_1-antagonist, atenolol or a selective β_2-antagonist, ICI 118,551 (Bilski *et al.*, 1983) were studied. Finally, effects of pretreatment with mabuterol on positive chronotropic and inotropic responses to dobutamine, procaterol, 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor), Bay K 8644 (a dihydropyridine calcium agonist) and $CaCl_2$ were examined in order to determine whether mabuterol possesses β_1- and/or β_2-receptor antagonistic activities.

Drugs

The drugs used in this study were (±)-1-(4-amino-3-chloro-5-trifluoromethyl-phenyl)-2-tert.-butyl-amino-ethanol hydrochloride (mabuterol, generously donated by Kaken Pharmaceutical Co. Ltd., Tokyo, Japan), (−)-isoprenaline hydrochloride (Nikken Kagaku, Tokyo, Japan), dobutamine hydrochloride (Shionogi Corp., Osaka, Japan), procaterol hydrochloride (Otsuka Pharmaceutical Corp., Tokushima, Japan), atropine sulphate (Wako Pure Chemical, Osaka, Japan). atenolol (Sigma Chemical Corp., St. Louis, MO, U.S.A.), ICI 118,551 (erythro-DL-1(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol generously donated by Imperial Chemical Industries, Macclesfield, England), 3-isobutyl-1-methylxanthine (IBMX, Aldrich Chemical, Milwaukee, WIS, U.S.A.) and Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-fluoromethylphenyl)-pyridine-5-carboxylate, generously donated by Dr F. Seuter, Bayer AG, Wuppertal-Elberfeld, F.R.G.).

Bay K 8644 was dissolved in ethanol and the other drugs were dissolved in physiological saline before the start of the experiment. Drugs were injected into the sinus node artery through a rubber tube with a microsyringe (Terumo Co.). The amount of drug solution injected was 0.01–0.03 ml over a period of 4 s.

The degree of chronotropic or inotropic effect was expressed as maximum percentage change of the basal atrial rate or developed tension. To examine any β-receptor antagonistic activity of mabuterol, the response to each agonist was assessed 1–1.5 min after pretreatment with mabuterol. Inhibition by an antagonist was expressed as percentage inhibition of the control value which was obtained just before treatment with an antagonist. Data were expressed as the mean ± s.e.mean and analysed by analysis of variance for multiple comparisons of data and Student's t test for unpaired data. A probability of

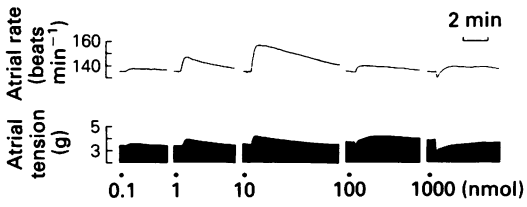

Figure 1 Changes in atrial rate and contractile force in response to 0.1, 1, 10, 100 and 1000 nmol mabuterol injected into the sinus node artery of an isolated, blood-perfused atrium of the dog.

less than 0.05 was considered as statistically significant.

Results

Effects of mabuterol on pacemaker activity and atrial contractility in the isolated, blood-perfused atrium of the dog

Figure 1 shows representative responses on atrial rate and atrial contractility induced by mabuterol at doses of 0.1–1000 nmol in an isolated, blood-perfused right atrium of the dog. When mabuterol was injected at doses of 0.1–10 nmol into the preparation, dose-dependent positive chronotropic and inotropic responses were evoked. However, at higher doses, chronotropic and inotropic responses became biphasic, i.e., negative followed by positive responses and the positive effects gradually decreased. At a dose of 1000 nmol, a biphasic chronotropic response and only a negative inotropic response was observed. Dose-response curves for atrial rate and atrial developed tension expressed as maximum percentage changes of the resting level in response to mabuterol ($n = 8$) and isoprenaline ($n = 6$) in isolated and blood-perfused right atria are shown in Figure 2. Mabuterol increased atrial rate and atrial tension dose-dependently over the range 0.01–10 nmol, and these positive chronotropic and inotropic responses gradually decreased at higher doses. Chronotropic (over 100 nmol) and inotropic responses (over 3 nmol) became biphasic and at doses of 300 and 1000 nmol, only a negative inotropic response was evoked. On the other hand, isoprenaline at doses of 0.001–0.3 nmol, dose-dependently increased atrial rate and atrial tension. The maximal positive chronotropic and inotropic responses to 10 nmol of mabuterol were 31.4 ± 2.8% and 32.4 ± 2.6%, respectively, and these responses

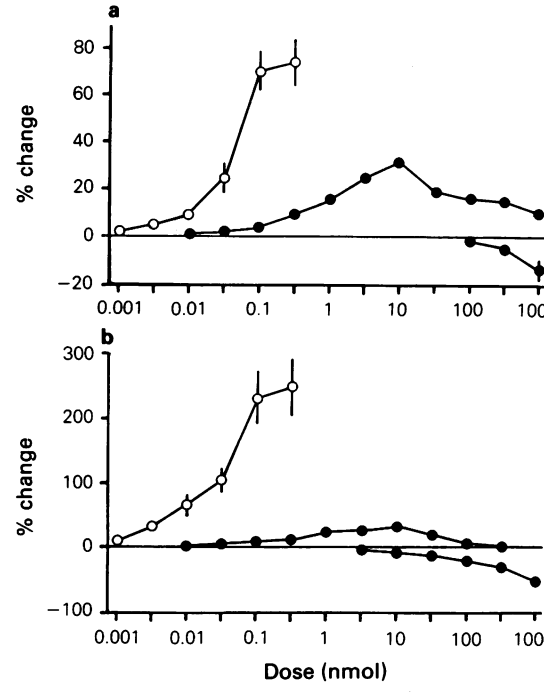

Figure 2 Mean dose-response curves for percentage changes in the maximum positive or negative chronotropic (a) and inotropic (b) responses to mabuterol (●, $n = 8$) and isoprenaline (○, $n = 6$) in isolated, blood-perfused atria of the dog. Vertical lines show s.e.mean. The basal atrial rate and contractile force in atrial preparations were 106.6 ± 4.4 (mean ± s.e.mean) beats min^{-1} and 1.6 ± 0.2 g, respectively.

were 41.4% and 12.9%, respectively, of the maximal positive chronotropic and inotropic responses induced by 0.3 nmol isoprenaline (Figure 2). The mabuterol-induced negative cardiac responses at higher doses were not inhibited by atropine ($n = 3$).

Effects of atenolol on positive chronotropic and inotropic responses to mabuterol, dobutamine and procaterol

A selective β_1-agonist, dobutamine and a selective β_2-agonist, procaterol were used for comparison. Mabuterol (1 nmol, $n = 8$), dobutamine (1 nmol, $n = 8$) and procaterol (1 nmol, $n = 7$) increased atrial rate by 13.8 ± 2.4%, 18.2 ± 6.1% and 21.5 ± 5.3%, respectively and atrial contractility by 29.2 ± 6.3%, 75.9 ± 11.8% and 41.0 ± 7.5%, respectively. In our previous study, dobutamine (0.1–10 nmol) and procaterol (0.03–30 nmol) dose-dependently increased atrial rate and contractile force in the isolated right

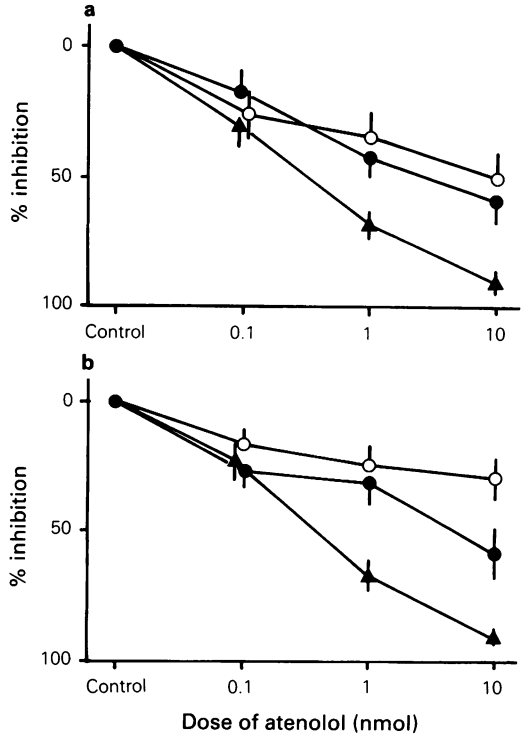

Figure 3 Effects of atenolol on increases in atrial rate (a) and atrial tension (b) in responses to mabuterol (●, $n = 8$), dobutamine (▲, $n = 8$) and procaterol (○, $n = 7$). The dose of each agonist was 1 nmol. Data are expressed as a percentage inhibition of the control value (Control) which was obtained just before treatment with atenolol. Points show mean values with s.e.mean.

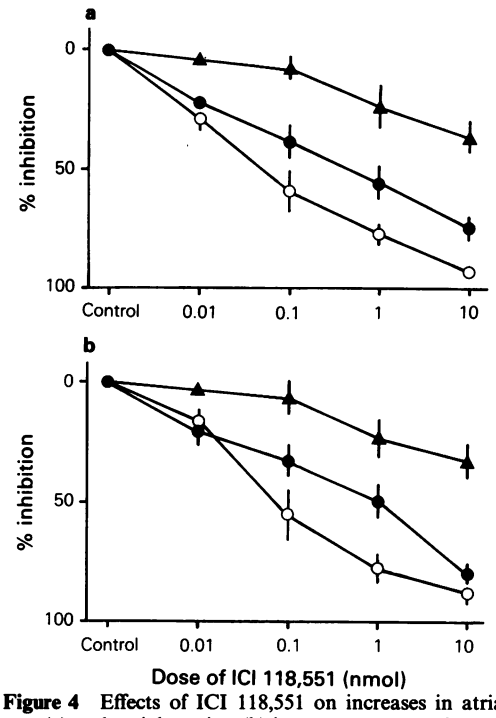

Figure 4 Effects of ICI 118,551 on increases in atrial rate (a) and atrial tension (b) in responses to mabuterol (●, $n = 8$), dobutamine (▲, $n = 7$) and procaterol (○, $n = 7$). The dose of each agonist was 1 nmol. Data are expressed as a percentage inhibition of the control value (Control) which was obtained just before treatment with ICI 118,551. Points show mean values with s.e.mean.

atrium of the dog (Akahane *et al.*, 1988; 1989). It has been reported that procaterol possesses β_1-agonist activities at high concentrations (Johansson & Persson, 1983; O'Donnell & Wanstall, 1985; Molenaar & Summers, 1987). Thus, the dose of each β-agonist which evoked about a 15–20% increase in atrial rate was selected for the control. Atenolol, a selective β_1-antagonist, at doses of 0.1–10 nmol dose-dependently inhibited dobutamine-induced positive chronotropic and inotropic responses. Atenolol also reduced the positive responses to mabuterol and procaterol. However, the inhibition by atenolol of the mabuterol- or procaterol-induced responses was much less than the inhibition of the dobutamine-induced responses. Summarized data are shown in Figure 3.

Effects of ICI 118,551 on the responses to β-agonists

Positive chronotropic and inotropic responses to mabuterol were dose-dependently inhibited by ICI 118,551 (0.01–10 nmol). Increases in atrial rate and atrial tension evoked by procaterol were more effectively inhibited by ICI 118,551 than those of mabuterol, and the difference in two inhibition curves for atrial rate and atrial tension were statistically significant by an analysis of variance ($P < 0.01$, respectively). Dobutamine-induced positive chronotropic and inotropic responses were only slightly attenuated by ICI 118,551 at doses of 0.01–10 nmol. The summarized data are shown in Figure 4.

Blocking effects of mabuterol on dobutamine-, procaterol-, IBMX-, Bay K 8644- and $CaCl_2$-induced positive cardiac responses

Pretreatment with mabuterol at doses of 3 and 30 nmol reduced to positive chronotropic and inotropic responses to both dobutamine and procaterol (Figure 5). Effects of mabuterol on increases in atrial rate and atrial tension evoked by 1 nmol dobutamine or 1 nmol procaterol in 6 right atrial preparations of the dog are shown in Figure 6. Both dobutamine- and procaterol-induced positive chrono- and inotro-

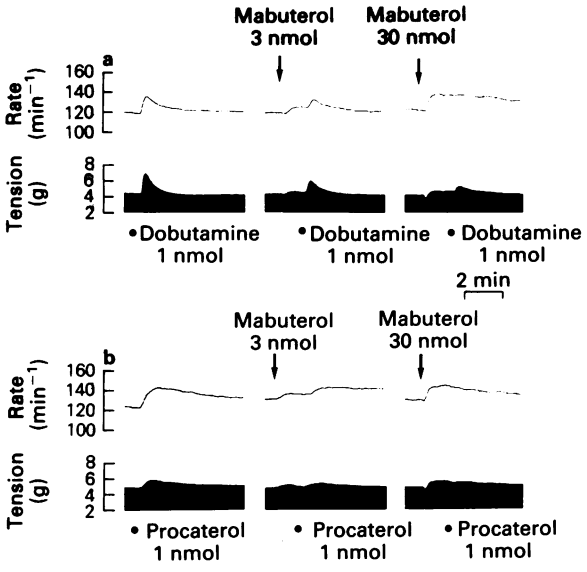

Figure 5 Effects of pretreatment with mabuterol (3 and 30 nmol) on changes in atrial rate and contractile force in response to 1 nmol dobutamine (a) and 1 nmol procaterol (b) in an isolated, blood-perfused atrium of the dog.

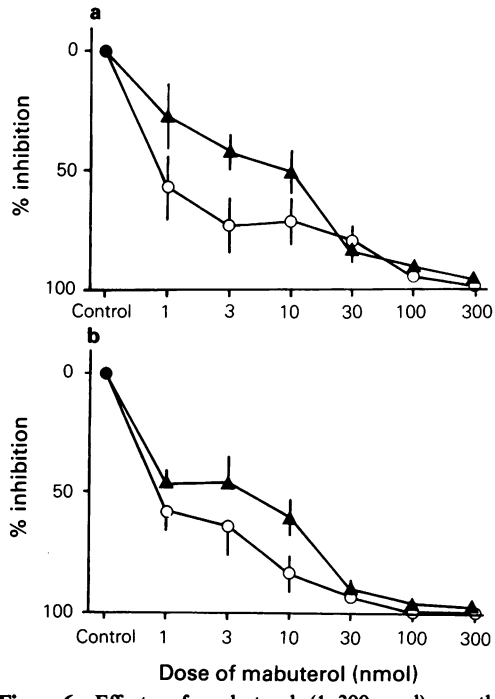

Figure 6 Effects of mabuterol (1–300 nmol) on the mean percentage changes in chronotropic (a) and inotropic responses (b) to 1 nmol of dobutamine (▲, $n = 6$) and 1 nmol of procaterol (○, $n = 6$) in canine isolated, blood-perfused atrial preparations. Data are presented as a percentage inhibition of the control value (Control) which was obtained just before treatment with mabuterol. Vertical lines represent s.e.mean.

pic responses were dose-dependently inhibited by mabuterol at doses of 1–300 nmol. Mabuterol at doses of 100–300 nmol completely suppressed dobutamine- and procaterol-induced responses. Mabuterol inhibited more effectively procaterol-induced atrial rate ($P < 0.05$) and atrial tension ($P < 0.01$) than dobutamine-induced responses. Fifty percent inhibitory doses (IC_{50}) of mabuterol for atrial rate and atrial tension to the effects of procaterol were 1.1 ± 0.4 nmol and 2.9 ± 1.3 nmol, respectively, and to the effects of dobutamine, 5.9 ± 2.0 nmol and 7.2 ± 2.0 nmol, respectively.

In order to examine whether mabuterol selectively inhibited β-adrenoceptors, effects of pretreatment with mabuterol on positive chronotropic and inotropic responses induced by a phosphodiesterase inhibitor, IBMX, a dihydropyridine calcium agonist, Bay K 8644, and $CaCl_2$ were studied in 5–9 isolated right atrial preparations of the dog. Since a direct injection of $CaCl_2$ into the preparation induced a much smaller chronotropic response than inotropic one (Furukawa *et al.*, 1988), the dose of a non-β-agonist which increased the atrial tension to almost the same extent (50–100%) as β-agonists with an obvious chronotropic response was selected, i.e., IBMX 135 nmol, Bay K 8644 0.8 nmol and $CaCl_2$ 3 μmol, respectively. The positive chronotropic and inotropic responses to IBMX, Bay K 8644 or $CaCl_2$ were not modified by pretreatment with 100 nmol mabuterol which completely suppressed the positive cardiac responses evoked by dobutamine or procaterol. Summarized data are shown in Table 1.

Discussion

Carlsson *et al.* (1972) reported that both β_1- and β_2-adrenoceptors mediate chronotropic responses in the cat heart. Carlsson *et al.* (1977) also showed that the increases in heart rate and ventricular contractility were mediated in part by β_2-adrenoceptors and suggested that the different responses might be responsible for differences in the relative distribution of β_1- and β_2-adrenoceptors in the sinus node and in the myocardium. Brodde *et al.* (1982) determined the amount of β_2-adrenoceptors in atria and ventricle of the rabbit using radioligand binding. As in animal hearts, the coexistence of β_1- and β_2-adrenoceptors has been reported in the receptor binding assay of human right atrium and left ventricle (Stiles *et al.*,

Table 1 Effect of pretreatment with mabuterol (100 nmol) on positive chronotropic and inotropic responses induced by several cardiotonics in the canine isolated, blood-perfused atrium

Compound	n	Control		Mabuterol	
		AR (%)	*CF* (%)	*AR* (%)	*CF* (%)
Dobutamine (1 nmol)	9	13.0 ± 3.2	113.3 ± 12.8	0.6 ± 0.2**	9.7 ± 2.9**
Procaterol (1 nmol)	7	18.9 ± 6.0	58.6 ± 15.0	0.1 ± 0.1*	2.9 ± 2.4**
IBMX (135 nmol)	5	23.3 ± 1.9	52.7 ± 6.5	30.3 ± 7.8	95.4 ± 19.5
Bay K 8644 (0.8 nmol)	7	9.5 ± 2.7	59.4 ± 13.8	7.8 ± 2.9	58.5 ± 12.9
$CaCl_2$ (3 μmol)	5	2.2 ± 1.0	94.4 ± 10.7	2.2 ± 0.3	68.5 ± 7.6

Each value indicates the mean ± s.e.mean.
AR, atrial rate; CF, contractile force; IBMX, 3-isobutyl-1-methylxanthine.
Significantly different from each control value at $^*P < 0.05$ and $^{**}P < 0.01$.

1983). Stimulation of β_2-adrenoceptors increases heart rate and cardiac contractility in human heart (Arnold *et al.*, 1985; Gille *et al.*, 1985; Kaumann & Lemoine, 1987), although β_1-receptor-mediated responses are predominant. Previous findings have indicated that β_2-selective drugs affected chronotropy more than inotropy (Carlsson *et al.*, 1977; Stiles *et al.*, 1983; Friedman *et al.*, 1987). Our present study clearly shows that mabuterol is a selective β_2-adrenoceptor agonist; it increased heart rate more markedly than atrial force (41.4% in atrial rate and 12.9% in atrial tension of the maximal responses to isoprenaline), though maximal positive chronotropic and inotropic responses induced by 10 nmol mabuterol were much smaller than those of isoprenaline. We have reported that atrial contractile force increases in parallel with the pacing rate when an isolated, blood-perfused right atrium of the dog was paced at a rate of 2–4 Hz (a positive frequency-force relationship, Chiba, 1976; Furukawa *et al.*, 1986). Since the mabuterol-induced positive inotropic response was greater than that induced by the increase in the pacing rate and almost equalled in extent the mabuterol-induced positive chronotropic response, mabuterol itself increased the atrial tension independently of the mechanism of a positive frequency-force relationship. Osada *et al.* (1984) reported that mabuterol increases the atrial tension of the isolated, electrically driven left atrium of guinea-pig. The mabuterol- and procaterol-induced positive chronotropic and inotropic responses were inhibited more by a β_2-selective antagonist, ICI 118,551, than by a selective β_1-antagonist, atenolol. In contrast, the positive chronotropic and inotropic responses induced by dobutamine were inhibited by atenolol much more than by ICI 118,551. These results suggest that mabuterol exhibits positive chronotropic and inotropic responses mainly through β_2-adrenoceptors in the isolated, blood-perfused dog atrium.

Murai *et al.* (1984) found that mabuterol has a high selectivity for bronchial smooth muscle and has little effect on cardiac muscle in conscious guinea-pigs. Although β_2-adrenoceptors have been identified by radioligand binding studies in the guinea-pig, rat and rabbit atria (Minneman *et al.*, 1979a,b; Hedberg *et al.*, 1980; Brodde *et al.*, 1982; Molenaar *et al.*, 1987), only β_1-, and not β_2-adrenoceptors, mediate positive chronotropic and inotropic responses in these species (O'Donnell & Wanstall, 1979; 1980; Wilson & Lincoln, 1984; Molenaar & Summers, 1987). Results obtained from these species might, therefore, tend to underestimate the chronotropic and inotropic responses to selective β_2-agonists in man. Manalan *et al.* (1981) reported that the β_1/β_2 adrenoceptor ratio was approximately 85%/15% for dog ventricular myocardium. In the anaesthetized dog, propranolol inhibits the increase in heart rate induced by exercise more markedly than atenolol, suggesting that sinoatrial β_2-adrenoceptors might be stimulated by circulating catecholamines (Friedman *et al.*, 1987). We have also shown the existence of β_2-adrenoceptor-mediated positive chronotropic and inotropic responses in the isolated, blood-perfused canine right atrium and left ventricle (Akahane *et al.*, 1988; 1989). Thus, dogs may be more suitable than guinea-pigs, rats or rabbits for predicting chronotropic and inotropic effects of β-adrenoceptor-agonist compounds in man.

In the dog isolated atrium, mabuterol, at high doses, induced negative cardiac responses. The negative cardiac responses were not mediated by muscarinic cholinoceptors because the negative cardiac responses were not modified by atropine.

Osada *et al.* (1984) found that more than 0.1 $\mu g\,ml^{-1}$ mabuterol inhibits the positive inotropic effect of isoprenaline and decreases the maximum driving frequency in guinea-pig isolated left atria, finally suggesting a β_1-blocking activity and a quinidine-like activity of mabuterol at higher doses. In our experiments, mabuterol suppressed not only dobutamine- but also procaterol-induced positive chronotropic and inotropic responses, whereas IBMX-, Bay K 8644- and $CaCl_2$-induced responses were not inhibited by mabuterol. These results indicate that mabuterol possesses both β_1- and β_2-adrenoceptor blocking activities at higher doses. Fifty percent inhibitory doses of mabuterol on the positive chronotropic and inotropic responses to procaterol were smaller than those to dobutamine. Osada *et al.* (1984) showed that the concentration of mabuterol that induces a 50% relaxation of the tracheal smooth muscle of the guinea-pig is one-two hundredth of the concentration that inhibits the β_1-receptor-mediated positive inotropic response to isoprenaline by 50%. However, in the present study, mabuterol inhibited both β_1- and β_2-receptor-mediated responses and was rather more effective in inhibiting β_2-receptor-mediated responses.

In conclusion, mabuterol is a selective β_2-agonist which evokes small positive chronotropic and much weaker positive inotropic responses, and mabuterol also possesses a non-selective β-antagonistic activity in isolated and blood-perfused canine right atrium. Caution may be required in the use of this drug when the dosage of mabuterol is very high or its excretion is disturbed in asthmatic patients.

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The effect of Paf antagonists on bronchial hyperresponsiveness induced by Paf, propranolol or indomethacin

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1 Intravenous administration of platelet activating factor, Paf (600 ng kg^{-1} h^{-1}) to ventilated anaesthetised guinea-pigs induced bronchial hyperresponsiveness to i.v. acetylcholine.

2 Pretreatment of ventilated, anaesthetised guinea-pigs with the β-adrenoceptor antagonist, propranolol, or the non-steroidal anti-inflammatory drug, indomethacin, induced bronchial hyperresponsiveness to i.v. histamine.

3 Paf-induced bronchial hyperresponsiveness was significantly attenuated by pretreatment with three different Paf antagonists, CV-3988, BN 52021 and WEB 2086.

4 Pretreatment of guinea-pigs with CV-3988, BN 52021 or WEB 2086 at doses inhibiting Paf-induced bronchial hyperresponsiveness, had no significant effect on propranolol or indomethacin-induced bronchial hyperresponsiveness.

5 It is suggested that bronchial hyperresponsiveness induced by propranolol or indomethacin is not secondary to Paf release in the guinea-pig.

Introduction

Increased airway responsiveness to a wide range of stimuli is one of the characteristic features of bronchial asthma (Hargreave *et al.*, 1986). Exposure to allergens, viral infections, oxidant gases such as ozone or nitrogen dioxide, or sensitising chemicals such as toluene diisocyanate can induce airway hyperresponsiveness in both man and experimental animals (Barnes, 1986). However, it is also recognised that certain classes of drugs administered to asthmatics will also induce bronchoconstriction and increase the responsiveness of the airways.

Shortly after their introduction into clinical use, β-adrenoceptor antagonists such as propranolol were found to cause bronchospasm (McNeil, 1964; McNeil & Ingram, 1966), an effect which also occurred in the guinea-pig (Maclagan & Ney, 1979). This was assumed to be a consequence of β-adrenoceptor blockade in the lungs, but even the use of cardioselective β-adrenoceptor antagonists did not reduce the incidence of bronchospasm (Skinner *et al.*, 1975). Additionally, β-adrenoceptor antagonists have been found to increase the responsiveness of the airways to other bronchoconstrictor agents such as histamine and acetylcholine (Ney, 1983).

Another class of drugs known to induce bronchoconstriction in a subset of asthmatics and to increase the responsiveness of the airways to standard spasmogens are the non-steroidal anti-inflammatory drugs (NSAIDs) (Adcock & Garland, 1980; Mitchell, 1983; 1988; Mitchell & Adcock, 1988). However, the mechanisms underlying this drug-induced bronchial hyperresponsiveness remain to be elucidated.

Recent evidence has suggested that an ether-linked phospholipid, platelet activating factor (Paf), is able to induce a long lasting, non-specific increase in bronchial responsiveness in experimental animals (Mazzoni *et al.*, 1985; Chung *et al.*, 1986; Christman *et al.*, 1987; Barnes *et al.*, 1987; Robertson & Page,

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1987; Coyle et al., 1988; Robertson et al., 1988) and man (Cuss et al., 1986). We have, therefore, investigated whether bronchial hyperresponsiveness induced by either indomethacin or propranolol is due to the release of Paf by the use of three distinct classes of Paf antagonists: a Paf analogue (CV-3988), a ginkgolide (BN 52021) and a triazolobenzodiazepine (WEB 2086).

Methods

Male Dunkin-Hartly guinea pigs (300–600 g) were anaesthetised with urethane (25% w/v, 7 ml kg^{-1}; i.p.) and the trachea, carotid artery and jugular vein cannulated for measurement of airway obstruction, systemic blood pressure and drug administration respectively. Guinea-pigs were ventilated with room air at 70 strokes min^{-1} with a stroke volume of 1 ml 100 g^{-1} body weight. Airway obstruction was measured using a differential pressure transducer connected to the side-arm of the tracheal cannula, and pulmonary inflation pressure (PIP) measured in mmHg.

Specificity of Paf antagonists

Acetylcholine (ACh) (5 $\mu g\,kg^{-1}$) or histamine (5 $\mu g\,kg^{-1}$) were administered intravenously as a bolus before and 30 min after treatment with BN 52021 (20 mg kg^{-1}, i.v.), CV-3988 (1 mg kg^{-1} i.v.) or WEB 2086 (1 mg kg^{-1} i.v.).

Airway hyperresponsiveness

Induced by Paf ACh (5 $\mu g\,kg^{-1}$) was administered intravenously as a bolus before, and 10 min after the infusion of Paf (600 ng kg^{-1} over 1 h). The total dose of Paf was infused at three increasing rates, (5% in 10 min, 20% in 20 min and 75% in 30 min) as described previously (Robertson & Page, 1987). The percentage increase in PIP induced by ACh (5 $\mu g\,kg^{-1}$, i.v.) was calculated before and after the infusion of Paf. Hyperresponsiveness was assessed by the incremental increase in response to ACh, determined by calculating the difference between the increase in PIP (%) induced by ACh before and after the infusion of Paf. Bronchial hyperresponsiveness was measured in animals pretreated with one of the various Paf antagonists or the appropriate vehicle control, according to the following protocol: WEB 2086 (1 mg kg^{-1}) or vehicle administered i.p. 1 h before anaethesia; CV 3988 (1 mg kg^{-1}) or vehicle administered i.v. immediatley before infusion of Paf; BN 52021 (20 mg kg^{-1}) or vehicle administered i.v. 30 min before anaesthesia.

Induced by propranolol or indomethacin Bronchoconstriction was induced by histamine (1–64 $\mu g\,kg^{-1}$, i.v.) and the dose required for a 50–100% increase in PIP was selected. This dose of histamine (2–8 $\mu g\,kg^{-1}$) was then given before and 15, 30 and 60 min after either propranolol (0.1 mg kg^{-1}, i.v.) or indomethacin (5 mg kg^{-1}, i.v.). Paf antagonists were administered according to the following protocol: WEB 2086 (1 mg kg^{-1}, i.p.) 1 h before propranolol or indomethacin; CV 3988 (1 mg kg^{-1}, i.v.) 10 min before propranol or indomethacin, and BN 52021 (20 mg kg^{-1}, i.p.) 30 min before propranolol or indomethacin.

Results are expressed as the percentage increase in the PIP response to histamine at each time interval after treatment with either indomethacin or propranolol.

Materials

Acetylcholine chloride, bovine serum albumin (BSA; fraction V, essentially fatty acid free) and urethane were purchased from Sigma and dissolved in physiological saline. Paf was obtained from Novabiochem Ltd and prepared in 0.25% BSA. (±)-Propranolol hydrochloride (Imperial Chemical Industries plc) was dissolved in physiological saline and indomethacin (Sigma) was dissolved in Na_2CO_3. WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo-[1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanon) (a gift from Dr Heuer, Boehringer Ingelheim KG, West Germany) was dissolved in saline, CV 3988 ((RS)-2-methoxy-3-(octadecylcarbamoyloxy) propyl-2-(3-thiazolo) ethyl phosphate) (a gift from Takeda Chemical Industries Ltd) was dissolved in heated saline and the pH adjusted to 7 by adding 1 M NaOH, BN 52021 ([3(1,1-dimethylethyl)hexahydro-1,4,4,7-B-trihydroxy-8-methyl 9H-1,7-(epoxymethanol) - 14,6 - alpha - H_2 - cyclopenta(c)furo [2,3b]furo[3',2',3,4] cyclo - penta [1,2 - d]furah-5,-9,12 (4H)-trione) (a gift from Dr P. Braquet, Institute Henri Beaufour, Le Plessis Robinson, France) was prepared as a suspension in acacia gum (10%) for i.p. administration and in 2% DMSO for i.v. administration.

Statistical analysis

Data are presented as the mean ± s.e.mean of n observations. Significance was assessed by the non-

Table 1 Specificity of Paf antagonists against histamine- and acetylcholine (ACh)-induced bronchoconstriction

		BN 52021 (20 mg kg^{-1} i.v. bolus)	WEB 2086 (1 mg kg^{-1} i.v. bolus)	CV-3988 (1 mg kg^{-1} i.v. bolus)
ACh	Before drug	56.25 ± 3.8	49.3 ± 8.7	47.9 ± 6.02
(5 μg kg^{-1} i.v.)	After drug	52.0 ± 9.6	49.2 ± 8.1	37.02 ± 3.3
Histamine	Before drug	146.0 ± 18.6	151.2 ± 26.5	141.4 ± 29.71
(5 μg kg^{-1} i.v.)	After drug	137.0 ± 22.7	152.5 ± 22.0	135.8 ± 26.7

The effect of pretreatment with Paf antagonists on % increase in pulmonary inflation pressure (PIP) induced by ACh or histamine. Results shown are mean ± s.e. % increase in PIP before and after Paf antagonists, $n = 5$–7 animals. None of the Paf antagonists utilised had any significant effect on ACh or histamine-induced increases in PIP.

parametric Wilcoxon matched-pairs signed-ranks test, with $P < 0.05$ being considered significant.

Results

The intravenous bolus administration of ACh (5 μg kg^{-1}) or histamine (5 μg kg^{-1}) induced significant increases in PIP which were not modified by any of the Paf antagonists utilised (Table 1).

The intravenous infusion of Paf in control guinea-pigs (treated with appropriate vehicles for antagonists) induced an increase in the airway responsiveness to ACh. This increase was significantly reduced by pretreatment with each of the Paf antagonists used (Figures 1–3).

Propranolol (0.1 mg kg^{-1} i.v.) and indomethacin (5 mg kg^{-1} i.v.) induced an increase in the airway responsiveness to histamine (Figure 4 and Table 2), and this effect was maximal at 30 min after the administration of each compound.

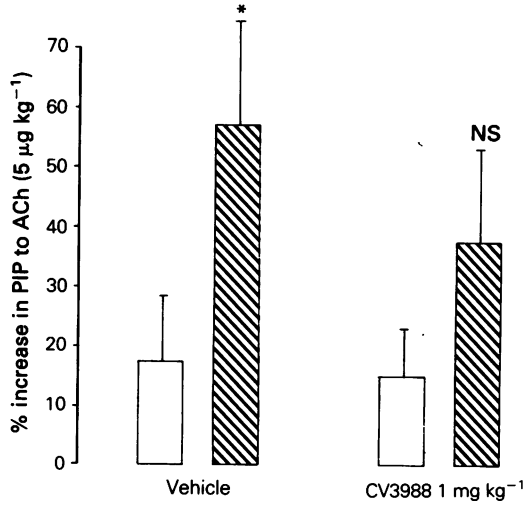

Figure 1 Airway responsiveness to acetylcholine (ACh, 5 μg kg^{-1}, i.v.) before (open columns) and after (hatched columns) an infusion of Paf (600 ng kg^{-1} h^{-1}, i.v.) in vehicle or CV-3988 (1 mg kg^{-1}, i.v.) treated animals. Results shown are the mean percentage increase in pulmonary inflation pressure (PIP) in 5–11 separate experiments and vertical lines show s.e.mean. Responses to ACh were significantly increased after Paf (*$P < 0.001$), but no significant increase was observed in animals pretreated with CV-3988 (NS).

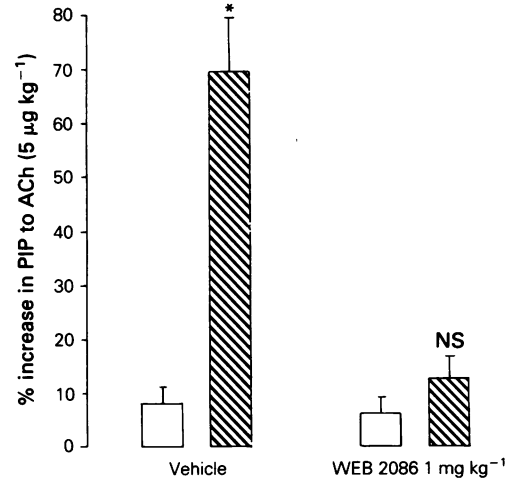

Figure 2 Airway responsiveness to acetylcholine (ACh, 5 μg kg^{-1}, i.v.) before (open columns) and after (hatched columns) an infusion of Paf (600 ng kg^{-1} h^{-1}, i.v.) in vehicle or WEB 2086 (1 mg kg^{-1}, i.p.) treated animals. Results shown are the mean percentage increase in pulmonary inflation pressure (PIP) of 5–11 separate experiments and vertical lines show s.e.mean. Responses were significantly increased after Paf (*$P < 0.001$), but no significant increase was observed in animals pretreated with WEB 2086 (NS).

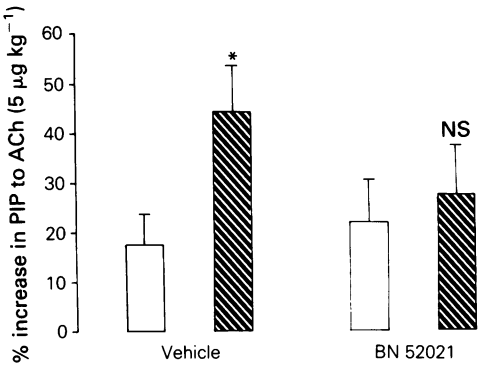

Figure 3 Airway responsiveness to acetylcholine (ACh, 5 $\mu g kg^{-1}$, i.v.) before (open columns) and after (hatched columns) an infusion of Paf (600 $ng kg^{-1}$, i.v.) in vehicle or BN 52021 (20 $mg kg^{-1}$, i.v.) treated animals. Results shown are the mean percentage increase in pulmonary inflation pressure (PIP) of 4 separate experiments and vertical lines show s.e.mean. Responses were significantly increased after Paf (* $P < 0.001$), but no significant increase was observed in animals pretreated with BN 52021 (NS).

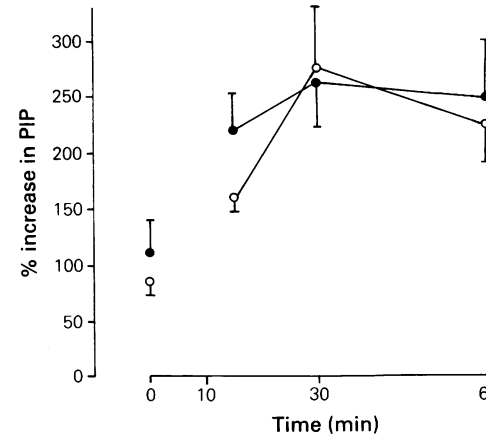

Figure 4 Percentage increase in pulmonary inflation pressure (PIP) induced by intravenous histamine before and at intervals after pretreatment with either propranolol (●) or indomethacin (○). Results are expressed as mean of 4–6 experiments and vertical lines indicate s.e.mean.

Table 2 Effect of Paf antagonists on propranolol and indomethacin-induced bronchial hyperresponsiveness

Drug	*% increase PIP*	
	Histamine 5 $\mu g kg^{-1}$	Histamine 5 $\mu g kg^{-1}$ after propranolol 0.1 $mg kg^{-1}$ i.v.
Saline	110 ± 3	263 ± 40
BN 52021 20 $mg kg^{-1}$ i.v.	92 ± 2	259 ± 14
WEB 2086 1 $mg kg^{-1}$ i.v.	96 ± 1	233 ± 26
CV 3988 1 $mg kg^{-1}$ i.v.	120 ± 2	264 ± 33
	Histamine 5 $\mu g kg^{-1}$	Histamine 5 $\mu g kg^{-1}$ after indomethacin 1 $mg kg^{-1}$ i.v.
Saline	85 ± 1	274 ± 56
BN 52021 20 $mg kg^{-1}$ i.v.	79 ± 2	230 ± 56
WEB 2086 1 $mg kg^{-1}$ i.v.	90 ± 2	220 ± 30
CV 3988 1 $mg kg^{-1}$ i.v.	80 ± 2	222 ± 52

The effect of pretreatment with Paf antagonists or saline on % increase in pulmonary inflation pressure (PIP) induced by intravenous histamine (5 $\mu g kg^{-1}$; $n = 5$) before and after treatment with propranolol (0.1 $mg kg^{-1}$ i.v.) or indomethacin (5 $mg kg^{-1}$ i.v.).

CV 3988, WEB 2086 and BN 52021 were all without significant effect on the airway hyperresponsiveness induced by propranolol and indomethacin (Table 2).

Discussion

Indomethacin has been shown to enhance the airway responsiveness to the spasmogen histamine both *in vitro* (Adcock & Garland, 1980) and *in vivo* (Mitchell, 1983; 1988). This phenomenon is not peculiar to indomethacin since other NSAIDs such as flufenamate, aspirin and phenylbutazone have also been shown to increase the bronchoconstrictor response to histamine in guinea-pigs (Mitchell & Adcock, 1987). Recent evidence has suggested that this phenomenon may involve an action of NSAIDs on the vagus nerve (Mitchell, 1988). In about 10% of patients with asthma, aspirin and other NSAIDs precipitate asthmatic attacks. Recently, platelets taken from aspirin-sensitive asthmatics have been demonstrated to release cytotoxic oxygen-free radicals when incubated with aspirin *in vitro*, whilst sodium salicylate, which is structurally related to aspirin but does not inhibit cyclo-oxygenase, does not activate such platelets (Ameisen *et al.*, 1985). These and other observations implicate the participation of platelets in the aetiology of this condition (Page, 1988a). Recently Schmitz-Schumann *et al.* (1987) have demonstrated that Paf release accompanies bronchoconstriction induced by NSAIDs in

aspirin-sensitive asthmatics. As Paf is able to induce bronchial hyperresponsiveness (Mazzoni *et al.*, 1985; Chung *et al.*, 1986; Barnes *et al.*, 1987; Robertson & Page, 1987; Robertson *et al.*, 1988; and present study), it was possible, therefore, that release of Paf could contribute to airway hyperresponsiveness induced by NSAIDs, particularly since Paf-induced bronchial hyperresponsiveness in the guinea-pig is known to be dependent on platelet activation (Mazzoni *et al.*, 1985).

However, the present results show that none of the Paf antagonists tested had any significant effect upon indomethacin-induced airway hyperresponsiveness in the guinea-pig, at concentrations that clearly inhibit Paf-induced bronchial hyperresponsiveness. The doses used to inhibit Paf-induced bronchial hyperresponsiveness were also comparable to those found previously to inhibit other biological actions of Paf (e.g. bronchoconstriction or hypotension) (Terashita *et al.*, 1983; Braquet *et al.*, 1985; Casals-Stenzel *et al.*, 1987) and our results indicate that at the concentrations employed, BN 52021, WEB 2086 and CV-3988 dδ not have muscarinic or H_1-receptor antagonist activity. These results suggest, therefore, that Paf does not play a central role in bronchial hyperresponsiveness induced by NSAIDs in the guinea-pig.

The mechanism of airway hyperresponsiveness induced by β-adrenoceptor antagonists is completely unknown. Ney (1983) demonstrated that it was not due to β-adrenoceptor blockade, since the dextrorotatory isomer of propranolol and the racemic mixture were equipotent at producing airway hyperresponsiveness, whilst the dextrorotatory isomer is considerably less potent as a β-adrenoceptor antagonist. Interestingly, the β-adrenoceptor agonist isoprenaline has also been demonstrated to induce airway hyperresponsiveness in the guinea-pig, this effect being additive with propranolol (Mazzoni *et al.*, 1987) and unrelated to occupancy of β-adrenoceptors. Ney (1983) showed that part of the propranolol-induced hyperresponsiveness in guinea-pigs could be inhibited by the leukotriene antagonist FPL-55712 or the combined lipoxygenase/cyclo-oxygenase inhibitor BW755C, which suggests a role for lipoxygenase-derived mediators in the pathogenesis of part of this phenomenon. We considered that Paf may also play a role in this phenomenon as it is often released from membrane phospholipids alongside arachidonic acid metabolites and is able to induce bronchial hyperresponsiveness. However, the present study clearly demonstrated that none of the Paf antagonists was able to inhibit significantly propranolol-induced bronchial hyperresponsiveness at doses that clearly inhibited Paf-induced bronchial hyperresponsiveness. Therefore, whilst Paf is able to induce many of the characteristic features of asthma, including bronchial hyperresponsiveness (Page, 1988b), our present data would suggest that this phospholipid is not involved with bronchial hyperresponsiveness induced by either NSAIDs or propranolol.

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Developmental disappearance of excitatory α_1-adrenoceptor function in the oesophagus of chick embryo

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1 Developmental change in the response to noradrenaline (NA) and 5-hydroxytryptamine (5-HT) was investigated in the chick oesophagus between 9 and 21 days of incubation and 4 days after hatching.

2 NA (5 μM) produced a significant contraction in the oesophagus at 9 days of incubation. The NA-induced contraction progressively decreased with development and changed to an inhibition of spontaneous contraction or a small relaxation by 17 days of incubation.

3 The NA-induced contractile response was inhibited by phentolamine (2.7 μM) and prazosin (0.55 μM). Phenylephrine (5 μM) but not clonidine (5–50 μM), also induced a contraction at early stages. The relaxation response to NA was sensitive to the β-receptor blocker, carteolol (3.4 μM).

4 Pretreatment with carteolol unmasked the contractile responses to NA in preparations at 17–19 days of incubation. However, even in the presence of carteolol, the contraction produced by NA decreased and disappeared by the time of hatching. This change in response to NA is accompanied by a decline in the pD_2 value. The response to phenylephrine (5 μM) followed the same pattern as that to NA.

5 The maximum binding sites of [^{3}H]-dihydroergocryptine to the crude membrane preparation from oesophagus changed little at 13, 17 and 21 days of incubation.

6 Isoprenaline (Iso, 0.01–20 μM) caused a carteolol-sensitive relaxation in the carbachol-contracted oesophagus after 13 days of incubation. The sensitivity (pD_2 value) to Iso decreased slightly up to 17 days of incubation.

7 5-HT (10 μM) caused a contraction in the oesophagus after 13 days of incubation and the amplitude of the response increased up to 17 days of incubation. The response to 5-HT was abolished by methysergide (1 μM) but not by tetrodotoxin (0.78 μM) or atropine (1 μM) at every stage tested.

8 These results suggest that the response to NA changed from an α_1-adrenoceptor-mediated contraction to a β-receptor-mediated relaxation during the embryonic period, resulting partly from the decline and disappearance of excitatory α_1-receptor function in the chick oesophagus.

Introduction

A few reports are available on the development of responsiveness to drugs in the smooth muscle during the early embryonic period (see Klinger, 1983). It has been realized that the responses of the smooth muscle mediated by muscarinic receptors consistently appear at early embryonic stages of development in the gastrointestinal tract of guinea-pigs (Gershon & Thompson, 1973) and rats (Miyazaki *et al.*, 1982; Ito *et al.*, 1988) and that the sensitivity to muscarinic drugs is relatively constant (Boréus & McMurphy, 1971; Miyazaki *et al.*, 1982) or progressively increased (Ito *et al.*, 1988) during the embryonic period.

There is a relative lack of systematic and quantitative analyses concerning developmental change of adrenoceptor functions. The few results that are available tend to be inconsistent. In human foetal intestine, the relaxation induced by catecholamines was reported to be mediated by only β-adrenoceptors (McMurphy & Boréus, 1968) or by

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both α- and β-receptors (Hart & Mir, 1971). On the other hand, in the lower part of colon (Ohkawa, 1978) and the duodenum (Munro, 1953) of guinea-pig foetus, an excitatory α-adrenoceptor, which does not function in mature animals, was also reported to be present. These findings suggest the possibility that adrenoceptors change during embryonic development. However, little is known about when the smooth muscle acquires responsiveness to catecholamines and whether the sensitivity to catecholamines remains constant throughout early embryonic and postnatal development.

Recently, we showed that the nature of the contractile response due to histamine changed from myogenic to neurogenic in the chick oesophagus between the embryonic and post-hatching periods (Miyazaki *et al.*, 1987).

In this study, we investigated the response induced by noradrenaline (NA) in the smooth muscle of chick oesophagus from 9 days of incubation to hatching (usually at 21 days) and 4 days after hatching to clarify the appearance and development of adrenoceptor functions. The responsiveness to 5-hydroxytryptamine (5-HT) was simultaneously studied as a control, since 5-HT has been found to produce a significant contraction in chick oesophagus (Bartlet & Hassan, 1968).

Methods

Animals

Incubated fertilized eggs and hatched chicks (Shaber Star Cross) were used. The eggs were maintained in a humidified incubator at 38 ± 0.5°C until the time of experiment. The age of chicks, which usually hatched at 21 days of incubation, was designated as day 0 at hatching. The chicks examined were from 9 days of incubation to 4 days after hatching.

Organ bath study

The segment of oesophagus upward from the crop, about 0.5–1.5 cm long, was isolated from the embryos or the chicks, which had been killed by stunning. The segment were placed in a 20 ml organ bath containing Krebs solution which was composed of (mM): NaCl 118.0, KCl 4.75, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.5. The solution was saturated with a mixture of 95% O_2 and 5% CO_2 (pH 7.4) and kept at 37 ± 1°C. The oesophageal segment was allowed to equilibrate for 45–60 min before any other experimental procedure was started. The longitudinal movement of the oesophagus was recorded by means of an isotonic transducer (Nihon Kohden, TD-112S) and a pen writing recorder (Nihon Kohden, RJG-3002). Drugs were applied to the organ bath directly with a pipette. As the amplitude of the responses induced by drugs greatly depended on the length of the oesophageal preparations, all responses were expressed as a percentage of the response caused by the application of potassium chloride (high K^+, 80 mM).

The tension applied to preparations was 10–20 mg from 9–11 days of incubation; 35–45 mg at 13 days of incubation; 100–120 mg at 15 days of incubation; 200–250 mg at 17 days of incubation; 450–500 mg at 19 days of incubation; 1–1.5 g from 21 days of incubation to 4 days after hatching.

Radioligand binding assay

The isolated oesophagus was homogenized in cold Tris buffer solution (Tris 50 mM, $MgCl_2$ 10 mM, pH 7.4) with a Polytron. The homogenate was filtered through nylon mesh and crude membrane suspensions were prepared from the filtrate according to the method of Williams *et al.* (1976). Binding of [^{3}H]-dihydroergocryptine ([^{3}H]-DHE) to α-receptors in crude membrane preparations was measured as follows. The membrane preparation, containing 150–400 μg protein, was incubated with [^{3}H]-DHE for 15 min at 25°C. The reaction was stopped by addition of 4 ml ice-cold buffer. The mixture was passed through a filter (Whatman GF/C) positioned over a vacuum and washed with 3 × 4 ml of ice-cold buffer. The radioactivity remaining on the filter was measured at about 40% efficiency. Specific binding was expressed as the differences between binding in the presence or absence of 10 μM phentolamine. The data were applied to the Scatchard plot and analyzed by the least squares method. The binding of [^{3}H]-DHE was linearly related to the amount of protein in the incubation mixture up to approximately 500 μg.

Protein in the homogenates and membrane preparations was measured as described by Lowry *et al.* (1951) with calf serum albumin used as a standard.

Drugs

Drugs used and their sources were; acetylcholine chloride (ACh, Wako), atropine sulphate (Wako), carbamylcholine chloride (carbachol; Merck), carteolol hydrochloride (Otsuka), clonidine hydrochloride (Sigma), 5-hydroxyptryptamine creatinine sulphate (5-HT, Aldrich), isoprenaline bitartrate (Iso, Sigma), methysergide hydrogen maleate (Sandoz), noradrenaline bitartrate (NA, Sigma), papaverine hydrochloride (Tokyo Kasei), phenylephrine tartrate (Tokyo Kasei), phentolamine methansulphonate (Ciba), prazosin hydrochloride (Pfizer Taito), tetrodotoxin (TTX, Sigma), yohimbine hydrochloride

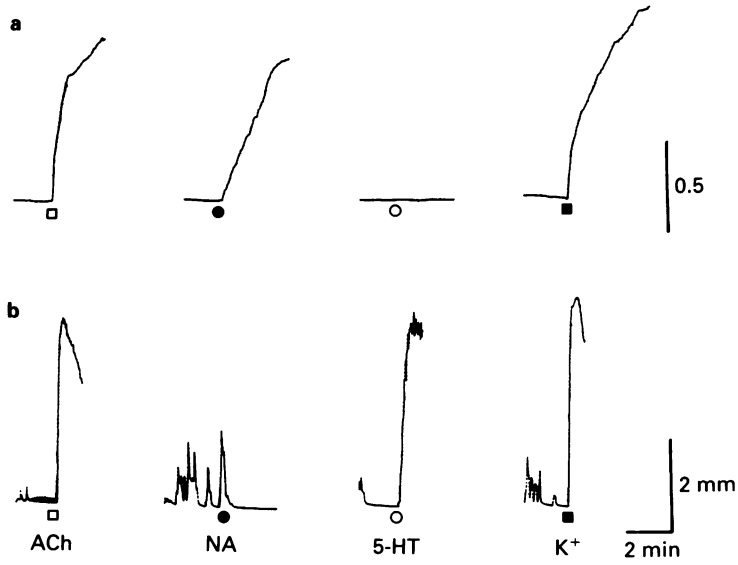

Figure 1 Responses induced by acetylcholine (ACh, 20 μM, □), noradrenaline (NA 5 μM, ●), 5-hydroxytryptamine (5-HT, 10 μM, ○) and high K^+ (K^+, 80 mM, ■) in the chick oesophagus at 11 days of incubation (a) and 17 days of incubation (b).

(Sigma) and [^{3}H]-dihdroergocryptine ([^{3}H]-DHE, New England Nuclear).

Statistical analysis

All results are expressed as means ± s.e.mean. The difference between the mean values for data were evaluated by Student's *t* test. A *P* value of 0.05 or less was considered statistically significant.

Results

Responses to noradrenaline and 5-hydroxytryptamine at different stages

The application of NA (5 μM) elicited a tonic contraction in preparations from embryos after 9–13 days of incubation (Figure 1a). The contractile response to NA progressively decreased in preparations from 9 to 15 days of incubation (Figure 2a). In most preparations after 17 days of incubation, the contractile response to NA disappeared and NA caused a small relaxation or inhibited the spontaneous contraction in some preparations which had a high tone and spontaneous activity (Figure 1b). In contrast to the NA-induced response, 5-HT (10 μM) did not cause any response in early stages (Figure 1a). The contractile responses to 5-HT (10 μM) first appeared at 13 days of incubation. The amplitude of 5-HT-induced contraction rapidly increased up to 17 days of incubation and remained unchanged to 4 days after hatching (Figure 1b and Figure 2b). During this period, the contractile response to ACh (20 μM) or high K^+ was consistently observed (Figure 1).

Pharmacological analysis of the responses to noradrenaline and 5-hydroxytryptamine

The NA-induced contraction was abolished or markedly inhibited by treatment with the α-adrenoceptor blocking agent, phentolamine (2.7–5.4 μM, $n = 11$), while the β-blocker carteolol (3.4 μM) potentiated the response to NA to different extents depending on the developmental stages (Figure 3a). Atropine (1 μM), which blocked the response to ACh (20 μM), did not affect the contraction induced by NA. In the preparation at later embryonic stages in which the response to NA was not observed, NA (5 μM) produced a relaxation when the tone of preparations was increased by adding carbachol (0.5 μM). The inhibitory response to NA (5 μM) was antagonized by carteolol but not by phentolamine ($n = 4$, Figure 3b). These findings suggest that the contraction and relaxation caused by NA was mediated by α- and β-adrenoceptors, respectively.

The contractile response to NA (5 μM) in preparations pretreated with carteolol was more inhibited by an α_1-adrenoceptor blocking agent prazosin (0.55 μM) than by an α_2-receptor blocker yohimbine (1 μM, Figure 4). Furthermore, phenylephrine (5 μM), an α_1 agonist, caused a contraction. On the other hand, clonidine (5–50 μM), an α_2 agonist, failed to

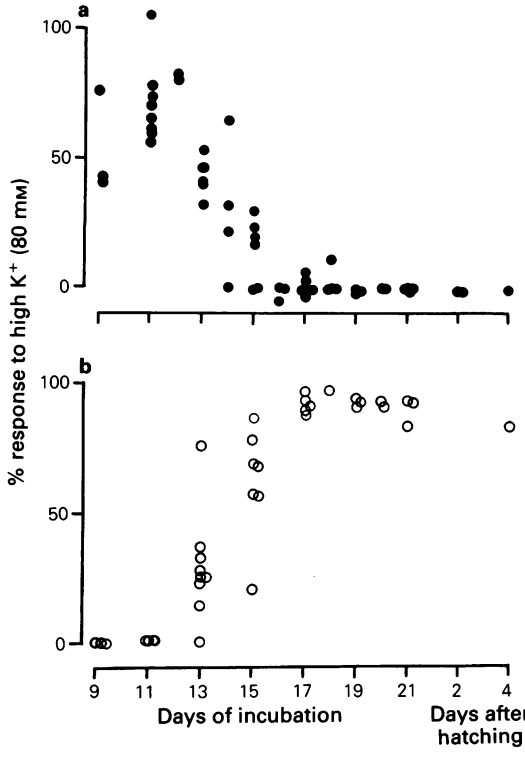

Figure 2 Developmental changes in the amplitude of responses to noradrenaline (NA) and 5-hydroxytryptamine (5-HT) in the chick oesophagus. Ordinate scales represent the relative amplitude of the contraction induced by NA (5 μM, ●, a) and 5-HT (10 μM, ○, b), expressed as a percentage of the high K^+ (80 mM)-induced contraction at various developmental stages.

induce a response in either preparations at 15 days of incubation or 2 days after hatching ($n = 10$). These results suggest that the receptor responsible for the contraction induced by NA was the α_1 subtype.

The contraction produced by 5-HT in preparations at various stages was consistently blocked by methysergide (1 μM, $n = 10$), but was not affected by atropine (1 μM, $n = 7$) or TTX (0.78 μM, $n = 6$), suggesting that 5-HT caused a contraction via the 5-HT receptors on the smooth muscle.

Contractile response mediated by α_1-adrenoceptors during development

To determine whether the change in response to NA from contraction to relaxation could be attributed to the decline of excitatory α_1-receptor function, NA responses after β-receptor blockade as well as phenylephrine responses were observed at various developmental stages (Figure 5). Pretreatment with carteolol (3.4 μM) augmented the contractile response to NA (5 μM) at early stages (13–15 days of incubation). However, even in the presence of carteolol, the NA-induced contraction decreased with development and finally disappeared by the time of hatching. The contractile responses induced by phenylephrine (5 μM) similarly declined and no appreciable contraction could be produced by phenylephrine after 21 days of incubation (Figure 5).

The developmental change in the receptor-mediated response to NA was estimated quantitatively by generating cumulative dose-response curves in preparations treated with carteolol from embryos after 13 days of incubation to 2 days after hatching. The dose-response curves to NA gradually shifted to the right with the decline of maximum response from 13 days of incubation (Figure 6). After 21 days of incubation the response to NA was slight (5–10% of high K^+ response) even at higher concentrations (200 μM). The calculated pD_2 values (a negative logarithm of 50% effective dose) also significantly declined from 6.55 ± 0.10 ($n = 5$) at 13 days of incubation to 5.39 ± 0.14 ($n = 6$) at 19 days of incubation, suggesting the decrease in sensitivity of α_1-receptors to NA.

Developmental change in α-receptor population

The binding of [^{3}H]-DHE to crude membrane preparations from chick oesophagus at different stages was saturable with a high affinity and the Scatchard plots derived from the specific binding of [^{3}H]-DHE were straight lines with a correlation coefficient of 0.948 ± 0.011 ($n = 13$).

The maximum binding sites (B_{max}; fmol mg^{-1} protein) of [^{3}H]-DHE, estimated from the Scatchard plot, did not significantly ($P > 0.05$) differ between 13 days of incubation and 21 days of incubation, though the B_{max} at 17 days of incubation tended to be greater than that at 21 days of incubation (Table 1). The apparent dissociation constant (K_d value) of [^{3}H]-DHE did not significantly change during the developmental stages tested (Table 1). This suggests that the number of [^{3}H]-DHE binding sites in the chick oesophagus change little during embryonic development. Thus, the disappearance of the contraction mediated by α_1-receptors during the embryonic period cannot be explained by the change in density of α_1-receptors in the oesophagus.

Relaxation responses mediated by β-adrenoceptors during development

The response to isoprenaline (Iso) in the presence of phentolamine (2.7 μM) was examined in preparations treated with carbachol, at a concentration that pro-

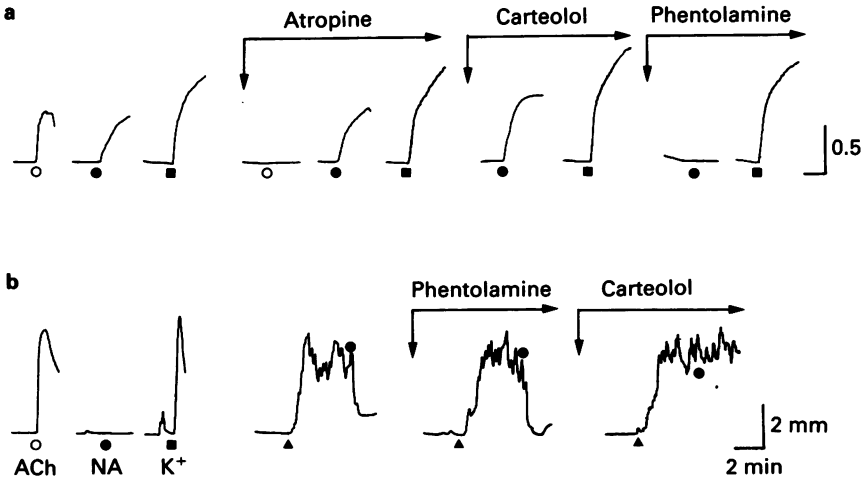

Figure 3 The effects of drugs on the contractile and relaxant responses to noradrenaline (NA) in the chick oesophagus preparations at 11 days of incubation (a) and 18 days of incubation (b). The responses to acetylcholine (ACh, 20 μM, ○), NA (5 μM, ●), and high K^+ (80 mM, ■) were observed in the presence (↓⟶) or absence of atropine (1 μM), carteolol (3.4 μM) or phentolamine (2.7 μM). In (b), the inhibitory response to NA was also observed in the preparation contracted by carbachol (0.5 μM, ▲).

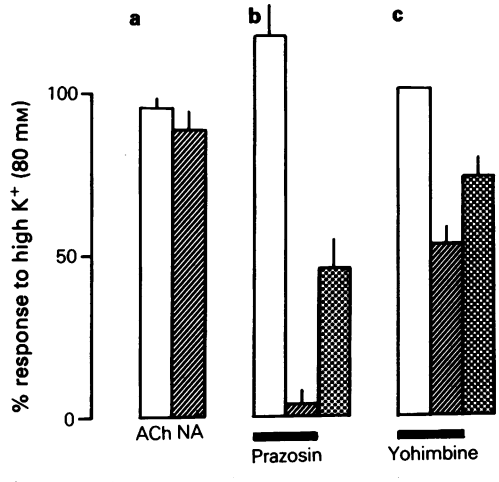

Figure 4 The effects of prazosin and yohimbine on the responses to noradrenaline (NA) and acetylcholine (ACh) in the presence of carteolol (3.4 μM). Ordinate scale represents the amplitude of responses to NA (5 μM) and ACh (20 μM) expressed as percentages of that induced by high K^+ (80 mM). Open and hatched columns show the mean (n = 2–7) of response to ACh (20 μM) and NA (5 μM) in the absence (a) or presence of prazosin (0.55 μM, b) or yohimbine (1 μM, c). Stippled columns represent the recovery responses after washing out prazosin (b) or yohimbine (c). Vertical lines show s.e.means.

duced a contraction of 70–80% of high K^+ contraction, from embryos at different stages. Iso (0.01–20 μM) did not cause a relaxation at 9 days of incubation (n = 3), while addition of papaverine

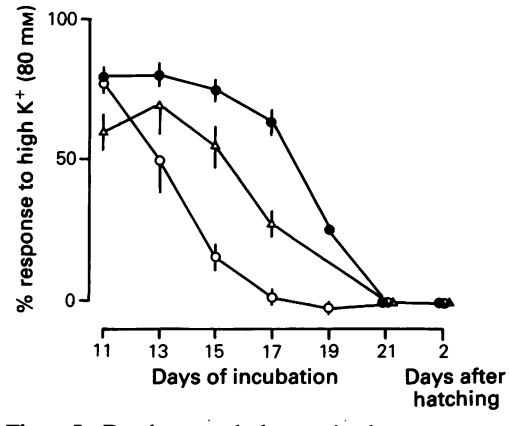

Figure 5 Developmental changes in the responses of the chick oesophagus to noradrenaline (NA) in the absence or presence of carteolol and to phenylephrine. Ordinate scale represents the relative amplitudes of responses to NA (5 μM) in the presence (●) or absence (○) of carteolol (3.4 μM) and to phenylephrine (5 μM, △), expressed as a percentage of the response to high K^+ (80 mM). Abscissa scale represents the age of chick in days. Each point shows the mean amplitude (n = 3–9); vertical lines represent s.e.means.

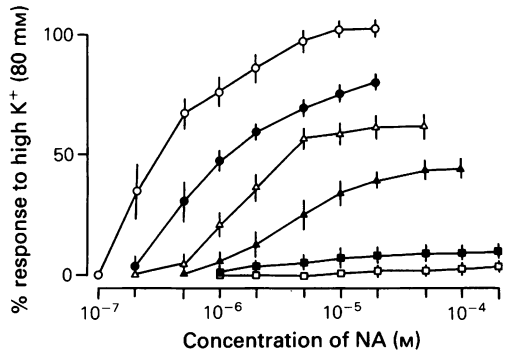

Figure 6 The concentration-response relationships to noradrenaline (NA) in the presence of carteolol in the chick oesophagus at different ages. Ordinate scale shows the relative amplitude of the response to NA in the presence of carteolol (3.4 μM) expressed as a percentage of the response to high K^+ (80 mM). Abscissa scale indicates the concentration of NA on a logarithmic scale. Symbols represent the mean amplitude (n = 3–6) of NA response at 13 (○), 15 (●), 17 (△), 19 (▲) and 21 days of incubation (□) and 2 days after hatching (■). Vertical lines represent s.e.means.

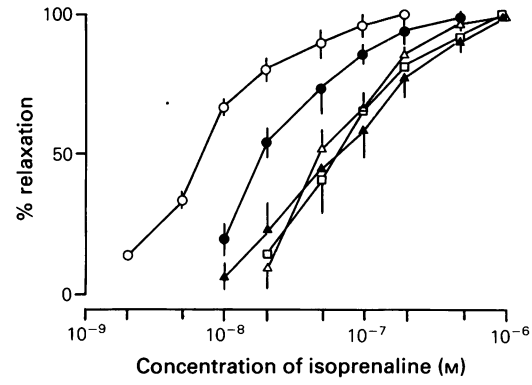

Figure 7 The concentration-response relationships to isoprenaline in the chick oesophagus at different stages. Ordinate scale shows the relative amplitude of the isoprenaline-induced relaxation as a percentage of carbachol (0.5–1 μM)-induced contraction. Abscissa scale shows the concentration of isoprenaline on a logarithmic scale. Symbols represent the mean amplitude (n = 3–4) of the response to isoprenaline at 13 (○), 15 (●), 17 (△), 19 (▲) and 21 days of incubation (□). Vertical lines represent s.e.means.

(100 μM) relaxed the preparations at this stage to a resting tone (n = 3). Distinct dose-dependent relaxations were caused by Iso (0.01–20 μM) in preparations after 13 days of incubation (Figure 7). The relaxation response to Iso was antagonized by carteolol (3.4–6.8 μM). The sensitivity (pD_2 value) to Iso tended to decrease from 13 days of incubation (8.12 ± 0.01, n = 3) up to 17 days of incubation (7.30 ± 0.06, n = 3) and thereafter remained unchanged up to 21 days of incubation (7.23 ± 0.15, n = 3, Figure 7). However, in about half of the preparations (n = 4) at 21 days of incubation, Iso failed to cause a relaxation to resting level even when used in high concentrations (10–20 μM).

Table 1 Parameters for the binding of α-adrenoceptor ligand to crude membrane preparations from chick oesophagus at different stages

	Days of incubation		
	13	*17*	*21*
[^{3}H]-DHE B_{max} (fmol mg^{-1} protein)	137.4 ±12.4	159.8* ±7.3	109.6* ±18.6
K_d (nM)	9.76 ±2.50 (4)	10.53 ±1.52 (6)	13.6 ±1.8 (3)

[^{3}H]-DHE = [^{3}H]-dihydroergocryptine. These parameters were calculated from a Scatchard plot. *P < 0.05 between preparations.

Discussion

The present experiments demonstrate that the response of the chick oesophageal smooth muscle to NA changes from a contraction to a relaxation during the embryonic period. The contraction induced by NA was observed in preparations taken at the earliest stage tested (9 days of incubation), at which the longitudinal muscle layer was observed to appear morphologically in the chick oesophagus (see Romanoff, 1960). The contraction induced by NA appeared to be mediated by α_1-adrenoceptors, as the response to NA was more sensitive to phentolamine or prazosin than to yohimbine. This is further supported by the fact that phenylephrine but not clonidine caused a similar contraction. These findings suggest that excitatory function mediated by α_1-adrenoceptors appears in the smooth muscle of chick oesophagus at early stages of development.

In stages after 17 days of incubation, NA produced a slight inhibitory effect, such as inhibition of spontaneous contraction or a small relaxation. Similar inhibitory effects of NA have been observed in the chick oesophagus 1–7 days after hatching (Bowman & Everett, 1964; Everett, 1966; Hassan & Osman, 1976). Following the blockade of β-receptors, the inhibitory effect of NA was prevented and a contraction occurred in the preparation up to 19 days of incubation, indicating that α_1- and β-receptors coexist during this period. The inhibitory β-receptor function may not develop in early stages,

because Iso caused a distinct relaxation in carbachol-contracted oesophagus only after 13 days of incubation and the contractile response to NA was not potentiated by carteolol up to this stage. Thus, it is likely that α_1- and β-receptors develop separately and the inhibitory β-receptor function lags behind. A similar change in the response to catecholamines was also found in the colonic smooth muscle of guinea-pigs (Ohkawa, 1978) and the tracheal smooth muscle of dogs (Pandya, 1977) during postnatal development. These findings, however, are not consistent with the observations in the ileum of human embryos. In these observations, catecholamines exclusively caused a relaxation mediated only by β-adrenoceptors (McMurphy & Boréus, 1968) and it was suggested that β-receptors developed earlier than α-inhibitory receptors (Hart & Mir, 1971); α-inhibitory receptors may be different from excitatory ones.

The potentiated contractile response to NA following β-receptor blockade decreased in amplitude together with the decline of sensitivity (pD_2 value) and almost disappeared by the time of hatching. The response to phenylephrine, α_1-agonist, also changed in parallel to the NA response. One reason for change in the type of NA responses seems to be the developmental decline and disappearance of α_1-excitatory function. The disappearance of receptor-mediated responses was observed in the expansor secundariorum muscle of developing chicks (Kuromi & Hasegawa, 1975). In this tissue, the contractile response to ACh decreased and disappeared during the postnatal periods. This phenomenon was explained by the decrease in density of muscarinic receptors (Crouch *et al.*, 1982). The ligand binding experiments in the present study showed that the α-receptor population of chick oesophagus, estimated by the binding of [^{3}H]-DHE, did not largely change between 13 days of incubation and 21 days of incubation, suggesting that the decrease and loss of contractile response via α-receptors may not necessarily be due to a change in receptor population.

It has been reported that α-receptor-mediated responses changed from excitatory to inhibitory in the guinea-pig colon during the postnatal period (Ohkawa, 1978). Our data may also be explained by the possible appearance of inhibitory α-adrenoceptors in the oesophagus after hatching. The report that the inhibitory effect of catecholamines in chick oesophagus was blocked only by the combination of α- and β-receptor blockers (Bowman & Everett, 1964) supports this idea.

In summary, the present study demonstrates that the response to NA is observed at 9 days of incubation and changes from α_1-receptor-mediated excitatory to β-receptor-mediated inhibitory in nature during embryonic development. The change in the response to NA seems to be in part attributable to the decline and disappearance of the excitatory function mediated by α_1-adrenoceptors during embryonic development. It seems unlikely that the decline in the α_1-excitatory function is due to the decrease in α-receptor population in the chick oesophagus.

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Effects of propafenone on electrical and mechanical activities of single ventricular myocytes isolated from guinea-pig hearts

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1 The effects of propafenone on the transmembrane action potential and sarcomere shortening during twitch contraction were investigated in single ventricular myocytes isolated from guinea-pig hearts.

2 Propafenone at low concentrations ($3–5 \times 10^{-7}$ M) slightly lengthened action potential duration (APD), but shortened it at higher concentrations. The shortening of APD was accompanied by an attenuation of sarcomere shortening during twitch contraction.

3 Propafenone ($>10^{-6}$ M) caused a concentration-dependent decrease in the maximum upstroke velocity ($\dot{V}_{max}$) of the action potential. In the presence of propafenone (3×10^{-6} M), trains of stimuli led to an exponential decline in $\dot{V}_{max}$. A time constant for the recovery of $\dot{V}_{max}$ from the use-dependent block was 4.8 s.

4 In myocytes treated with propafenone (3×10^{-6} M), the $\dot{V}_{max}$ of test action potentials preceded by the conditioning clamp pulses to 0 mV was progressively decreased by increasing the duration of single clamp pulse or by increasing the number of multiple brief clamp pulses.

5 These findings suggest that propafenone has use-dependent inhibitory action on the sodium channel by binding to the channel during both activated and inactivated states, and that the unbinding rate is comparable to that of Class-I antiarrhythmic drugs with intermediate kinetics. Propafenone may also have an inhibitory action on calcium and potassium channels.

Introduction

Propafenone is a newly introduced antiarrhythmic drug. Clinical studies have demonstrated that propafenone given by the oral or parenteral route has potent inhibitory actions against both supraventricular and ventricular tachyarrhythmias under various pathological conditions (Seipel & Breithardt, 1980). Experiments by previous investigators have shown that propafenone reduces the maximum upstroke velocity ($\dot{V}_{max}$) of action potential without affecting resting membrane potential (Kohlhardt & Seifert, 1980; 1983; 1985; Ledda *et al.*, 1981; Dukes & Vaughan Williams, 1984). In animals *in vivo*, and in patients, propafenone has been shown to suppress conduction velocity in all cardiac tissues (Connolly *et al.*, 1983). These findings suggest that primary electrophysiological effects of propafenone in the heart are similar to those of local anaesthetic-type (Class-I) antiarrhythmic drugs: inhibition of the sodium current. However, the precise mode of action of propafenone on the cardiac sodium channel in relation to the cellular antiarrhythmic mechanism remains to be elucidated.

In the present study, the effects of propafenone on the transmembrane action potentials were investigated in single ventricular myocytes isolated from guinea-pig hearts. The modulation of drug-induced $\dot{V}_{max}$ inhibition by stimulation frequency or by various clamp pulses was studied extensively to compare with the characteristics of its sodium channel blocking action with other Class I drugs. Mechanical activity of single cells was also obtained by measurement of average single sarcomere length during twitch contractions through the use of our original image processing system (Sato *et al.*, 1988).

In experiments to assess the drug action on cardiac cellular properties, such single myocytes are considered to be more favourable than conventional

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multicellular preparations, because various influences of conduction and of ion accumulation or depletion in the narrow extracellular spaces can be eliminated.

Methods

Cell isolation

Single ventricular myocytes were enzymatically isolated by a procedure similar to that described previously (Watanabe *et al.*, 1985). In brief, hearts were quickly removed from guinea-pigs (200–300 g) and perfused by the Langendorff method with the following solutions in sequence: (1) Ca^{2+}-free Krebs solution for 5 min, (2) enzyme solution containing either collagenase (2 mg ml^{-1}, Sigma, type V), trypsin (0.01 mg ml^{-1}, Sigma, type III-S) and 4×10^{-5} M Ca^{2+}, or collagenase (2 mg ml^{-1}, Sigma, type V) and protease (0.2 mg ml^{-1}, Sigma, type XIV), for 5 min, and (3) Ca^{2+}-free Krebs solution for 5 min. The left ventricle was then cut into small pieces in Ca^{2+}-free Krebs solution to disperse myocytes. A few drops of cell suspension were placed in a recording chamber attached to an inverted microscope. The chamber was perfused with normal Krebs-bicarbonate solution at a rate of 2 ml min^{-1}. In experiments using suction pipette electrodes, Krebs-HEPES solution was employed instead of Krebs-bicarbonate solution. The temperature of the perfusate was maintained at 35°C.

Following the stepwise increase in calcium concentration of the medium to 1.8 or 2.0 mM (normal Krebs solution), 30 to 40% of myocytes had deteriorated into round-shaped cells due to irreversible contracture, whereas the remaining cells were tolerant to calcium; the cells maintained rod-shapes without spontaneous beating. The experiments were carried out only on the latter type of myocyte.

Electrophysiological set-up

Membrane potential was recorded through conventional glass microelectrodes filled with 3 M KCl (20–50 MΩ). The membrane potential was electronically differentiated to obtain the maximum upstroke velocity ($\dot{V}_{max}$) of action potential. Cells were stimulated by application of short current pulses (1 ms in duration) through the recording microelectrode. Intensity of the stimulation was adjusted to obtain a constant latency (2–4 ms).

Mechanical activities of single myocytes were characterized by measuring average single sarcomere length by the microcomputer-based image processing system. Details of the system may be obtained from our recent paper (Sato *et al.*, 1988).

The whole-cell clamp method (Hamill *et al.*, 1981) was used for voltage clamp parts of the experiments. Pipettes were fire-polished and filled with internal solution to have a resistance ranging from 2 to 3 MΩ. Action potentials were recorded in current-clamp mode through the pipette by passing short (<4 ms) stimulus current. Transition from the voltage-clamp mode to the current-clamp mode was regulated by a pulse generator through an electronic relay. Details of the experimental protocol are given in the Results section.

Solutions

Composition of the Krebs-bicarbonate solution was as follows (mM): NaCl 120, KCl 4.0, $CaCl_2$ 2.0, $MgSO_4$ 1.3, NaH_2CO_3 25.2 and glucose 5.0. The solution was equilibrated with 95% O_2:5% CO_2 to maintain a pH of 7.4. The Ca^{2+}-free Krebs solution had the same composition as that of Krebs-bicarbonate solution without $CaCl_2$. The Krebs-HEPES solution contained (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, HEPES 5.0 and glucose 5.0. The pH was adjusted to 7.4 by adding NaOH, and the solution was equilibrated with 100% O_2. The pipette internal solution consisted of (mM): KCl 120.0, NaH_2PO_4 10.0, EGTA 1.0, MgATP 5.0 and HEPES 10.0. The pH was adjusted to 7.2 by adding KOH.

Propafenone (Yamanouchi Pharmaceutical Co. Ltd.) was dissolved in deionized water to make a stock solution (10^{-1} M), and desired drug concentrations were obtained by adding a small amount of stock solution to the perfusate.

Data analysis

Values were presented as means ± s.e. with number unless otherwise specified. Data were analyzed by use of the *t* test, analysis of variance, Dunnett's test and regression analysis. Significance level was set at $P < 0.05$.

Results

Effects of propafenone on membrane action potential and twitch contraction

The electromechanical effects of propafenone were examined in guinea-pig ventricular myocytes constantly stimulated at 1.0 Hz. Transmembrane action potential accompanied by sarcomere shortening (twitch contraction) was recorded by use of glass microelectrodes and the image-processing system (Figure 1). Control values of action potential parameters before drug application were as follows: resting potential (RP), -90.3 ± 0.3 mV; action potential duration at 75% repolarization (APD_{75}), 204 ± 6 ms; and $\dot{V}_{max}$, 262 ± 6 V s^{-1} ($n = 18$). The resting sarcomere length was 1.86 ± 0.02 μm

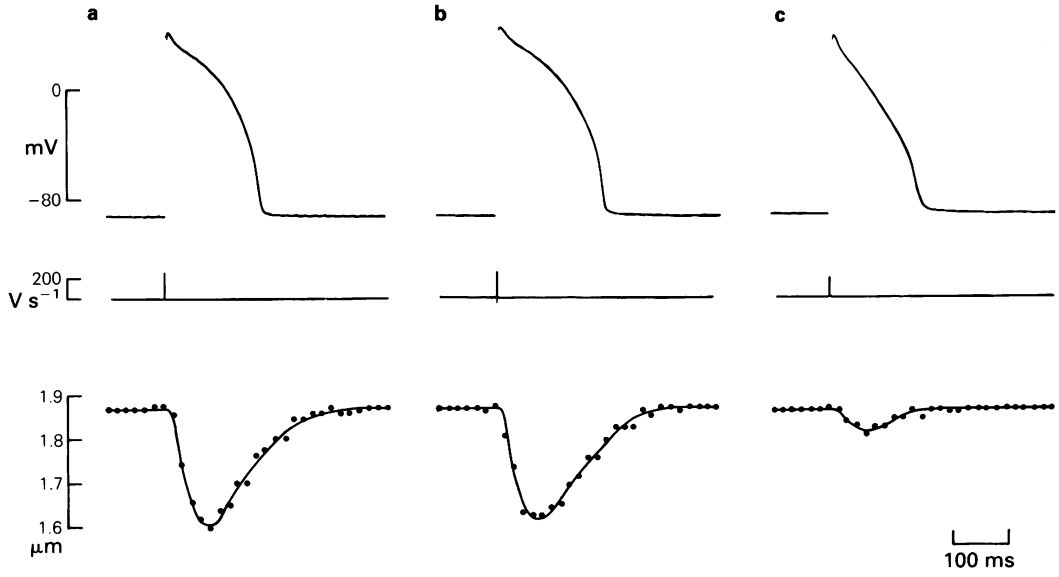

Figure 1 Effects of propafenone of the transmembrane action potential and accompanied sarcomere shortening in guinea-pig ventricular myocyte: (a) control; (b) propafenone (3×10^{-7} M); (c) propafenone (3×10^{-6} M). The top trace is the membrane potential and the middle trace is its first derivative. The bottom trace shows average single sarcomere shortening during twitch contraction accompanied by action potential. The myocyte was constantly driven at 1.0 Hz. Data were obtained before and 30 min after the drug application at each concentration.

($n = 10$). Twitch contraction was initiated soon after the fast upstroke of the action potential, and the shortest sarcomere length ($1.57 \pm 0.02\,\mu m$, $n = 10$) was achieved approximately 80–100 ms after the stimulus. Relaxation progressed slowly; and the sarcomere length returned to the resting level at 100–200 ms after full repolarization of the action potential.

Following exposure to propafenone at 10^{-7} M for 30 min, there were no significant changes in action potential configuration and sarcomere shortening traces. Propafenone (3×10^{-7} M) caused a slight prolongation of APD_{75} without affecting other parameters (Figure 1). At concentrations above 10^{-6} M, however, APD_{75} was shortened, and the $\dot{V}_{max}$ was decreased in a dose-dependent manner (Figures 1, 2). The shortening of sarcomere length during twitch contraction was also decreased. At 3×10^{-6} M, APD_{75}, $\dot{V}_{max}$ and the peak shortening of sarcomere length were decreased to 87%, 65%, and 19% of the respective control values. RP and the resting sarcomere length were unaffected even at the highest concentration tested (10^{-5} M).

Stoichiometry of drug-receptor complex was applied for the inhibition curves of $\dot{V}_{max}$ (Figure 2a) under the assumption of the law of mass action. An apparent K_D value at 1.0 Hz (a concentration required for half reduction of $\dot{V}_{max}$) was 4.4×10^{-6} M with Hill's coefficient (n_H) of 1.8. The $\dot{V}_{max}$ inhibition by propafenone was enhanced with a higher stimulation frequency (2.5 Hz) and the curve was shifted to the left with a K_D value of 2.0×10^{-6} M and n_H of 1.4.

Use-dependent block of $\dot{V}_{max}$ by propafenone

The enhancement of inhibition of $\dot{V}_{max}$ by propafenone at higher stimulation frequencies may reflect the use-dependent block of the sodium channel as with other Class-I antiarrhythmic drugs. This characteristic was investigated further by applying stimulation trains (1.0 or 2.5 Hz), which were separated from each other by a rest period of 120 s (Figure 3). In untreated control myocytes, the $\dot{V}_{max}$ was almost unchanged during such stimulation trains. Following treatment with propafenone (3×10^{-6} M), $\dot{V}_{max}$ of the first action potential in each train was slightly decreased; there was a tonic block of $\dot{V}_{max}$ by $2.3 \pm 0.2\%$ ($n = 5$). Further decline of $\dot{V}_{max}$ during the stimulation train (use-dependent block) depended on the stimulation frequency; the decrease of $\dot{V}_{max}$ from the first action potential to the new steady-state was $27.2 \pm 2.4\%$ at 1.0 Hz ($n = 5$), and $60.1 \pm 2.3\%$ at 2.5 Hz ($n = 4$).

Beat to beat decline of $\dot{V}_{max}$ during stimulation trains was expressed as a single exponential curve, so that the onset rate per action potential (AP^{-1}) at which the $\dot{V}_{max}$ fell to the new steady state level was calculated in each experiment. The average values

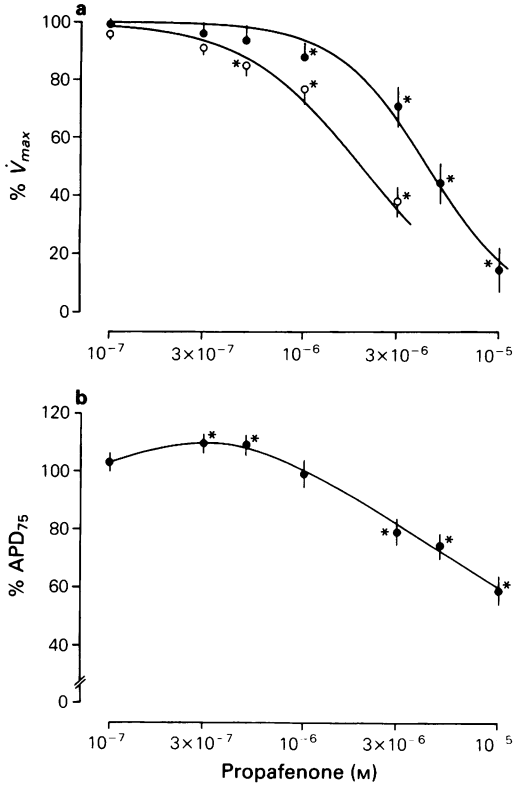

Figure 2 Concentration-dependent effects of propafenone on $\dot{V}_{max}$ (a) and APD_{75} (b). All values are normalized by the reference value under drug-free control conditions. Data were obtained before and 30 min after the drug application at each concentration. Stimulation frequencies were 1.0 Hz (●) and 2.5 Hz (○). Values are indicated as mean with s.e. shown by vertical lines ($n = 5$). *Significantly different from the reference value at $P < 0.05$. The curves in (a) represent nonlinear least-squares fits to the equation:

$$\%\dot{V}_{max} = (1 + [\text{drug}]^{nH}/K_D)^{-1}$$

where n_H is Hill's coefficient and K_D is the apparent dissociation constant. The K_D value was 4.4×10^{-6} M (n_H 1.8) at 1.0 Hz and 2.0×10^{-6} M (n_H 1.4) at 2.5 Hz.

were $0.33 \pm 0.03\,AP^{-1}$ at 1.0 Hz ($n = 5$), and $0.20 \pm 0.02\,AP^{-1}$ at 2.5 Hz ($n = 4$).

Effects of conditioning clamp pulses on $\dot{V}_{max}$ inhibition

The effects of depolarizing clamp pulses on the $\dot{V}_{max}$ of subsequent test action potentials were examined in order to determine whether the marked use-dependency of $\dot{V}_{max}$ inhibition induced by propafenone is due to blockade of the activated or inactivated sodium channel (Kodama *et al.*, 1987).

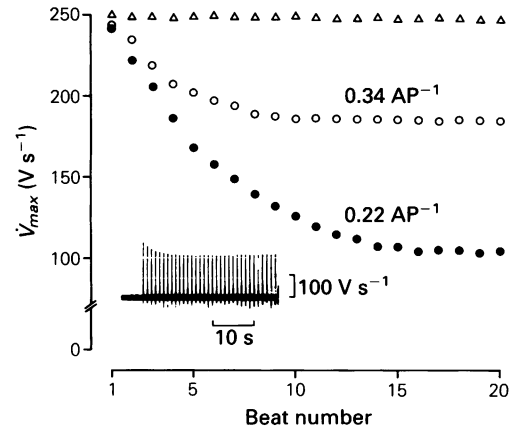

Figure 3 Rate-dependent decrease in $\dot{V}_{max}$ (use-dependent block) by propafenone. Inset shows superimposed record of differentiated upstrokes of the action potentials during stimulation train at 1.0 Hz in a previously quiescent myocyte. The record was obtained 30 min after the drug application at 3×10^{-6} M. The graph shows the beat-to-beat change in $\dot{V}_{max}$ at the start of stimulation trains. Ordinate scale indicates $\dot{V}_{max}$, and abscissa scale indicates number of beats (action potentials) from the initiation of the stimulation train. Frequencies of stimulation were 1.0 Hz under control conditions (△), and 1.0 Hz (○) and 2.5 Hz (●) in the presence of propafenone.

Figure 4 shows the experiment with a single clamp pulse of 0 mV. Following a rest period of 120 s, the membrane potential was clamped from the resting level (holding potential of −82 mV) to 0 mV for 10 to 1000 ms. At the end of the conditioning clamp pulse, membrane potential was clamped back to the holding potential for 100 ms, which is long enough for a drug-free channel to reactivate fully (Carmeliet & Vereecke, 1979), but short enough so that only partial dissociation of drugs from blocked channel occurs (Grant *et al.*, 1984). The voltage-clamp was then released, and a stimulus was applied to elicit a test action potential.

In untreated control myocytes, such a clamp pulse with a duration less than 200 ms had no significant effect on the $\dot{V}_{max}$ of the test action potential. However, further prolongation of the clamp pulse duration resulted in a slight but significant decrease in $\dot{V}_{max}$, suggesting a slow inactivation of the sodium channel. A clamp pulse of 1000 ms in duration decreased $\dot{V}_{max}$ by $6.2 \pm 1.0\%$ ($n = 5$) from the value of the action potential without conditioning clamp pulse (reference level). In myocytes treated with propafenone (3×10^{-6} M) similar clamp pulses caused more remarkable $\dot{V}_{max}$ reduction of the test action potential. Thus, a significant $\dot{V}_{max}$ decrease ($6.6 \pm 1.1\%$, $n = 5$) from the reference value was

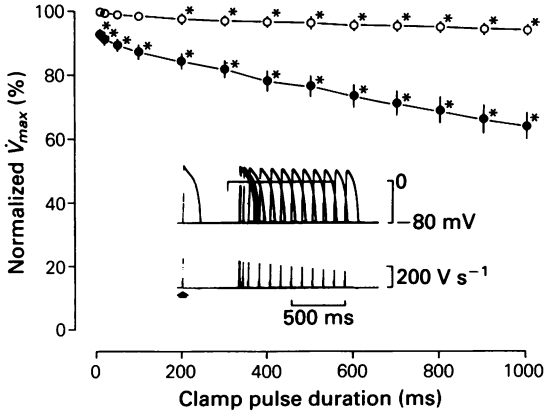

Figure 4 Influence of clamp pulse duration on $\dot{V}_{max}$ inhibition by propafenone. Inset shows superimposed record of action potentials (upper trace) and their differentiated upstroke spikes (lower trace) 30 min after addition of propafenone at 3×10^{-6} M. Action potentials were elicited without clamp pulse as a reference value of $\dot{V}_{max}$ (arrow at left) or 100 ms after a single clamp pulse to 0 mV having a duration of 10, 20, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 ms. Each conditioning clamp pulse was preceded by a 2 min rest period. The results obtained from five myocytes are summarized in the graph. Ordinate scale, $\dot{V}_{max}$ of test action potential normalized by the reference value. Abscissa scale, clamp pulse duration. Data were obtained before (○) and 30 min after the drug application (●). Values are indicated as mean with s.e. shown by vertical lines ($n = 5$). *Significantly different from the reference value at $P < 0.05$.

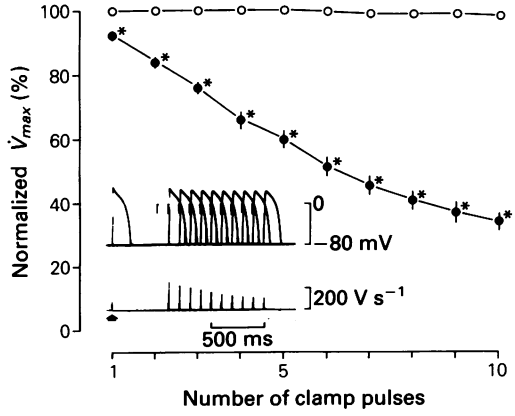

Figure 5 Influence of multiple clamp pulses on $\dot{V}_{max}$ inhibition by propafenone. Inset shows superimposed record of action potentials (upper trace) and their differentiated upstroke spikes (lower trace) 30 min after addition of propafenone at 3×10^{-6} M. Action potentials were elicited without clamp pulse as a reference value of $\dot{V}_{max}$ (arrow at left) or 100 ms after the last clamp pulse to 0 mV. The duration of each clamp pulse was 10 ms and the interval between the clamp pulses, during which the membrane potential was clamped to the resting potential level (−82 mV), was 100 ms. The number of clamp pulses was varied from one to ten. Each conditioning clamp pulse train was preceded by a 2 min rest period. The results obtained from five myocytes are summarized in the graph. Ordinate scale, $\dot{V}_{max}$ normalized by the reference value. Abscissa scale, number of clamp pulses. Data were obtained before (○) and 30 min after the drug application (●). Values are indicated as mean with s.e. shown by vertical lines ($n = 5$). *Significantly different from the reference value at $P < 0.05$.

observed even with 10 ms clamp pulse. The $\dot{V}_{max}$ reduction was further enhanced as the clamp pulse duration was prolonged and reached 36.4 ± 3.1% ($n = 5$) at 1000 ms.

Figure 5 illustrates the effects of multiple brief clamp pulses. The duration of each clamp pulse to 0 mV was 10 ms and the interval between clamp pulses, during which membrane potential was clamped at the resting level (−82 mV), was set to 100 ms. The test action potential was elicited 100 ms after termination of the last pulse. In untreated control cells, such multiple clamp pulses up to 10 had no significant effect on the $\dot{V}_{max}$ of the test action potential. In myocytes treated with propafenone at 3×10^{-6} M, the $\dot{V}_{max}$ of the test action potential was decreased progressively as the number of pulses was increased. At the 10th pulse, the decrease of $\dot{V}_{max}$ from the reference level reached 63.6 ± 2.9% ($n = 5$).

In four myocytes, the recovery process of $\dot{V}_{max}$ following the use-dependent block was examined by introducing a single test action potential following 20 clamp pulses to 0 mV (each clamp pulse duration was 10 ms and the interval between the pulses was 100 ms) with various coupling intervals. Under drug-free control conditions, the $\dot{V}_{max}$ recovered almost completely within 100 ms after the termination of the last clamp pulse. After treatment with propafenone (3×10^{-6} M), much slower $\dot{V}_{max}$ recovery was observed. Representative results are shown in Figure 6, where fractional $\dot{V}_{max}$ reduction was plotted against the coupling interval in a semilogarithmic graph. In the presence of propafenone, the recovery time course of $\dot{V}_{max}$ with a coupling interval longer than 1 s was approximated by a single exponential function. The average time constant (τ_R) was calculated as 4.8 ± 0.2 s ($n = 4$).

Discussion

The present study on guinea-pig ventricular myocytes indicates that propafenone above 10^{-6} M causes a concentration-dependent decrease in $\dot{V}_{max}$ of action potential, and that this $\dot{V}_{max}$ inhibition is

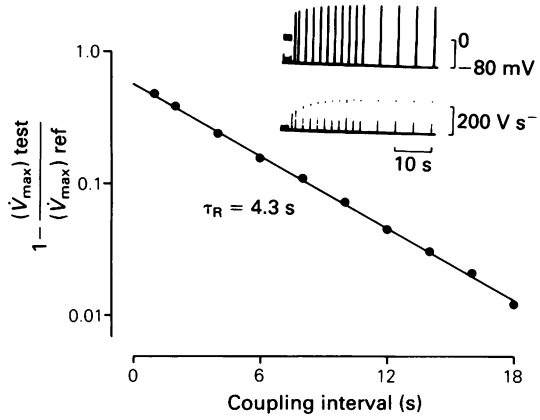

Figure 6 Recovery of $\dot{V}_{max}$ from a use-dependent block by propafenone. Inset shows superimposed record of action potentials (upper trace) and their differentiated upstroke spikes (lower trace). Following 20 conditioning clamp pulses to 0 mV (each clamp pulse duration was 10 ms and the interval between the pulses was 100 ms), a test action potential was elicited with various coupling intervals, during which the membrane potential was clamped to the resting potential (−82 mV). Each conditioning clamp pulse train was preceded by a 2 min rest period. Data were obtained 30 min after addition of propafenone at 3×10^{-6} M. The graph indicates recovery process of the $\dot{V}_{max}$ of the test action potential. Ordinate scale, fractional $\dot{V}_{max}$ reduction of test action potential compared to the $\dot{V}_{max}$ value of action potential without conditioning clamp pulse (reference value): $1 - (\dot{V}_{max})\text{test}/(\dot{V}_{max})\text{ref}$. Abscissa scale, coupling interval between the last clamp pulse and the test action potential. The time course of $\dot{V}_{max}$ recovery with a coupling interval longer than 1 s was approximated by a single exponential function with a time constant of 4.3 s.

enhanced at higher stimulation frequencies. Propafenone at low concentrations ($3–5 \times 10^{-7}$ M) slightly prolonged action potential duration (APD), but shortened that at the higher concentrations. The resting membrane potential (RP) was not affected even at 10^{-5} M. These findings are more or less in agreement with those reported previously by Kohlhardt & Seifert (1980; 1983; 1985) in guinea-pig papillary muscles. Dukes & Vaughan Williams (1984) also reported similar $\dot{V}_{max}$ inhibition in rabbit ventricular and atrial muscles as well as in Purkinje fibres.

In mammalian ventricular muscle cells, most of the ionic current flowing across the cell membrane at the time of $\dot{V}_{max}$ is the sodium current; other membrane currents make no significant contributions (Carmeliet & Vereeck, 1979). The decrease in $\dot{V}_{max}$ by propafenone without any change in RP may, therefore, reflect an inhibitory effect of this drug on the fast sodium inward current (I_{Na}). In the following discussion, we used $\dot{V}_{max}$ as an approximate index of sodium channel availability to infer changes in the drug-blocked sodium channels. For quantitative measurements, however, it seems important to note that the probably convex-shaped non-linear relationship between $\dot{V}_{max}$ and the maximal limiting Na conductance (Bean *et al.*, 1982; Sheets *et al.*, 1988) might introduce a variable error on the precise amount of g_{Na} depression so that I_{Na} block will be increasingly overestimated at higher drug concentrations.

The present experiments using stimulation trains showed a marked use-dependent block of $\dot{V}_{max}$ by propafenone with little tonic block. Such characteristics are consistent with those observed in guinea-pig papillary muscles (Kohlhardt & Seifert, 1983; 1985). According to the 'modulated receptor hypothesis' proposed by Hondeghem & Katzung (1977, 1980) to explain the interaction between local anaesthetic type (Class I) antiarrhythmic drugs and cardiac sodium channels, the reduction of I_{Na} is due to accumulation of drug-associated nonconducting channels (blocked channels). If propafenone, like most Class I antiarrhythmic drugs (Grant *et al.*, 1984; Hondeghem & Katzung, 1984) has a higher affinity for the receptor of an activated and inactivated channel than for a resting channel, the accumulation of blocked channels during the stimulation train leading to a marked use-dependent inhibition of $\dot{V}_{max}$ would be expected.

The onset rate of the use-dependent block by propafenone in the present experiments ($0.33\,AP^{-1}$ at 1.0 Hz, 3×10^{-6} M propafenone) is comparable to that observed in guinea-pig papillary muscles (Kohlhardt & Seifert, 1985) and those for quinidine, disopyramide, which have been classified as intermediate kinetic Class I drugs by Campbell (1983).

Recently, we (Kodama *et al.*, 1987) have shown that Class I antiarrhythmic drugs currently available can be divided into two groups in terms of their sodium channel blocking phase during the conditioning clamp pulse to 0 mV; one 'transient' and one 'maintained' (Courtney, 1988). The former group of drugs (quinidine and disopyramide) may block the sodium channel mainly during its activated state corresponding to the upstroke phase of the action potential, while the latter group (lignocaine, mexiletine, tocainide and aprindine) may act predominantly during the inactivated state, corresponding to the plateau phase of the action potential. In the present experiments with propafenone we examined such a 'state-dependency' of sodium channel block by using the suction pipette whole-cell clamp technique. Protocols employed are similar to our previous experiments with single sucrose-gap voltage clamp techniques (Kodama *et al.*, 1987). In the presence of propafenone (3×10^{-6} M), a single conditioning clamp to 0 mV resulted in a substantial

transient decrease in $\dot{V}_{max}$ (6.6% at 10 ms pulse) followed by an additional maintained decrease in $\dot{V}_{max}$ with further prolongation of the clamp pulse duration. It was also shown that multiple brief clamp pulses caused much more remarkable decrease of $\dot{V}_{max}$ than single prolonged clamp pulse even when the total amount of time clamped to 0 mV level of the former one was much less than the latter one. These findings suggest that propafenone may block sodium channels by binding both activated and inactivated states. In other words this drug has intermediate binding characteristics between activated channel blockers and inactivated channel blockers.

The recovery process of $\dot{V}_{max}$ from the use-dependent block following multiple clamp pulses was expressed by a single exponential function with a time constant (τ_R) of 4.8 s. This value is similar to that reported by Kohlhardt & Seifert (1985) in guinea-pig papillary muscle preparations. It is therefore suggested that the propafenone molecule may dissociate from sodium channel receptors at resting or inactivated state with rates comparable to quinidine or disopyramide (Campbell, 1983).

The biphasic change of APD may reflect multiple modes of action of propafenone on membrane ionic currents. The shortening of APD by propafenone at $>10^{-6}$ M, which was accompanied by an attenuation of sarcomere shortening during twitch contraction, may probably be explained by a decrease of slow calcium inward current (I_{Ca}), because activation of I_{Ca} is of prime importance for the genesis of the plateau phase of the action potential as well as of twitch contraction in mammalian ventricular muscle cells. In voltage clamp experiments on cat papillary muscles, Kohlhardt (1977) demonstrated that propafenone (6.5×10^{-5} M) inhibited both I_{Na} and I_{Ca}. The inhibition of I_{Ca} by propafenone was also shown in rabbit sinus node cells (Satoh & Hashimoto, 1984). These reports seem to lend support to the above explanation. However, we cannot eliminate other possible mechanisms for the shortening of APD. For instance, propafenone might decrease the slowly inactivating sodium current during the plateau phase of the action potential (tetrodotoxin-sensitive sodium window current) (Clarkson *et al.*, 1984). APD prolongation by low concentrations of propafenone might be due to a decrease of the potassium outward current by this drug. Further experimental studies are required to determine these points and to obtain a complete profile of the drug action.

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The effect of arginine and nitric oxide on resistance blood vessels of the perfused rat kidney

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1 The vasodilator effects of arginine, nitric oxide (NO), acetylcholine (ACh) and sodium nitroprusside (NP) in the noradrenaline-preconstricted ('high tone') perfused rat kidney have been examined.

2 L-Arginine (0.6–23 μmol) caused a biphasic change in renal perfusion pressure. D-Arginine (0.6–23 μmol) was without effect. The second vasodilator component was abolished and the first vasoconstrictor effect augmented following CHAPS-induced removal of the vascular endothelium suggesting that vasodilatation was endothelium-dependent.

3 L-Arginine salts produced transient and dose-related vasodilatation. L-Arginine methylester was the most potent with an ED_{50} of 2.2 ± 0.4 μmol ($n = 6$). The rank order of potency of the salts tested was: methylester > hydroxamate > chloride. L-Homoarginine chloride was also vasodilator (ED_{50}, 12.0 ± 1.3 μmol, $n = 5$). D-Arginine chloride was without effect at doses up to 170 μmol. Responses to L-arginine chloride were endothelium-derived relaxing factor (EDRF)-dependent being abolished by CHAPS (4.7 mg ml^{-1}, 30 s) and significantly inhibited (>70%) by gossypol (3 μM) and nordihydroguaiaretic acid (NDGA, 10 μM).

4 Vasodilatation due to NO was unaffected by CHAPS and gossypol treatment but inhibited by NDGA. NO was approximately 3 times less potent than ACh but 3000 times more potent than L-arginine methylester.

5 Kidneys perfused for 1 h with Krebs solution containing L-arginine chloride (100 μM) or L-canavanine (50 μM) showed no change in sensitivity towards ACh or NP. Higher concentrations of L-arginine chloride (500 μM) or L-canavanine (150 μM) significantly reduced the response to both vasodilators.

6 L-Arginine salts dilate resistance blood vessels of the perfused rat kidney by a mechanism which may involve the release of EDRF from vascular endothelial cells of the perfused rat kidney.

Introduction

Endothelium-derived relaxing factor (EDRF) is a chemically unstable, locally acting vasodilator released from the vascular endothelium of intact blood vessels (Furchgott, 1984). EDRF mediates the relaxation of preconstricted rings or bands of isolated arteries and veins in response to acetylcholine (ACh) and is responsible for the vasodilator effect of this substance on resistance blood vessels of several perfused organs including rat lung (Cherry & Gillis, 1987), mesentery (Furchgott *et al.*, 1987) and kidney (Bhardwaj & Moore, 1988). Recently evidence has been presented that EDRF is identical with nitric oxide (NO) (Ignarro *et al.*, 1987; Palmer *et al.*, 1987) although the existence of an additional EDRF which relaxes blood vessels by hyperpolarizing membranes of vascular smooth muscle cells has also been proposed (Taylor & Weston, 1988).

Despite progress in the identification of EDRF the biochemical pathway for its formation remains obscure. Recent work has implicated L-arginine as the physiological precursor of NO (Palmer *et al.*, 1988; Schmidt *et al.*, 1988a,b). We have now investigated the vasodilator effect of L-arginine and a selection of its salts as well as its putative metabolite, NO, in the perfused rat kidney in an attempt to gain further insights into the biochemical mechanism of EDRF formation.

Methods

The experimental procedures employed in this study have been detailed elsewhere (Bhardwaj & Moore, 1988). Briefly, rats (male Sprague-Dawley, 250–350 g) were stunned by a blow to the head and exsanguinated. The left kidney was removed and perfused with warmed (37°C), oxygenated (95% O_2: 5% CO_2) Krebs solution containing indomethacin (8 μM) to inhibit prostanoid formation. Perfusion pressure was constantly monitored by means of a Bell & Howell pressure transducer connected to a Devices pen recorder. Drugs were injected in volumes less than 20 μl to prevent vascular effects due to an injection artefact. Vasodilator responses to ACh, L-arginine base and salts, NO and NP were assessed in so-called 'high tone' preparations which were partially preconstricted by addition to the perfusing Krebs solution of a concentration of noradrenaline (0.1–0.5 μM) that increased basal perfusion pressure by 130–160 mmHg. This increase represents approximately 60–80% of the maximal response. No significant loss of vasoconstrictor tone was observed in such preparations for periods of up to 4 h. A dose cycle time of 5 min was employed for all vasodilator drugs.

In some experiments, endothelial cells lining resistance blood vessels in the non-constricted (i.e. 'low tone') rat kidney were removed by addition to the perfusing Krebs solution of the detergent, 3,3 cholamidopropyl dimethylammonio 1-propanesulphonate (CHAPS, 4.7 mg ml^{-1}, 30 s). In other experiments, L-arginine chloride (100–700 μM), L-canavanine (50–250 μM), gossypol (3 μM) or nordihydroguaiaretic acid (NDGA, 10 μM) were added to the Krebs solution and allowed to perfuse the pre-constricted (i.e. 'high tone') kidney for a minimum of 1 h before further challenge with vasodilator drugs. Kidneys were weighed before and at the end of the experiment to determine the extent of oedema formation.

Solutions of NO (BDH, 99.9%) were prepared essentially as described by Shikano *et al.* (1988). Briefly, NO (10 ml) was transferred without contact with air into rubber-sealed, Wheatman flasks containing 25 ml nitrogen-degassed distilled water and kept on ice throughout the experiment. Assuming complete saturation with gas the concentration of NO in solution is approximately 3.3 mM. This concentration was used to calculate the doses of NO administered. Indomethacin was dissolved in 0.5% Na_2CO_3. Other drugs were dissolved in saline. All drugs were purchased from Sigma Ltd and kept on ice throughout the experiment. NDGA was dissolved in Krebs solution. Solutions of NDGA and NO were prepared fresh each day. NDGA was used within 1 h.

Data are presented as mean ± s.e.mean. Statistical analysis was performed by use of Student's *t* test.

Results

Effects of acetylcholine, nitric oxide and sodium nitroprusside on renal perfusion pressure

The basal perfusion pressure of rat kidney preparations used in this study was 148.5 ± 7.2 mmHg ($n = 18$). No significant change in kidney weight was observed following perfusion for up to 4 h (2.9 ± 0.6 g, $n = 18$, at the start compared with 3.3 ± 0.2 g, $n = 18$, at the end of the experiment) suggesting the absence of significant oedema formation over this period.

ACh, NO and NP produces dose-related vasodilatation of the noradrenaline-preconstricted, perfused rat kidney. Responses to ACh and NO were transient whilst responses to similarly effective doses of NP were characteristically more prolonged. Representative traces are shown in Figure 1. The doses of each drug required to produce 50% of the maximal response (ED_{50}) were 0.18 ± 0.05 nmol, $n = 6$, 0.66 ± 0.11 nmol, $n = 5$ and 1.3 ± 0.3 nmol, $n = 6$, while the maximal falls in perfusion pressure which could be achieved were 61.5 ± 4.5 mmHg, $n = 6$, (1.1 nmol), 53.2 ± 4.5 mmHg, $n = 6$, (3.3 nmol) and 70.6 ± 4.1 mmHg, $n = 6$, (7.6 nmol) respectively.

Removal of endothelial cells lining resistance blood vessels within the kidney abolished the vasodilator effect of ACh without influencing responses to NO or NP. Similarly, responses to approximate ED_{70} doses of ACh (0.22 nmol, 5.5 ± 3.4 mmHg, cf. 45.7 ± 4.4 mmHg, $n = 5$, $P < 0.05$) but not NO (1.65 nmol, 38.9 ± 6.7 mmHg, cf. 41.4 ± 4.2 mmHg, $n = 6$, $P > 0.05$) or NP (2.2 nmol, 51.2 ± 4.5 mmHg, cf. 45.6 ± 6.7 mmHg, $n = 6$, $P > 0.05$) were reduced by gossypol (3 μM). In contrast, in separate preparations NDGA (10 μM) reduced the response to ACh (0.22 nmol, 4.4 ± 2.3 mmHg, cf. 48.7 ± 3.2 mmHg, $n = 5$, $P < 0.05$) and NO (1.65 nmol, 6.7 ± 3.3 mmHg, cf. 45.4 ± 4.5 mmHg, $n = 6$, $P < 0.05$) and partially blocked vasodilatation due to NP (2.2 nmol, 34.4 ± 2.2 mmHg, cf. 43.3 ± 1.9 mmHg, $n = 6$, $P < 0.05$).

Effect of arginine base and salts on renal perfusion pressure

In preliminary experiments, L- and D-arginine base (0.6–23 μmol) were assessed for renal vasodilator activity. In 'high tone' perfused rat kidney preparations, L-arginine produced a biphasic effect viz. vasoconstriction followed by vasodilatation (Figure 2). For example, administration of 11.5 μmol L-arginine caused an increase in perfusion pressure of 53.9 ± 7.5 mmHg followed immediately thereafter by a fall of 16.9 ± 5.0 mmHg (both $n = 8$). In contrast,

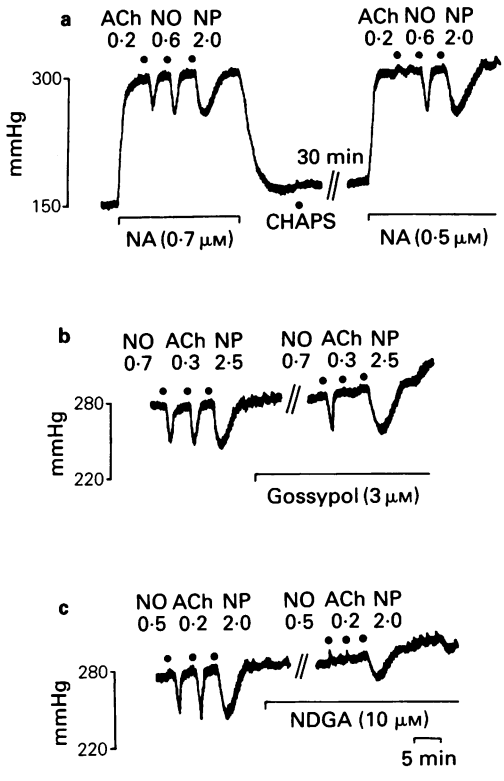

Figure 1 Representative traces showing the vasodilator effect of approximate ED_{50} doses (nmol) of acetylcholine (ACh), nitric oxide (NO) and nitroprusside (NP) in noradrenaline-preconstricted, perfused rat kidneys. (a) CHAPS (4.7 mg ml^{-1}, 30 s) was administered in a 'low tone' perfused rat kidney preparation and the effect on vasodilatation due to ACh, NO and NP assessed 30 min thereafter following noradrenaline (NA) addition to the perfusing Krebs solution. (b and c): Effect of gossypol (3 μM) and nordihydroguaiaretic acid (NDGA) (10 μM) administered to 'high tone' rat kidneys on vasodilatation due to ACh, NO and NP. Preparations were exposed to the inhibitor for 1 h before further injection of vasodilator drug. Vertical bar indicates perfusion pressure in mmHg. Horizontal bar shows time in min.

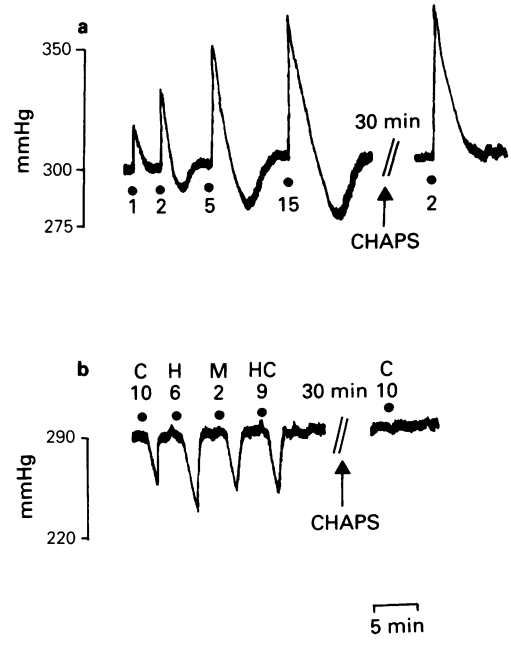

Figure 2 Representative traces showing the biphasic effect of L-arginine base (μmol) on perfusion pressure in the noradrenaline-preconstricted, perfused rat kidney (a) and vasodilator effect of approximate ED_{50} doses (μmol) of L-arginine chloride (C), L-arginine hydroxamate (H), L-arginine methylester (M) and L-homoarginine chloride (HC) (b). The effect of CHAPS (4.7 mg ml^{-1}, 30 s) administered to 'low tone' perfused rat kidney preparations (as in Figure 1) on responses to L-arginine base and L-arginine chloride are also shown. Vertical bar indicates perfusion pressure in mmHg. Horizontal bar shows time in min.

D-arginine base had no effect on kidney perfusion pressure over the dose-range used.

Unlike L-arginine base, bolus injection of L-arginine chloride, L-arginine hydroxamate, L-arginine methylester or L-homoarginine chloride resulted in dose-related vasodilatation of similar duration to that observed with ACh (Figure 2). The ED_{50} values (maximum response in brackets) for each of these L-arginine salts are: 14.0 ± 1,2 μmol (55.2 ± 6.4 mmHg, 95.2 μmol), 4.4 ± 0.5 μmol (67.0 ± 9.2 mmHg, 70 μmol), 2.2 ± 0.4 μmol (76.5 ± 2.3 mmHg, 51.2 μmol) and 12.0 ± 1.3 μmol (73.3 ± 10.0 mmHg, 96 μmol) (all n = 4–7). No vasoconstrictor response was observed with any of these L-arginine salts at doses up to 100 μmol. Of all the arginine salts tested, only D-arginine chloride failed to affect perfusion pressure even at doses as high as 170 μmol (results from 6 experiments).

Removal of endothelial cells from intra-renal blood vessels by CHAPS perfusion augmented the vasoconstrictor component whilst abolishing the vasodilator effect of injected L-arginine base (Figure 2). Similarly, removal of endothelium with CHAPS abolished and exposure of perfused kidneys to either gossypol (3 μM) or NDGA (10 μM) significantly reduced renal vasodilatation due to an approximate ED_{50} dose of L-arginine chloride (10 μmol) by 84.8 ± 7.6% and 81.9 ± 6.6% (n = 6).

Effect of arginine 'loading' on the response to acetylcholine and sodium nitroprusside

Attempts were also made to 'load' rat kidney preparations with L-arginine chloride by addition of this amino acid to the Krebs perfusate. 'High tone' perfused rat kidneys exposed for 1 h to a high concentration of L-arginine chloride (700 μM) responded with a slowly developing fall in perfusion pressure which resulted in complete loss of noradrenaline-induced tone within 15–25 min. Kidneys routinely perfused with Krebs containing a lower concentration (100 μM) of L-arginine chloride exhibited no reduction in vascular tone over a 2 h period. In these circumstances no change in the vasodilator potency of ACh (ED_{50}, 0.21 ± 0.07 nmol, maximum response, 64.3 ± 3.9 mmHg, 1.2 nmol, $n = 5$, both $P > 0.1$) or NP (ED_{50}, 1.6 ± 0.6 nmol, maximal response, 66.6 ± 7.8 mmHg, 7.6 nmol, $n = 5$, both $P > 0.1$) was observed. Increasing the concentration of L-arginine even further (500 μM) produced in the first instance a small (approx. 12 mmHg) fall in perfusion pressure which reversed within 5 to 10 min to restore full 'tone'. Thereafter, in the continued presence of this concentration of L-arginine chloride responses to both ACh (ED_{50}, 1.1 ± 0.05 nmol, maximum response 28.7 ± 3.2 mmHg, 9.6 nmol, $n = 5$, both $P < 0.05$) and NP (ED_{50}, 4.7 ± 0.4 nmol, maximum response, 36.1 ± 4.6 mmHg, 30.4 nmol, $n = 5$, both $P < 0.05$) were non-selectively reduced.

Effect of L-canavanine on the response to acetylcholine and sodium nitroprusside

Addition of L-canavanine (50 μM) to the Krebs perfusate did not affect responses to any of the vasodilator drugs tested. Increasing the concentration of L-canavanine (150 μM) non-selectively antagonized the renal vasodilator effect of approximate ED_{70} doses of ACh (e.g. 0.22 nmol, 26.3 ± 6.2 mmHg, cf. 48.3 ± 8.1 mmHg, $n = 5$, $P < 0.05$) and NP (e.g. 2.2 nmol, 24.5 ± 3.4 mmHg, cf. 46.5 ± 4.5 mmHg, $n = 6$, $P < 0.05$). Even higher concentrations of L-canavanine (250 μM) added to the Krebs perfusate reduced or more often totally abolished the tone of kidney preparations and thus could not be used.

Discussion

The vasodilator effect of ACh in the perfused rat kidney is reduced by removal of the endothelium with CHAPS or exposure to gossypol or NDGA and is thus dependent upon the formation of EDRF which confirms the results of a previous study (Bhardwaj & Moore, 1988). In addition we describe here the powerful vasodilator activity of NO on renal resistance blood vessels. The response to NO is unaffected by CHAPS or gossypol but reduced by NDGA treatment and in consequence is most likely a direct effect on vascular smooth muscle.

A major aim of the present investigation was to clarify the part played by L-arginine in the formation of EDRF within the kidney. To this end we have attempted to duplicate some of the experiments performed either in large arteries (Schmidt *et al.*, 1988a) or on cultured endothelial cells (Palmer *et al.*, 1988) which have suggested that L-arginine is the physiological precursor of EDRF.

L-Arginine salts (chloride, hydroxamate, methylester) as well as L-homoarginine chloride were vasodilators in the perfused rat kidney, albeit some 3 orders of magnitude less potent than either NO or ACh. D-Arginine chloride was inactive. Like ACh, responses to L-arginine were reduced or abolished by removal of the endothelium with CHAPS, or exposure to gossypol or NDGA, implying that the response of the rat renal vasculature to L-arginine salts is EDRF-dependent. By similar reasoning the secondary vasodilator component of the response to L-arginine base is also EDRF-dependent. The renal vasoconstriction that occurred following injection of L-arginine base is not endothelium-dependent since it is augmented by CHAPS. Furthermore, renal vasoconstriction is unrelated to the pH of the injection medium since D-arginine base, which exhibits an identical pH to that of L-arginine base, is devoid of activity in the perfused rat kidney.

That L-arginine and its salts are vasodilator in the perfused rat kidney by a mechanism involving EDRF is in accord with recently published evidence that this amino acid is the physiological precursor of NO in vascular endothelial cells. Schmidt and colleagues (1988a) came to this conclusion when they demonstrated that L-canavanine, which prevents conversion of L-arginine to nitrite by macrophages (Iyengar *et al.*, 1987), antagonized ATP- and ACh-induced relaxation of the isolated rat aorta. In the present study, L-canavanine non-selectively blocked responses to both endothelium-dependent (ACh) and endothelium-independent (NP) vasodilators indicating a depressant effect on vascular smooth muscle reactivity, rather than or in addition to an effect on EDRF biosynthesis. That L-canavanine prevents NO release has been confirmed in the perfused rabbit aorta by a selective chemiluminescence technique (Schmidt *et al.*, 1988b). Whether L-canavanine inhibits NO release by inhibiting arginine catabolism is not clear. For example, L-canavanine exhibits potent cytotoxic activity which underlies its clinical efficacy in the treatment of certain cancers in man (Green & Ward, 1983). Similar cytotoxicity directed towards

vascular endothelial cells may also contribute to the diminished responses to ACh observed in both the rat kidney (this study) and aorta (Schmidt *et al.*, 1988a). For these reasons L-canavanine may not be a suitable tool for investigating the biosynthesis of EDRF.

Additional evidence that L-arginine is the precursor for EDRF has been obtained in cultured endothelial cells (Palmer *et al.*, 1988). Cells grown in culture containing L-arginine were shown to release larger amounts of NO following bradykinin challenge than did similar cells cultured in the absence of L-arginine. In contrast, Thomas & Ramwell (1988) have recently demonstrated that L-arginine does not potentiate the relaxant effect of ACh on isolated arteries such as the rat pulmonary artery and furthermore has no vasodilator activity in the perfused rat mesentery. These authors conclude that an arginine-containing peptide rather than L-arginine itself is the natural precursor to NO. In the present experiments we have confirmed, in the perfused rat kidney, that arginine does not potentiate renal vasodilatation due to ACh although, following bolus injection, it does dilate resistance blood vessels of the perfused rat kidney (this study) and mesentery (Al-Swaiyeh & Moore, unpublished work).

The present results do not allow us to confirm the precise mechanism of action of L-arginine and its salts in the rat kidney. The rapidity with which L-arginine salts produce vasodilatation following injection makes it most unlikely that this amino acid is incorporated into a peptide prior to enzyme attack and NO biosynthesis as suggested by Thomas & Ramwell (1988). Additionally, if L-arginine is enzymatically converted to NO in the rat kidney then, based upon their relative potency as vasodilators, the rate of conversion is likely to be minimal (i.e. $<0.01\%$). Furthermore, the nonselective effect of L-canavanine, the failure to increase EDRF release by ACh following arginine loading and the vasodilator effect of L-homoarginine – not a substrate for EDRF formation in cultured endothelial cells (Palmer *et al.*, 1988) – all suggest that exogenous L-arginine is not converted into NO in the kidney. Thus, we propose that L-arginine and its salts release EDRF from rat renal endothelial cells i.e. an effect similar to that of ACh. In this context it may be significant that L-arginine stimulates calcium influx into β cells of rat isolated pancreatic islets (Herchuelz *et al.*, 1984). A similar action in vascular endothelial cells would be expected to trigger EDRF efflux (see Luckhof *et al.*, 1988). Further experiments are necessary to determine whether L-arginine indeed releases EDRF or is converted into EDRF by the rat kidney. The possibility that L-arginine exhibits both such activities cannot be discounted.

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Effect of chronic intra-accumbens administration of the TRH analogue CG3509 on histamine-induced behaviour in the rat

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1 The present study has investigated the effect of chronic intra-accumbens administration of the thyrotrophin-releasing hormone (TRH) analogue, CG3509, on CG3509- and histamine-induced spontaneous motor activity and brain TRH-like immunoreactive (TRH-LI) levels in the rat.

2 Chronic intra-accumbens administration of CG3509 ($5 \times 5\,\mu g$ over 3 days) induced: (a) a significant ($P < 0.05$) reduction in intra-accumbens CG3509 ($0.5\,\mu g$)-induced hyperactivity, (b) reduced levels of TRH-LI in the nucleus accumbens but not other brain regions, (c) a marked increase (107%, $P < 0.01$) in histamine-induced non-locomotor hyperactivity.

3 The present results demonstrate that alteration of central TRH function following treatment with a TRH analogue enhances the effect of intra-accumbens histamine on behavioural hyperactivity, possibly via changes in H_1 receptors and suggest that the neuropeptide, TRH and histamine interact in behavioural arousal mechanisms in rat brain.

Introduction

Accumulating evidence supports a role for histamine as a neurotransmitter/neuromodulator in the CNS (see reviews Prell & Green, 1986; Pollard & Schwartz, 1987). Furthermore, recent studies (Bristow & Bennett, 1988a) suggest that the behavioural arousal mechanisms of this amine (Kalivas, 1982) may reside, in part, within the nucleus accumbens, since marked hyperactivity occurs in response to intra-accumbens administration and the histamine H_1-receptors mediating this response have been identified (Bristow & Bennett, 1988a). Interestingly, histamine-induced hyperactivity shows many similarities to that previously described following intra-accumbens administration of the neuropeptide, thyrotrophin releasing hormone (TRH) (Heal & Green, 1979; Sharp *et al.*, 1984; Kalivas et *al.*, 1987). Similarly, specific TRH receptor binding sites are present within the nucleus accumbens (Taylor & Burt, 1982; Sharif & Burt, 1983; Rostene *et al.*, 1984) and TRH release from rat accumbens slices has been described (Bennett *et al.*, 1981). The present study has therefore investigated possible intra-accumbens interactions between TRH and the histaminergic system in rats by use of behavioural techniques. Previous evidence indicates that histamine may interact with TRH in the accumbens, since histamine induces TRH release from synaptosomal preparations (Barraclough *et al.*, 1983) and manipulation of endogenous brain histamine levels is associated with marked changes in accumbens levels of TRH (Bennett *et al.*, 1983).

In an attempt to counter the poor biological stability and short-lasting actions of TRH, several structural analogues have been produced by modifications to the N-terminal pyroGlu and C-terminal Pro-NH_2 residues of the parent peptide. Various neuropharmacological studies have shown that these analogues are more potent than TRH on account of their enhanced biological stability (Metcalf, 1983). Thus microinjection of TRH analogues into the nucleus accumbens induces a greater and longer-lasting hyperactivity response than that seen with TRH (Sharp *et al.*, 1984). Furthermore, chronic treatment with TRH analogues results in the development of a behavioural tolerance to the peptide (Simasko & Horita, 1985; Fone *et al.*, 1988), which may be a consequence of a down regulation of central TRH receptors (Ogawa *et al.*, 1983; Simasko & Horita, 1985). In the present study, rats were chronically treated with the analogue CG3509

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(orotyl-L-histidyl-L-prolineamide), as a method for manipulating central TRH receptor-mediated function, and the effects on the hyperactivity response induced by CG3509 and on histamine-induced behaviour were investigated. The effect of chronic TRH analogue treatment on the levels of TRH-like immunoreactivity (TRH-LI) in selected brain regions was also determined.

Methods

Bilateral cannulation of the nucleus accumbens

Male Wistar rats (300 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and bilaterally implanted with stainless steel guide cannulae (23 gauge, 15 mm length) according to the atlas of Paxinos & Watson (1982); A/P + 1.7; L/R ± 1.4; V − 3 mm. A minimum of 7 days was allowed before the experiments were commenced, during this time cannulae were kept patent by a removable stainless steel stylet (31 gauge, 15 mm length).

Activity measurements

Drug-induced changes in activity were monitored using the Actimat doppler shift radar activity meter (Marsden & King, 1979), as described previously (Bristow & Bennett, 1988a). This system measures both the speed and duration of rat activity by directing a 10 W energy microwave beam (10.5 GH_z) from a doppler radar module into an experimental enclosure (60 × 70 × 65 cm) and analyses the small frequency changes of the reflected signals. Activity occurring in the frequency band of 0.4–4 Hz largely represents movements of the head and body without actual locomotion. Conversely, movements in the frequency band 4–100 Hz reflect high speed activity consisting largely of locomotion around the cage.

Individual rats were allowed an hour to acclimatize to the system after which they were removed and lightly restrained by hand. Drug injections into each cannula were administered over a 1 min period, the injection needle was removed after a further 30 s and animals were returned to the enclosure. A 1 min recovery period was then allowed before activity measurements commenced. Behaviour was monitored in 15 min intervals for 1 or 2 h and expressed as activity counts, with one count representing 1 s of activity. Activity counts occurring in both high and low frequency wavelengths are presented.

Experimental procedures

All experiments were carried out between 09 h 00 min and 12 h 00 min and rats were familiarised with the experimental environment on at least 2 separate occasions before behavioural responses were tested. All rats were initially tested for their behavioural responses following intra-accumbens injection of (a) 0.9% saline (2 × 1 μl, day 1), (b) histamine (10 μg μl^{-1}, 2 × 1 μl, day 3) and CG3509 (0.5 μg μl^{-1}, 2 × 0.5 μl, day 6). Rats were then chronically treated twice daily (09 h 00 min and 18 h 00 min) with either 0.9% saline (2 × 0.5 μl) or CG3509 (5 μg μl^{-1}, 2 × 0.5 μl), 5 bilateral intra-accumbens injections given over 3 days (days 8–10). The behavioural response to CG3509 (0.5 μg μl^{-1}, 2 × 0.5 μl) was then retested 24 h later (day 11). Similarly, histamine (10 μg μl^{-1}, 2 × 1 μl)-induced activity was retested on day 12.

Measurement of tissue TRH levels

On completion of activity measurements (day 12) rats were stunned and decapitated and the nucleus accumbens, septal nuclei, corpus striatum and hypothalamus dissected according to Craigies anatomical atlas (1963) (Lighton *et al.*, 1984). Tissue samples were extracted in 1 ml 90% methanol, the supernatants dried down in a Buchler vortex evaporator at 60°C and stored at −80°C for radioimmunoassay. The tissue pellets were retained for protein analysis by the method of Lowry *et al.* (1951).

TRH-like immunoreactivity was measured by the radioimmunoassay method of Lighton *et al.* (1984) using ethanol precipitation to separate bound from free peptide. The antiserum, raised in sheep at Nottingham by the method of Bassiri & Utiger (1972), was used at a working assay dilution of 1:20 K and shown to cross-react only with peptides containing both the N- and C-terminal residues of synthetic TRH. Assay sensitivity was 28 fmol per tube and the intra-assay and inter-assay coefficients of variation were 2.1% and 12% respectively.

Peptide recovery through the extraction, drying and assaying procedures was assessed by spiking bilateral tissue halves with 10 ng exogenous synthetic TRH. A mean tissue recovery of 61 ± 6% ($n = 4$) was calculated.

Statistical analysis

The effects of chronic treatment with CG3509 on the behavioural responses induced by CG3509 and histamine were analysed by use of Student's paired t test to compare pre- and post-treatment responses in the same group. Data presented as time courses were analysed by 2-way analysis of variance for repeated measures (BMDP statistical software) and F values, degrees of freedom (d.f.) and P values presented where appropriate. The hyperactivity response induced by various CG3509 doses (Figure 2) was analysed by one way analysis of variance using

Peritz's f-test for multiple comparisons (Harper, 1984). Tissue TRH levels measured in various brain regions from rats chronically treated with CG3509 or saline were analysed by Student's t tests.

Drugs

Histamine dihydrochloride (Sigma) and CG3509 (Grunenthal GmbH, Aachen) were dissolved in 0.9% saline solution for intra-accumbens injections.

Results

Effect of intra-accumbens administration of saline, histamine and CG3509 on rat activity

Bilateral microinjection of histamine ($10\,\mu g\,\mu l^{-1}$) into the nucleus accumbens induced marked biphasic changes in behavioural activity as demonstrated previously (Bristow & Bennett, 1988a,b). Thus in the first 15 min rats showed a significant ($P < 0.01$) reduction in activity counts, compared to saline controls, followed by a marked hyperactivity response (Figure 1). Intra-accumbens administration of the TRH analogue CG3509 ($0.01–1\,\mu g\,\mu l^{-1}$, $2 \times 0.5\,\mu l$) induced a marked dose-dependent increase in activity counts, without an initial hypoactivity phase (Figure 2), similar to that described previously (Sharp *et al.*, 1984). Furthermore, subdivision of activity counts into those representing low speed activity (i.e. small body movements, Figure 2b) and high speed activity (i.e. locomotion, Figure 2c) showed that an increase in activity occurred in both these wavelengths. However, changes in the low frequency band accounted for some 80% of the total activity changes recorded in 2 h.

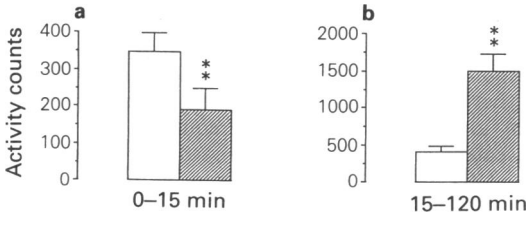

Figure 1 Effect of intra-accumbens injection of saline ($2 \times 1\,\mu l$) (open columns) or histamine ($10\,\mu g$, $2 \times 1\,\mu l$) (hatched columns) on activity counts accumulated from (a) 0–15 min and (b) 15–120 min. Columns represent mean activity counts and bars show s.e.mean ($n = 7$); data analysed by Student's paired t test. $^{**}P < 0.01$ compared to saline controls.

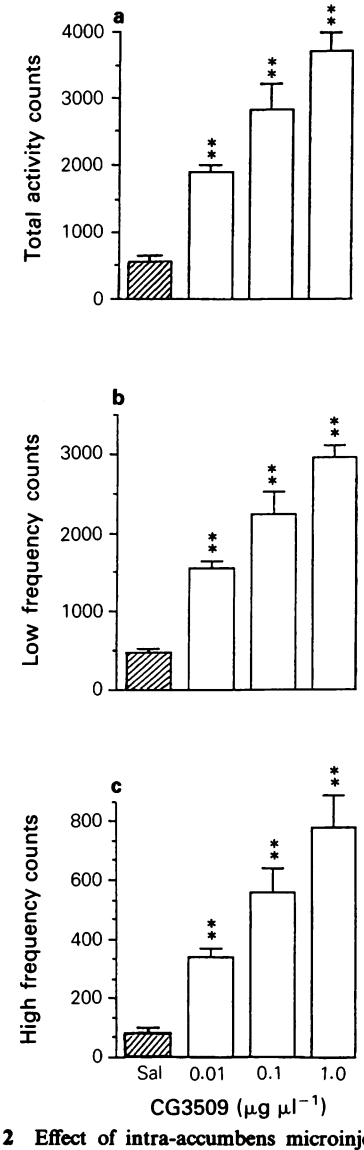

Figure 2 Effect of intra-accumbens microinjection of saline ($2 \times 0.5\,\mu l$) (hatched columns) or CG3509 ($0.01–1\,\mu g\,\mu l^{-1}$, $2 \times 0.5\,\mu l$) (open columns) on (a) total activity counts, (b) low frequency counts and (c) high frequency counts accumulated from 0–120 min. Columns represent mean activity counts and bars show s.e.mean ($n = 7$). Data were analysed by one way analysis of variance using Peritz's f test for multiple comparisons. $^{**}P < 0.01$ compared to saline controls.

Effect of chronic treatment with CG3509 on CG3509-induced behaviour

After pretreatment testing of the behavioural responses to histamine and CG3509, rats were

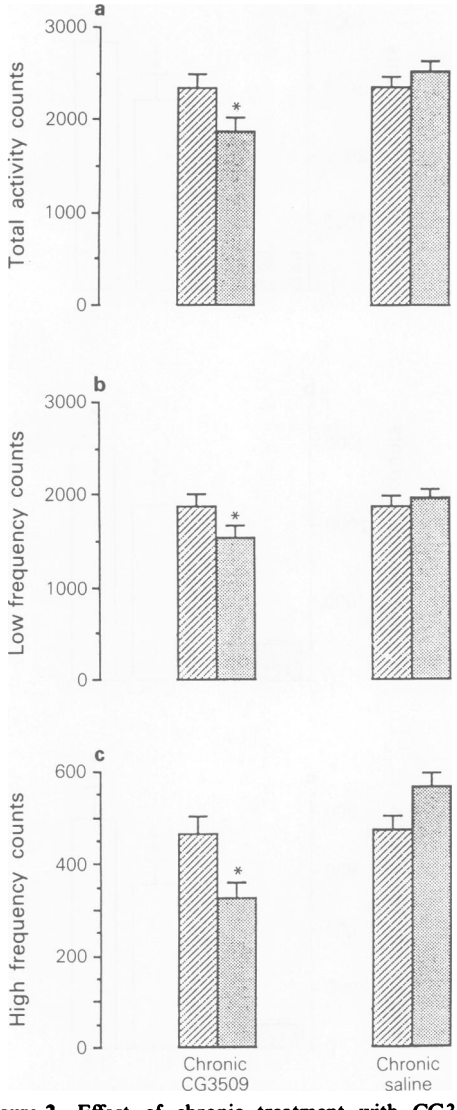

Figure 3 Effect of chronic treatment with CG3509 ($5\,\mu g\,\mu l^{-1}$, $2 \times 0.5\,\mu l$, $n = 7$) or saline ($2 \times 0.5\,\mu l$, $n = 7$) on CG3509 ($0.5\,\mu g\,\mu l^{-1}$, $2 \times 0.5\,\mu l$)-induced hyperactivity responses measured before (hatched columns) and after (stippled columns) chronic treatment. Columns represent mean activity counts accumulated from 0–60 min postinjection for (a) total activity counts, (b) low frequency counts and (c) high frequency counts; bars show s.e.mean. Data for each group were analysed by Student's paired t test. $^{*}P < 0.05$ compared to pretreatment CG3509 response.

chronically treated twice daily with either 0.9% saline ($2 \times 0.5\,\mu l$) or CG3509 ($5\,\mu g\,\mu l^{-1}$, $2 \times 0.5\,\mu l$), five intra-accumbens injections being given over 3 days. The behavioural response to CG3509 was then

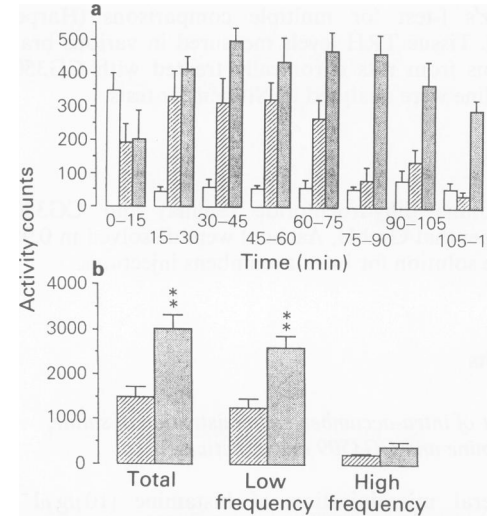

Figure 4 Effect of chronic treatment with CG3509 on histamine ($10\,\mu g\,\mu l^{-1}$, $2 \times 1\,\mu l$)-induced behavioural changes (a) Histamine-induced activity counts recorded for 15 min intervals measured before (hatched columns) and after (stippled columns) chronic treatment. Open columns show activity counts following intra-accumbens saline ($2 \times 1\,\mu l$) injection. In (a) and (b) columns represent mean activity counts ($n = 7$) and bars show s.e.mean. (a) Data (i.e. pretreatment vs post-treatment response) were analysed by 2 way analysis of variance for repeated measures: $F_{treatment} = 19.76$, d.f. 1, 6, $P = 0.0044$; $F_{time} = 10.07$, d.f. 7, 42, $P < 0.0001$; $F_{interaction} = 2.93$; d.f. 7, 42, $P = 0.0136$. (b) Total, low frequency and high frequency activity counts accumulated from 15–120 min following histamine administration before (hatched columns) and after (stippled columns) chronic CG3509 treatment. Data were analysed by Student's paired t test. $^{**}P < 0.01$ compared to pretreatment histamine response.

retested 24 h later. Rats chronically treated with the TRH analogue showed a small but significant ($P < 0.05$) reduction (20%) in CG3509-induced hyperactivity (Figure 3). In contrast, chronic treatment with saline did not alter the hyperactivity response induced by CG3509. Subdivision of total activity counts into those occurring in low (Figure 3b) and high (Figure 3c) frequency wavelengths revealed a significant ($P < 0.05$) reduction in CG3509 induced hyperactivity in both wavelengths, of 18% and 30% respectively.

Effect of chronic treatment with CG3509 on histamine-induced behaviour

Histamine ($10\,\mu g\,\mu l^{-1}$, $2 \times 1\,\mu l$)-induced behaviour following intra-accumbens injection was retested 48 h after the final chronic treatment with either

saline ($2 \times 0.5\,\mu l$) or CG3509 ($5\,\mu g\,\mu l^{-1}$, $2 \times 0.5\,\mu l$). Chronic TRH analogue administration resulted in a marked increase (107%, $P < 0.01$, Figure 4) in histamine-induced hyperactivity represented by activity counts accumulated from 15–120 min, while activity counts recorded in the first 15 min were not significantly altered, suggesting that histamine-induced hypoactivity was unaffected (Figure 4a). Furthermore, subdivision of the total activity counts into those occurring in low and high frequency wavelengths showed that the increase in histamine-induced hyperactivity resulted from a significant ($P < 0.01$) increase in low frequency activity i.e. small body movements (Figure 4b). In contrast, locomotor behaviour represented by counts occurring in the high frequency wavelengths was not significantly altered (Figure 4b). Chronic treatment with saline did not alter either histamine induced hypo- or hyperactivity (Figure 5).

Effect of chronic CG3509 treatment on brain tissue TRH levels

On completion of activity testing, rats were killed and TRH content measured in (a) nucleus accumbens, (b) septal nuclei, (c) corpus striatum and (d) hypothalamus. Rats treated with chronic intra-accumbens CG3509 ($5\,\mu g\,\mu l^{-1}$, $2 \times 0.5\,\mu l$) showed a significant ($P < 0.05$) reduction in TRH levels in the nucleus accumbens (Table 1). In contrast, TRH levels measured in the remaining brain regions were not significantly different in chronic saline- or CG3509-treated rats.

Discussion

Chronic treatment with the TRH analogue CG3509 (5 intra-accumbens injections over 3 days) resulted in a significant ($P < 0.05$) reduction in CG3509-induced hyperactivity responses compared to those measured before treatment. In contrast, chronic saline injection did not alter the behavioural response induced by the analogue. These results are consistent with those of previous studies which demonstrated the development of a behavioural tolerance following treatment with TRH analogues (Simasko & Horita, 1985; Fone *et al.*, 1988). Furthermore, this reduced behavioural response may be a consequence of a down regulation of TRH receptors, since Simasko & Horita demonstrated a significant reduction in [^{3}H]-3MeHis^{2}TRH binding to brain homogenates on the third day of chronic treatment with the TRH analogue MK771.

After the behavioural responses had been measured in the present study the rats were killed, the brains removed and the nucleus accumbens and adjacent regions assayed for TRH content by radioimmunoassay. In rats chronically treated with

Table 1 Effect of chronic treatment with saline or CG3509 ($5 \times 5\,\mu g\,\mu l^{-1}$) on regional brain levels of thyrotrophin-releasing hormone-like immunoreactivity (TRH-LI) in the rat

	TRH ($pg\,\mu g^{-1}$ protein)	
Brain region	*Saline treated*	*CG3509 treated*
Nucleus accumbens	1.1 ± 0.3 (6)	0.4 ± 0.1** (7)
Septum	1.3 ± 0.1 (5)	1.1 ± 0.2 (6)
Corpus striatum	0.3 ± 0.2 (3)	0.3 ± 0.1 (6)
Hypothalamus	2.6 ± 0.1 (7)	2.5 ± 0.1 (7)

After completion of behavioural measurements rats were killed and brain tissue TRH-LI levels measured by radioimmunoassay. Results are expressed as the mean tissue TRH levels ± s.e.mean; n = number in parentheses. **$P < 0.01$, Student's t test.

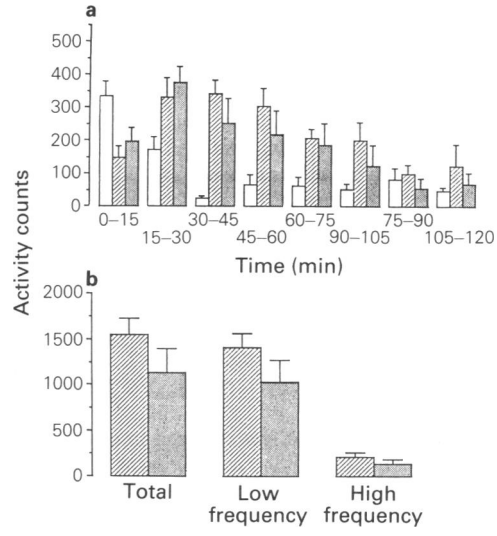

Figure 5 Effect of chronic treatment with saline on histamine ($10\,\mu g\,1\,\mu l^{-1}$, $2 \times 1\,\mu l$)-induced behaviour. (a) Histamine-induced activity counts recorded in 15 min intervals were measured before (hatched columns) and after (stippled columns) chronic saline treatment. Open columns show activity counts following saline control injections. In (a) and (b) columns represent mean activity counts ($n = 7$) and bars show s.e.mean. (a) Data (pretreatment response vs posttreatment response) were analysed by 2-way analysis of variance for repeated measures: $F_{treatment} = 0.38$, d.f. 1, 6, $P = 0.56$; $F_{time} = 6.37$, d.f. 7, 42, $P = 0.098$. (b) Total, low frequency and high frequency counts measured from 15–120 min following histamine administration before (hatched columns) and after (stippled columns) CG3509 treatment. Data were analysed by Student's paired t test and were not significantly different compared to pretreatment response in each frequency range.

the TRH analogue the levels of TRH in the nucleus accumbens were significantly reduced ($P < 0.05$) compared to control saline-treated animals. In contrast, the levels of TRH measured in the septum, striatum and hypothalamus were not altered following CG3509 treatment. It is unlikely that this reduction in TRH results from interference of CG3509 in the assay procedure since TRH analogues show negligible cross-reactivity with the TRH-antisera preparation (Lighton *et al.*, 1984). Furthermore, since the change in endogenous peptide was restricted to the nucleus accumbens, this may suggest that selective changes in TRH synthesis and/or release may occur in addition to the down regulation of TRH receptors, predicted by the development of a behavioural tolerance to CG3509.

In addition to the changes induced in central TRH function, chronic CG3509 treatment was also associated with a marked selective increase in histamine-induced hyperactivity, shown previously to be mediated by H_1 receptors (Bristow & Bennett, 1988a). Interestingly, subdivision of the activity analysis into the major non-locomotor and minor locomotor components showed that this potentiation in histamine-induced hyperactivity was largely an effect on non-locomotor behaviour i.e. head and forepaw movements, sniffing and grooming (Bristow & Bennett, 1988a). In contrast, the transitory histamine-induced hypoactivity response observed in previous studies (Bristow & Bennett, 1988a,b) was unaltered by treatment with the TRH analogue. This suggests that presynaptic events, i.e. histamine synthesis and release, may not be modulated by TRH, since previous evidence (Bristow & Bennett, 1988b) would suggest that the hypoactivity is mediated by H_3 autoreceptors which are intimately involved with these processes (Arrang *et al.*, 1983; 1987). The present results, then, suggest that TRH present within and released from (Bennett *et al.*, 1981) the accumbens specifically modulates the arousal action of histamine at postsynaptic H_1 receptors. That neuropeptides are able to modulate the behavioural effects induced by neurotransmitter substances in the nucleus accumbens has been demonstrated previously, e.g., neurotensin blocks, whereas substance P enhances, dopamine mediated hyperlocomotion (Kalivas *et al.*, 1984; Kalivas & Miller, 1984).

Various previous studies have shown that intra-accumbens injection of TRH or its more stable analogues induces a marked hyperactivity response in rats (Miyamoto & Nagawa, 1977; Heal & Green, 1979; Heal *et al.*, 1981; Sharp *et al.*, 1984; Miyamoto *et al.*, 1984; Kalivas *et al.*, 1987). This behaviour, like that following intra-accumbens histamine, consists of changes in several different behaviours including locomotion, sniffing, grooming, and rearing, but also includes forepaw licking and chewing, wet dog shakes and straub tail (Heal *et al.*, 1981; Sharp *et al.*, 1984). While the stereotypy and locomotor behaviours apparently involve accumbens and other forebrain mechanisms (Sharp *et al.*, 1984), recent evidence suggests that wet dog shakes, forepaw licking and tail elevation result from actions on spinal or rhombencephalon neurones (Fone *et al.*, 1987; Johnson *et al.*, 1989). Thus the latter behaviours, observed following intra-accumbens injection of TRH analogues, possibly result from diffusion to more caudal brain regions.

There is a large body of evidence suggesting that TRH-induced hyperlocomotion is mediated via an increase in mesolimbic dopamine release (Miyamoto & Nagawa, 1977; Heal & Green, 1979; Sharp *et al.*, 1984), but other neurotransmitter systems have also been implicated in the hyperactivity response induced by this peptide. In particular, evidence suggests the involvement of the noradrenergic system in both rats (Lin *et al.*, 1983; Miyamoto *et al.*, 1984) and mice (Heal *et al.*, 1987). The present results now indicate that the histaminergic pathway from the mammillary bodies to the accumbens (Pollard & Schwartz, 1987) is also associated with mechanisms of arousal and may be modulated by changes in TRH receptor function.

Although the present and previous studies support an interaction between histamine and TRH in arousal mechanisms, the precise nature of the interaction remains unclear. Although histamine stimulates release of TRH from sheep accumbens synaptosomal preparations through actions on H_2 receptors (Barraclough *et al.*, 1983), it is unlikely that histamine-induced hyperactivity responses in the rat are directly mediated through stimulation of TRH release, since the potent and selective H_2 agonist dimaprit did not induce any behavioural effects and the response to histamine was not attenuated following pretreatment with the H_2 antagonist SKF93479 (Bristow & Bennett, 1988a). Moreover, if TRH-induced hyperactivity is mediated by dopaminergic mechanisms (see above) and histamine-induced hyperactivity were mediated via an increase in TRH release, then pretreatment with dopamine antagonists would be expected to block the effects of histamine. However, preliminary studies indicate that pretreatment with sulpiride, at doses shown to be effective in blocking amphetamine-induced locomotion (Sharp *et al.*, 1986), have no effect on histamine-induced behaviour (unpublished observations). In contrast, the selective modulation of histamine H_1 receptor-mediated hyperactivity responses, observed in the present study, may suggest interactions at the level of the H_1 receptor itself and preliminary binding studies have demonstrated increases in H_1 receptor number following

presentation with CG3509 (Bristow & Bennett, 1988c). It thus remains to be determined whether chronic TRH analogue treatment induces any changes in H_1 receptor sensitivity and whether this relates to the changes occurring in the histamine H_1 receptor-mediated hyperactivity.

In summary, chronic intra-accumbens treatment with the TRH analogue CG3509 is associated not only with the development of a behavioural tolerance to the peptide, but also with a marked increase in histamine H_1 receptor-mediated hyperactivity. These results provide evidence suggesting that histamine and TRH are closely linked in arousal mechanisms associated with the nucleus accumbens.

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Interaction of potassium channel openers and blockers in canine atrial muscle

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1 The possibility that the interaction between potassium channel openers, e.g. cromakalim, pinacidil and nicorandil, and some potassium channel blockers involves a common site was investigated in canine atrial muscle.

2 Cromakalim, pinacidil and nicorandil produced a negative inotropic effect, their pD_2 ($-\log EC_{50}$) values being 6.11 ± 0.07, 5.37 ± 0.09 and 4.55 ± 0.07, respectively.

3 The potassium channel blockers, tetraethylammonium (TEA), tetrabutylammonium (TBA), 3,4-diaminopyridine (DAP), CsCl and $BaCl_2$ all produced a positive inotropic effect.

4 The concentration-effect curves for the negative inotropic actions of pinacidil were shifted in a parallel way to the right by low concentrations of TEA, TBA or $BaCl_2$. Maximum responses to pinacidil were depressed by higher concentrations of the blockers. An analysis of the non-competitive antagonism by TEA yielded pK_A ($-\log K_A$) values of 4.00–4.05 for pinacidil.

5 The concentration-effect curves for cromakalim and nicorandil were shifted by TEA similarly to those for pinacidil, and a similar analysis yielded pK_A values of 4.47–4.68 for cromakalim and 3.47–3.74 for nicorandil.

6 The K_A values of cromakalim, pinacidil and nicorandil were about 10–30 times greater than their EC_{50} values, indicating that there are non-linear stimulus-effect relationships between the binding of the three potassium channel openers to their binding sites at potassium channels and their negative inotropic effects.

7 The dissociation constants for TEA could also be estimated from pA_2 and pK_B values for antagonizing competitively and non-competitively the negative inotropic effects of the three potassium channel openers; they were 3.47–3.89, and did not differ between the potassium channel openers.

8 The concentration-effect curves for the three potassium channel openers were not affected by DAP or CsCl.

9 These results suggest the following: (i) quaternary ammonium compounds like TEA and TBA antagonize the negative inotropic effect of cromakalim, pinacidil and nicorandil by binding to potassium channels, thus preventing binding of the channel openers to the same sites or closely related sites in canine right atrial muscles. (ii) The potassium channels responsible for the negative inotropic effects of the three potassium channel openers seem to be akin to apamin-insensitive and calcium-insensitive potassium channels in smooth muscle and are opened at the resting membrane potential.

Introduction

Recently increasing attention has been paid to vasodilator drugs which activate or open potassium channels in vascular smooth muscle and eventually lower blood pressure. These drugs called 'potassium channel activators or openers' include nicorandil (Taira *et al.*, 1979), pinacidil (Arrigoni-Martelli *et al.*, 1980; Bray *et al.*, 1987) and cromakalim (BRL 34915; Hamilton *et al.*, 1986; Weir & Weston, 1986a,b), although nicorandil seems also to have an additional mechanism of action similar to classical nitrates (Taira, 1987). Potassium channel opening as one of the mechanisms underlying the cardiac action of vasodilator drugs was first suggested for nicorandil by us (Yanagisawa *et al.*, 1979) and also demonstrated by us (Yanagisawa & Taira, 1980). Nonetheless, studies of the cardiac effects of potassium channel openers are scant compared with those of their vasodilator effects. In a previous study (Yanagisawa *et al.*, 1988), we investigated the effects of cromakalim, pinacidil and nicorandil on the force

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of contraction of canine atrial muscles in relation to their effects on cyclic nucleotides levels in canine atrial muscle, because nicorandil had been shown to increase guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels and a possible involvement of an increase in cyclic GMP levels in the negative inotropic effect of nicorandil had been suggested (Endoh & Iijima, 1983). We concluded that these three potassium channel openers reduce the force of contraction in canine atrial muscles exclusively by opening potassium channels. The increases in cyclic GMP were not involved in the negative inotropic effect, because their negative inotropic effects were blocked by the potassium channel blocker, tetraethylammonium (TEA; Coraboeuf & Vassort, 1968; Harder & Sperelakis, 1978), whereas the increase in cyclic GMP levels produced by nicorandil remained unchanged. It has also been shown that in trachealis muscle the relaxant and hyperpolarizing effects of cromakalim are suppressed by potassium channel blockers such as TEA, 4-aminopyridine and procaine but not by apamin (Allen *et al.*, 1986). Thus, it was of interest to know whether the negative inotropic effects of the three potassium channel openers were specifically blocked by potassium channel blockers. If the effects are specifically blocked by a certain class of potassium channel blockers, it is possible that the three potassium channel openers and their specific blockers would interact at the same site or closely related sites at potassium channels. The present experiments were designed to explore this possibility. For this purpose, we investigated how the negative inotropic effects of the three potassium channel openers on the force of contraction in canine atrial muscles could be modified by various potassium channel blockers, TEA, tetrabutylammonium (TBA; French & Shoukimas, 1981), CsCl (Hagiwara *et al.*, 1976; Isenberg, 1976), $BaCl_2$ (Harder & Sperelakis, 1978; Ehara & Inazawa, 1980), and 3,4-diaminopyridine (DAP). We further analysed the antagonism of TEA and TBA against potassium channel openers by applying Schild analysis (Arunlakshana & Schild, 1959) or procedures devised to analyse non-competitive antagonism (Kenakin, 1987).

Methods

Hearts were excised from mongrel dogs of either sex, (weight 5 to 13 kg) anaesthetized with pentobarbitone sodium (30 mg kg^{-1} i.v.). Trabecular muscles of the right atrial wall were isolated from the heart in oxygenated cold (ca. 7°C) Krebs-Henseleit solution and mounted in 20 ml organ baths. The composition (mmol l^{-1}) of the solution was as follows: NaCl 118, $NaHCO_3$ 24.9, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 11.1, ascorbic acid 0.057 and Na_2EDTA, 0.027. The solution was equilibrated with 95% O_2 and 5% CO_2 at a temperature of 37°C (pH 7.4). Muscles were stretched to a resting tension of about 0.5 g and were stimulated by square wave pulses of twice threshold voltage and 5 ms duration at a frequency of 0.5 Hz. During an equilibration period of about 1 h, the length of the muscle was adjusted to that producing maximum contractile force. The force of isometric contraction was recorded on a thermal pen-writing oscillograph (NEC San-ei, Recti-Horiz-8K) by means of strain-gauge transducers (Shinkoh, UL-10230). The concentrations of nicorandil or pinacidil were increased at 5 min intervals and those of cromakalim and nifedipine at 10 or 30 min intervals, respectively. Usually 4–8 muscles were isolated from each heart and run in parallel, one of them being used as control. All of the potassium channel blockers were administered 20 min before the potassium channel openers were applied. All muscles were treated with both 10^{-6} M nadolol and 10^{-6} M atropine to eliminate possible effects of noradrenaline and acetylcholine released from autonomic nerve terminals by electrical stimuli or indirectly by potassium channel blockers (Harvey & Marshall, 1977; Yanagisawa *et al.*, 1978).

Drugs and chemicals were obtained from the following sources: cromakalim (Beecham Research Laboratories), pinacidil (Shionogi), nicorandil hydrochloride (Chugai), nifedipine (Bayer), tetraethylammonium chloride (TEA, Wako), tetrabutylammonium chloride (TBA, Wako), 3,4-diaminopyridine (DAP, Wako), (±)-nadolol base (Squibb) and atropine sulphate (Wako). Cromakalim was dissolved in 70% ethanol to give a concentration of 10 mmol l^{-1}. Pinacidil was dissolved in 0.1 N HCl to give a concentration of 200 mmol l^{-1}. Nifedipine concentration 289 μmol l^{-1}. Nadolol base was dissolved in 0.5 N HCl to give a concentration of 32 mmol l^{-1}. Other drugs were dissolved in distilled water in the desired concentrations. From these stock solutions the desired concentrations were obtained by diluting with distilled water.

The concentration-negative inotropic effect curves for potassium channel openers were expressed as % reduction in the basal force and computer fitted to a logistic equation:

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

where E is normalized response, M is the maximum response of each drug, A is drug concentration, K is EC_{50} value of each drug and p is the slope parameter (Parker & Waud, 1971). EC_{50} values were presented as pD_2 ($pD_2 = -\log EC_{50}$).

The antagonism by TEA and TBA of the negative inotropic effects of the three potassium channel openers was analysed in the following way: Schild analysis (Arunlakshana & Schild, 1959) was performed at the concentration range of TEA (10^{-4} to 10^{-3} M) which produced parallel rightward shifts of the concentration-negative inotropic effect curves and at that of TBA (3×10^{-5} to 3×10^{-4} M) which produced parallel rightward shifts, although the maxima were suppressed only slightly at 3×10^{-4} M TBA. With a concentration of TEA (3×10^{-3} M) which produced a suppression of the maxima of the concentration-negative inotropic effect curves, the procedures were employed which analyse non-competitive antagonism (Kenakin, 1987).

Experimental values are given as mean or mean ± s.e.mean. Statistical significance of differences between mean values was estimated by Student's t test. A t test for the paired comparison was used when it was applicable. A P value less than 0.05 was considered to be significant.

Results

Antagonism by TEA of the negative inotropic effects of potassium channel openers and nifedipine

As in a previous study (Yanagisawa *et al.*, 1988), cromakalim (10^{-8} to 10^{-4} M), pinacidil (10^{-7} to 3×10^{-4} M) and nicorandil (10^{-6} to 10^{-3} M) caused a concentration-dependent decrease in the force of contraction (up to 90%) in canine right atrial muscles. Nifedipine (10^{-8} to 3×10^{-6} M) produced a negative inotropic effect leading to complete abolition of contractions. Figure 1 shows the modification by TEA of concentration-negative inotropic effect curves for cromakalim, pinacidil, nicorandil and nifedipine. TEA (10^{-4} to 10^{-2} M) *per se* produced a concentration-dependent positive inotropic effect which amounted to 140% at its maximum effect in canine atrial muscles (Table 1), in which the pD_2 value of TEA was 3.33. These concentrations of TEA antagonized the negative inotropic effects of the potassium channel openers and nifedipine (Figure 1, Table 2). However, the mode and magnitude of antagonism by TEA were different between the potassium channel openers and nifedipine. TEA at 10^{-4} to 10^{-3} M shifted the concentration-negative inotropic effect curves for the three potassium channel openers in a parallel fashion to the right; the maxima and slope factors remained unchanged (Figure 1, Table 2). With 10^{-3} to 10^{-2} M TEA, however, the curves were shifted to the right and downward. In contrast TEA only shifted the concentration-negative inotropic effect curves for nifedipine to the right. The pD_2 values of nifedipine in producing a negative inotropic effect in the absence and presence of 10^{-3} and 10^{-2} M TEA were 7.14 ± 0.08, 6.78 ± 0.08 and 6.60 ± 0.08, respectively. Thus, although the shift was indeed produced by TEA, its magnitude was very small and the maxima were not changed.

Quantitative analysis of the antagonism by TEA of the negative inotropic effects of potassium channel openers

Since the concentration-negative inotropic effect curves for the three potassium channel openers were shifted to the right in a parallel way by 10^{-4} to 10^{-3} M TEA, the data obtained with these concentrations of TEA were subjected to Schild analysis. Each Schild regression was linear and had a slope of unity (Figure 2). This analysis yielded pA_2 values of 3.64, 3.47 and 3.66 for TEA against cromakalim, pinacidil and nicorandil, respectively. Since the pA_2 values of TEA were not different for the three potassium channel openers, it seemed that TEA competed for the same binding sites with the three potassium channel openers to antagonize their negative inotropic effect.

TEA at 3×10^{-3} M suppressed the maximum effects of the potassium channel openers (Figure 1, Table 2). To analyse the non-competitive antagonism by TEA, equieffective concentrations of pinacidil were obtained from the concentration-effect curves in the absence and presence of 3×10^{-3} M TEA and the dissociation constant (K_A) of pinacidil and the binding constants (K_B) of TEA were estimated (Figure 3, Table 3). The pK_A ($= -\log K_A$) values of pinacidil estimated in three ways were 4.05, 4.00 and 4.03. These K_A values were about 20 times greater than EC_{50} values of pinacidil (Table 2). The pK_B ($= -\log K_B$) values of TEA estimated in three ways were 3.84, 3.89 and 3.86. Similar analyses were performed for the concentration-effect curves of cromakalim and nicorandil in the absence and presence of 3×10^{-3} M TEA (Table 3). The K_A values determined for cromakalim and nicorandil were also 10 to 30 times greater than their EC_{50} values (Table 3). The pK_B values of TEA against cromakalim and nicorandil were 3.86 and 3.51, respectively. These pK_B values of TEA were very close to the pA_2 values of TEA.

Modification by other potassium channel blockers of the negative inotropic effect of pinacidil

The potassium channel blockers, TBA (3×10^{-5} to 3×10^{-4} M), $BaCl_2$ (3×10^{-4} and 10^{-3} M), DAP (10^{-5} to 3×10^{-3} M) and CsCl (10^{-2} M) all produced a positive inotropic effect in canine atrial muscles,

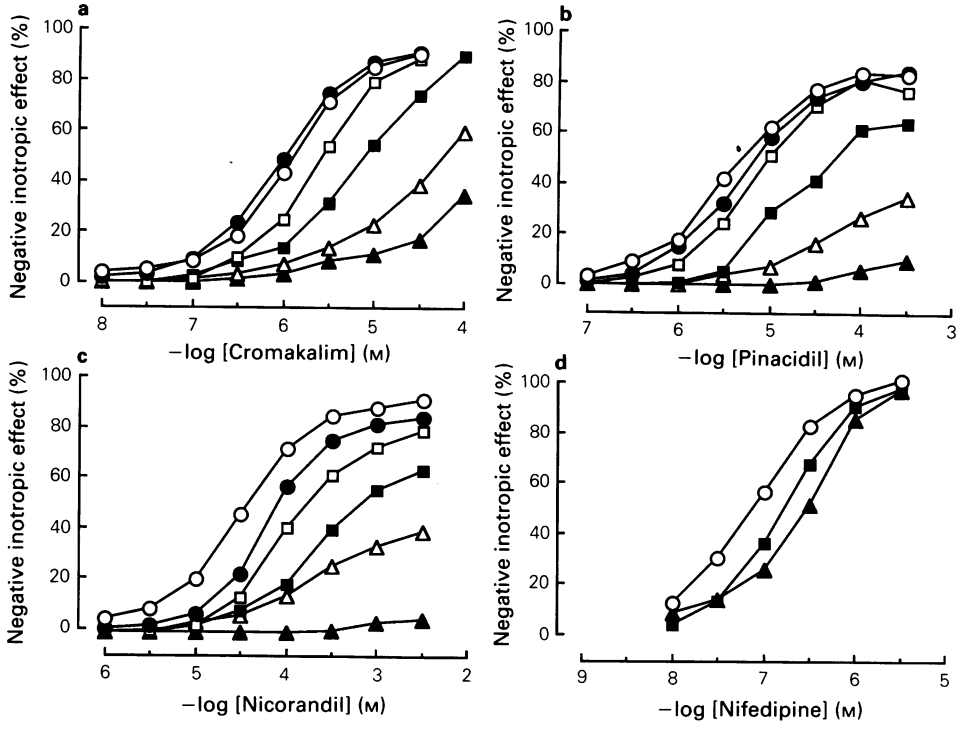

Figure 1 Modification by tetraethylammonium (TEA) of the concentration-negative inotropic effect curves for (a) cromakalim, (b) pinacidil, (c) nicorandil and (d) nifedipine in canine atrial muscles. (○) Control, (●) 10^{-4} M, (□) 3×10^{-4} M, (■) 10^{-3} M, (△) 3×10^{-3} M, (▲) 10^{-2} M TEA.

although their potencies and maximum effects differed (Table 1). Figures 4 and 5 show differential modification by these potassium channel blockers of the negative inotropic effect of pinacidil. TBA (3×10^{-5} to 3×10^{-4} M) and $BaCl_2$ (3×10^{-4} and 10^{-3} M), like TEA, shifted the concentration-effect curves of pinacidil to the right and slightly downward in a concentration-dependent manner (Figure 4, Table 2). Since the suppression of the maximum effect of pinacidil by 3×10^{-4} M TBA was marginal,

Table 1 Positive inotropic effects of various potassium channel blockers in canine atrial muscles

	Concentrations (M)						
	10^{-5}	3×10^{-5}	10^{-4}	3×10^{-4}	10^{-3}	3×10^{-3}	10^{-2}
TEA			(16)	(16)	(28)	(16)	(19)
			7.4 ± 2.3	41.7 ± 9.4	137.2 ± 32.7	171.7 ± 38.9	142.7 ± 32.2
TBA		(5)	(6)	(6)			
		31.3 ± 12.4	41.1 ± 10.2	43.5 ± 7.7			
DAP	(7)		(8)		(6)	(7)	
	49.2 ± 2.3		69.7 ± 9.4		55.1 ± 32.7	120.6 ± 38.9	
$BaCl_2$				(5)	(5)		
				35.0 ± 16.3	75.1 ± 17.6		
CsCl							(17)
							99.5 ± 26.9

Positive inotropic effects expressed as % increase above basal force are given as means ± s.e.mean. Numbers of muscles are given in parentheses.
TEA, tetraethylammonium; TBA, tetrabutylammonium; DAP, 3,4-diaminopyridine.

Table 2 Influences of tetraethylammonium (TEA), tetrabutylammonium (TBA) or $BaCl_2$ on the negative inotropic effects of potassium channel openers in canine atrial muscles

		Concentrations of potassium channel blockers (M)				
	Control	3×10^{-5}	10^{-4}	3×10^{-4}	10^{-3}	3×10^{-3}
TEA						
Cromakalim	(6)		(5)	(5)	(12)	(6)
Max	93.7 ± 2.4		94.5 ± 1.5	94.5 ± 0.9	93.5 ± 2.0	71.1 ± 7.4*
pD_2	6.11 ± 0.07		5.97 ± 0.05	5.74 ± 0.08*	5.27 ± 0.13*	4.69 ± 0.13*
Pinacidil	(17)		(6)	(6)	(5)	(5)
Max	87.9 ± 2.1		82.9 ± 4.0	86.9 ± 2.0	75.3 ± 4.6*	44.2 ± 9.4*
pD_2	5.37 ± 0.09		5.31 ± 0.07	5.09 ± 0.07*	4.57 ± 0.14*	4.17 ± 0.07*
Nicorandil	(12)		(5)	(5)	(5)	(5)
Max	91.6 ± 1.8		85.4 ± 3.7	79.3 ± 5.0	64.0 ± 7.2*	47.8 ± 9.4*
pD_2	4.55 ± 0.07		4.19 ± 0.05*	3.99 ± 0.07*	3.76 ± 0.16*	3.34 ± 0.19*
TBA						
Pinacidil	(4)	(5)	(6)	(6)		
Max	87.7 ± 5.2	80.8 ± 3.6	76.8 ± 3.6	72.8 ± 5.0*		
pD_2	5.10 ± 0.15	4.75 ± 0.07*	4.26 ± 0.13*	3.67 ± 0.09*		
$BaCl_2$						
Pinacidil	(6)			(5)	(5)	
Max	88.1 ± 4.7			90.6 ± 2.8	79.7 ± 4.1*	
pD_2	5.31 ± 0.08			4.80 ± 0.11*	4.61 ± 0.19*	

Maximum effects (Max) and pD_2 values were obtained by computer fitting to the logistic equation of the concentration-effect curves for cromakalim, pinacidil and nicorandil (see Methods), and are given as means ± s.e.mean.

* $P < 0.05$ compared with values in the absence of potassium channel blockers (control). Numbers of muscles are given in parentheses.

the data obtained with TBA (3×10^{-5} to 3×10^{-4} M) were subject to Schild analysis. The analysis yielded a pA_2 value of TBA of 4.70 (Figure 4b). DAP (10^{-5} to 3×10^{-3} M) and CsCl (10^{-2} M) failed to affect the concentration-effect curves of pinacidil (Figures 4 and 5).

No modification by CsCl of the negative inotropic effects of nicorandil and cromakalim

As with pinacidil, the concentration-negative inotropic effect curves for nicorandil and cromakalim in atrial muscles were not modified by 10^{-2} M CsCl (Figure 5).

Discussion

As previously observed (Yanagisawa *et al.*, 1988), cromakalim, pinacidil and nicoradil, whose mechanism of action is thought to be the activation or opening of potassium channels in vascular (Weston & Abbot, 1987) and cardiac muscle (Yanagisawa & Taira, 1980; Cain & Metzler, 1985; Iijima & Taira, 1987; Scholtysik, 1987), produced a negative inotropic effect in canine atrial muscles. Their maximum negative inotropic effects were about 90% as against 100% with the calcium channel blocker, nifedipine (Yanagisawa *et al.*, 1988). The mechanism for such negative inotropic effects is thought to be due secondarily to the shortening of the action potential of atrial muscle produced by increased outward potassium currents (Yanagisawa & Taira, 1980). As discussed in our previous article (Yanagisawa *et al.*, 1988), the decreased force of contraction and duration of action potential obtained with nicorandil were consistent with what has previously been demonstrated in voltage-clamp experiments (Morad & Trautwein, 1968).

It is known that potassium channel blockers produce a positive inotropic effect by prolongation of the action potential duration due to blockade of potassium channels in cardiac muscle (Coraboeuf & Vassort, 1968 for TEA; Yanagisawa & Taira, 1979 for 4-aminopyridine; Ehara & Inazawa, 1980 for $BaCl_2$). Indeed, in the present study all the potassium channel blockers used, TEA, TBA, $BaCl_2$, DAP and CsCl, produced a positive inotropic effect which, however, differed in magnitude. The negative inotropic effects of three potassium channel openers were antagonized differently by these potassium channel blockers; the negative inotropic effect of pinacidil, which was subject to all of the potassium channel blockers used, was antagonized by TEA, TBA and $BaCl_2$ but not by DAP and CsCl. Thus,

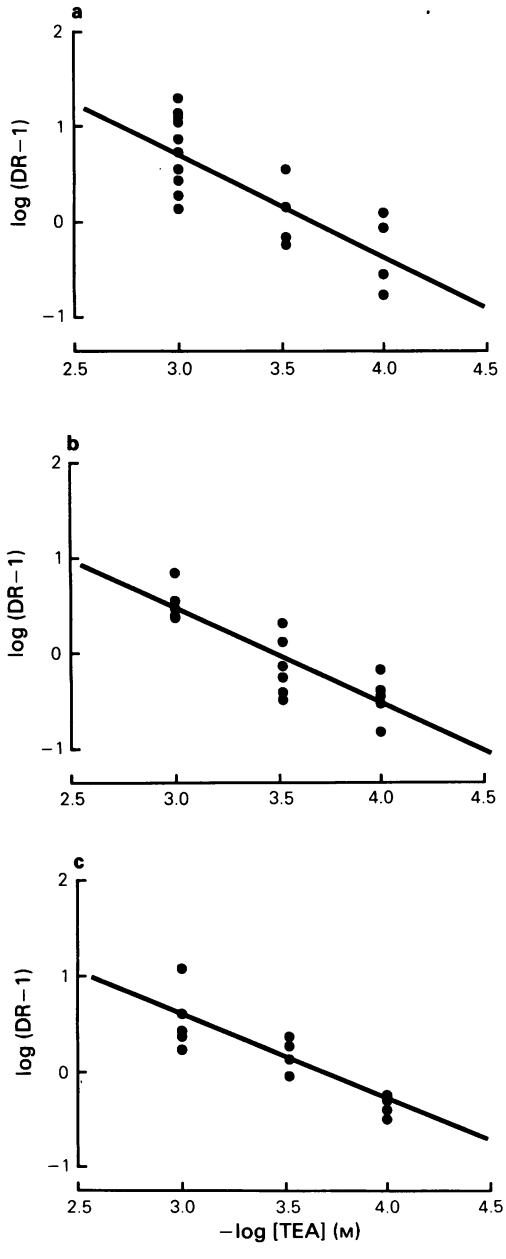

Figure 2 Schild plots of the antagonism of the negative inotropic effects of (a) cromakalim, (b) pinacidil and (c) nicorandil by tetraethylammonium (TEA) in canine atrial muscles. (a) Cromakalim: pA_2 value, 3.64; the slope of regression line, −1.07; the correlation coefficient, −0.78 ($n = 21$). (b) Pinacidil: pA_2 value, 3.47; the slope of regression line, −1.00; the correlation coefficient, −0.85 ($n = 16$). (c) Nicorandil: pA_2 value, 3.66; the slope of regression line, −0.88; the correlation coefficient, −0.87 ($n = 15$).

Table 3 Dissociation constants (K_A), pD_2 values and ratios of K_A to EC_{50} of the three potassium channel openers and the binding constants (K_B) and pA_2 values of tetraethylammonium (TEA)

	Potassium channel openers			*TEA*	
	pK_A	pD_2	K_A/EC_{50}	pK_B	pA_2
Cromakalim	4.68			3.78	
	4.47	6.11	35	3.86	3.64
	4.57			3.86	
Pinacidil	4.05			3.84	
	4.00	5.37	22	3.89	3.47
	4.03			3.86	
Nicorandil	3.74			3.47	
	3.47	4.55	9	3.65	3.66
	3.58			3.51	

K_A of the three potassium channel openers and K_B of TEA were estimated by the analysis of non-competitive antagonism in three ways. The pA_2 values of TEA were obtained by Schild analysis (Figure 2). Ratios of K_A (mean of the values) to the EC_{50} (pD_2; Table 2) of the potassium channel openers indicate that there are nonlinear stimulus-effect relationships between the binding of the three potassium channel openers to their binding sites and their negative inotropic effects.

the antagonism by TEA, TBA or $BaCl_2$ of the negative inotropic effect of pinacidil, and that by TEA of the negative inotropic effects of cromakalim and nicorandil, should not simply be ascribed to functional antagonism between the positive inotropic effects produced by potassium channel blockade on the one hand and the negative inotropic effect caused by potassium channel opening on the other. Indeed, the specific antagonism between TEA and the three potassium channel openers and that between TBA and pinacidil suggests that TEA and TBA interact with the same or closely related sites of actions as the potassium channel openers. In the present study Schild analysis applied to the concentration range of TEA (10^{-4} to 10^{-3} M) which produced rightward parallel shifts of the concentration-negative inotropic effect curves for the three potassium channel openers yielded pA_2 values of 3.47–3.66 for TEA which were not different for the three potassium channel openers (Figure 2 and Table 3). Analysis of the non-competitive antagonism by the high concentration of TEA (3×10^{-3} M) of the concentration-effect curves of the three potassium channel openers yielded pK_B values of 3.47–3.89 for TEA which were also not different for the three potassium channel openers (Table 3). Thus, the dissociation constants for TEA were determined to be $1.3–3.4 \times 10^{-4}$ M. The values were close to EC_{50} values (4.7×10^{-4} M; $pD_2 = 3.33$) which caused a positive inotropic effect but far lower than the concentrations of TEA usually used

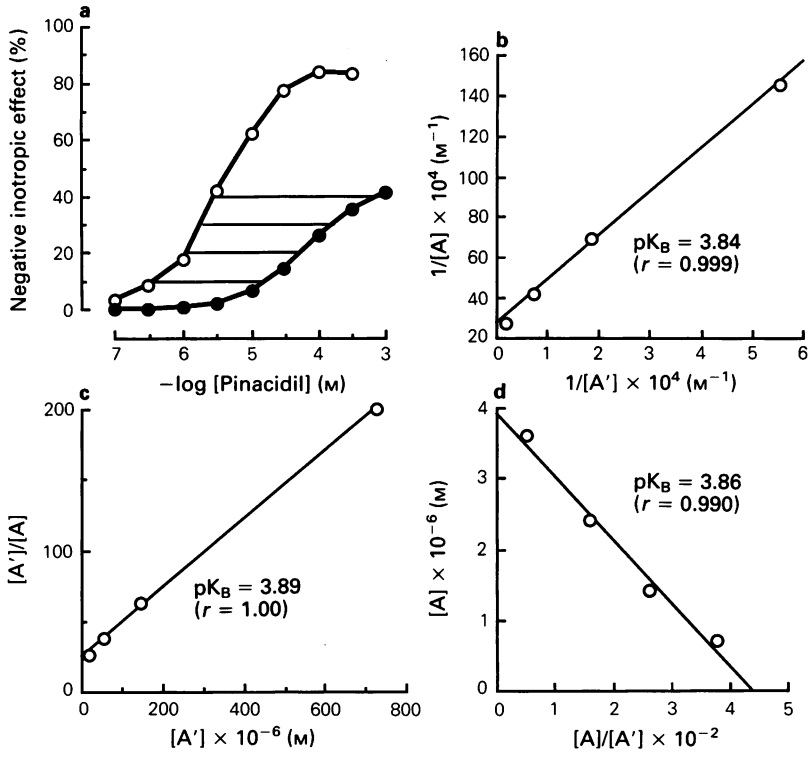

Figure 3 The non-competitive antagonism of the negative inotropic effects of pinacidil by tetraethylammonium (TEA, 3×10^{-3} M) in canine atrial muscles. (a) Concentration-negative inotropic effect curves for pinacidil in the absence (○) and presence (●) of 3×10^{-3} M TEA. Equieffective concentrations of pinacidil expressed by horizontal lines were obtained from the logistic equation of the concentration-effect curves for pinacidil in the absence and presence of 3×10^{-3} M TEA. (b) Double-reciprocal plot of equieffective concentrations of pinacidil in the absence (ordinate scale, 1/[A]) and presence (abscissa scale, 1/[A']) of 3×10^{-3} M TEA. Slope, 21.8; intercept, 2.70×10^5; dissociation constant (K_A) of pinacidil, 8.9×10^{-5} M; dissociation constant (K_B) of TEA, 1.45×10^{-4} M. (c) Regression of [A']/[A] vs. [A']. Slope, 2.43×10^5; intercept, 24.2; dissociation constant (K_A) of pinacidil, 9.9×10^{-5} M; dissociation constant (K_B) of TEA, 1.29×10^{-4} M. (d) Regression [A]/[A'] vs. [A]. Slope, −89.7; intercept, 3.94×10^{-6}; dissociation constant (K_A) of pinacidil, 9.4×10^{-5} M; dissociation constant (K_B) of TEA, 1.38×10^{-4} M.

(more than 10^{-2} M) to block potassium conductance by external application. The dissociation constants of TEA determined in the present study were also close to those (K_D) for the reaction between TEA and its receptors to inhibit potassium conductance ($K_D = 4 \times 10^{-4}$ M, $-\log K_D = 3.40$) in the frog node or molluscan neurone (Stanfield, 1983). TBA was about 10 times more potent than TEA in shifting the concentration-effect curves of pinacidil to the right. A similar difference in potency between TEA and TBA has been obtained for blockade of potassium channels in neurones of various species (French & Shoukimas, 1981; Stanfield, 1983). Thus, the binding of quaternary ammonium compounds to their binding sites at potassium channels could preclude potassium channel openers from binding to the same sites or closely related sites.

The analysis of non-competitive antagonism by TEA yielded dissociation constants (K_A) of 8.9–9.9×10^{-5} M ($-\log K_A = 4.0$–4.1) for pinacidil (Figure 3, Table 3) which were about 20 times greater than the EC_{50} of the negative inotropic effect of pinacidil ($4.3 \pm 0.1 \times 10^{-6}$ M, $pD_2 = 5.37 \pm 0.09$; Table 2). There were also similar differences between K_A values and EC_{50} values for cromakalim and nicorandil (Table 3). These results suggest that there is nonlinear stimulus-effect relationships between the binding of the three potassium channel openers to their binding sites at potassium channels and their negative inotropic effects. Thus, the possibility that

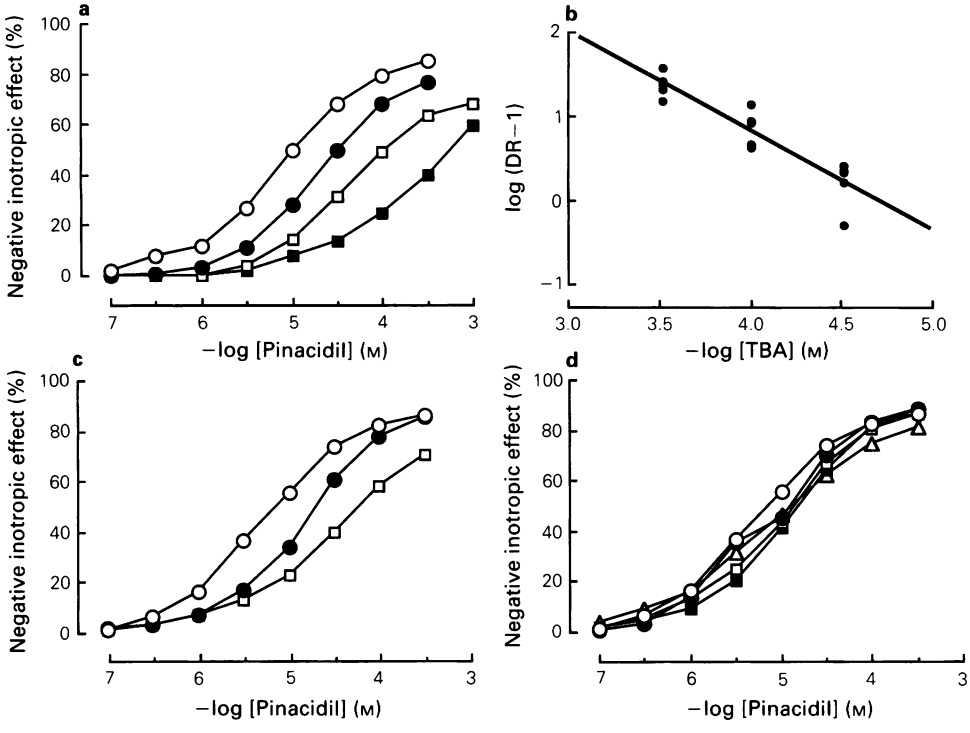

Figure 4 Differential modification by (a) tetrabutylammonium (TBA), (c) $BaCl_2$ and (d) 3,4-diaminopyridine (DAP) of the concentration-negative inotropic effect curves for pinacidil in canine atrial muscle. Each point represents mean value of 4–8 muscles. (a) (○) Control. (●) 3×10^{-5} M, (□) 10^{-4} M, (■) 3×10^{-4} M TBA. (b) Schild plot of the antagonism of the negative inotropic effects of pinacidil by TBA in canine atrial muscles. pA_2 value, 4.70; the slope of regression line, −1.17; the correlation coefficient, −0.93 ($n = 17$). (c) (○) Control. (●) 3×10^{-4} M, (□) 10^{-3} M $BaCl_2$. (d) (○) Control. (●) 10^{-5} M, (□) 10^{-4} M, (■) 10^{-3} M, (△) 3×10^{-3} M DAP.

the three potassium channel openers bind to only a fraction of these channels to produce their negative inotropic effects seems to be expressed by the preclusion of potassium channel openers from binding to the same channels previously bound by TEA.

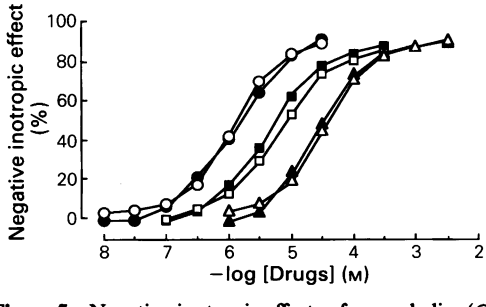

Figure 5 Negative inotropic effects of cromakalim (○, ●), pinacidil (□, ■) and nicorandil (△, ▲) in canine atrial muscle in the absence (○, □, △) and presence (●, ■, ▲) of 10^{-2} M CsCl. Each point represents mean value of 5–6 muscles.

Specific antagonism of the negative inotropic effects of the three potassium channel openers by quaternary ammonium compounds, demonstrated in the present study, is consistent with the finding that in guinea-pig trachealis muscle the relaxant and hyperpolarizing effects of cromakalim are suppressed by TEA (Allen *et al.*, 1986). The failure of DAP, a congener of 4-aminopyridine (4-AP, Harvey & Marshall, 1977), to block the negative inotropic effect of pinacidil in the present study, however, is inconsistent with the finding that in trachealis muscle the effects of cromakalim were suppressed by 4-AP. In cardiac muscles, 4-AP has been shown to block the transient outward current which is activated partly by an increase in intracellular Ca (Kenyon & Gibbons, 1979; Simurda *et al.*, 1988). The failure of DAP to antagonize the negative inotropic effects of pinacidil suggests that the potassium channels opened by pinacidil are different from channels for 4-AP- and DAP-sensitive transient outward current.

Although quaternary ammonium compounds are known to block potassium currents in cardiac muscle (Coraboeuf & Vassort, 1968), the kind of potassium channels they block has not been elucidated; because they are usually effective when applied internally (Stanfield, 1983). In the guinea-pig superior mesenteric artery both $BaCl_2$ and TEA are known to reduce resting potassium conductance (Harder & Sperelakis, 1978). As is the case with quaternary ammonium compounds, the kind of potassium channels opened by the potassium channel openers in cardiac sarcolemma has not been elucidated; opening of the inward rectifying potassium channels (I_{K1} channels) (Taira, 1987) and opening of a new class of potassium channels (Kakei *et al.*, 1986) are currently suggested. In intestinal smooth muscle of the guinea-pig, nicorandil has been shown to activate apamin-insensitive and Ca-insensitive potassium channels (Yamanaka *et al.*, 1985). In the guinea-pig taenia caeci, too, cromakalim and nicorandil are capable of opening apamin-insensitive potassium channels (Weir & Weston, 1986a). Thus, the potassium channels in canine atrial muscles opened by cromakalim, pinacidil and nicorandil seem to be akin to apamin-insensitive and Ca-insensitive channels in smooth muscles and to be opened at the resting membrane potential by these drugs (Kakei *et al.*, 1986; Iijima & Taira, 1987). The binding of these potassium channel openers to their binding sites at these potassium channels seems to be modified either directly or indirectly by quaternary ammonium compounds, like TEA and TBA.

In the present study CsCl was entirely ineffective in antagonizing the negative inotropic effects of the three potassium channel openers. This is understandable since potassium channel openers are effective when potassium ions are present, even if caesium ions as an impermeable potassium ion substitute (Hagiwara *et al.*, 1976) are included in the medium.

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Central nervous system kinin receptors and the hypertensive response mediated by bradykinin

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1 Bradykinin (Bk) administered intracerebroventricularly to the rat causes an increase in arterial pressure.

2 Analogues of Bk with agonist and antagonist activity were injected, over a wide dose-range, into the posterior region of the fourth ventricle of unanaesthetized rats implanted with permanent ventricular canullae, and blood pressure was measured directly from the abdominal aorta.

3 The analogues Ile-Ser-Bk (T-kinin) and Lys-Lys-Bk, which interact with both B_1 and B_2 Bk receptors, produced pressor effects similar to those of Bk, although of greater duration, whereas des-Arg^9-Bk, a B_1-receptor agonist, had no effect.

4 The B_1-antagonist des-Arg^9-[Leu^8]-Bk did not alter the Bk pressor response, but D-Arg-[Hyp^3, Thi^{5,8},D-Phe^7]-Bk, which interacts both with B_1- and B_2-receptors blocked the responses to Bk, T-kinin and Lys-Lys-Bk and caused parallel shifts to the right of the Bk dose-response curves. Neither antagonist, by itself, had any effect on blood pressure.

5 It is concluded that the central pressor response to Bk is mediated by receptors of the B_2 subtype.

Introduction

Bradykinin (Bk), when injected into the systemic circulation, usually causes vasorelaxation and consequently, a temporary decrease in blood pressure (Rocha e Silva *et al.*, 1949). However, the same peptide administered to the cerebral ventricles (Graeff *et al.*, 1969; Pearson *et al.*, 1969) or into the cerebral circulation (Lang & Pearson, 1968), causes a marked and sustained increase in the mean arterial pressure. This hypertensive response to Bk has been documented in rabbits (Graeff *et al.*, 1969), rats (Pearson *et al.*, 1969), cats (Lang & Pearson, 1968) and dogs (Pearson & Lang, 1969). In rats, the pressor response to intraventricularly injected Bk has been ascribed to the stimulation of Bk receptors in the lateral septal area (Correa & Graeff, 1975), or alternatively, in areas adjacent to the ventral portion of the third ventricle (Lewis & Phillips, 1984). However, there are data which suggest that the pressor response to the peptide results from the stimulation of sites on the dorsal surface of the medulla (Lindsey *et al.*, 1988 and unpublished observations), probably in the area of the fourth ventricle. In fact, Bk injected into the posterior region of the fourth ventricle is about twenty times as potent, has a considerably shorter latency and a larger maximal effect than when injected into the lateral ventricle (Lindsey *et al.*, 1988).

Only recently have specific antagonists been developed for the Bk receptors. With the advent of antagonists, however, two subpopulations of Bk receptors have been described. The B_1-receptor which is present in the rabbit aorta (Regoli & Barabé, 1980) and the rat renal artery (Guimarães *et al.*, 1986), is stimulated selectively by des-Arg^9-Bk (DABk) and is blocked by des-Arg^9-[Leu^8]-Bk (DALBk). The B_2-receptors, present in arteries and other tissues are stimulated by Bk and many analogues and are blocked by a series of Bk derivatives with a D-phenylalanine substitution in position 7 (Vavrek & Stewart, 1985), such as D-Arg-[Hyp^3, Thi^{5,8},D-Phe^7]-Bk (DAHTDBk). In contrast to the B_1-agonists and antagonists, the B_2-agonists or antagonists are not selective and in general also interact with the B_1-receptors (Regoli *et al.*, 1986).

Other kinins of physiological or practical interest have also been developed. For example, Lys-Lys-Bk (LLBk), a kinin agonist which is exceptionally resistant to degradation by kininase activity (Roblero *et*

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Table 1 Kinin receptor agonists and antagonists employed in the present study

Peptides	*Agonist activity*	*Antagonist activity*	*Amino acid sequence*
Bradykinin (Bk)	B_1 B_2		Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Lys-Lys-Bk (LLBk)	B_1? B_2		Lys-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
des-Arg^9-Bk (DABk)	B_1		Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe
T-kinin (TK)	B_1? B_2		Ile-Ser-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
des-Arg^9-[Leu^8]-Bk (DALBk)		B_1	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu
D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-Bk (DAHTDBk)		B_1 B_2	D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Phe-Thi-Arg

al., 1973; Lindsey *et al.*, 1987), is a potential tool to detect *in vivo* kininase activity in experimental situations where the use of kininase inhibitors is precluded. Another agonist of interest, Ile-Ser-Bk or T-kinin (TK) was first obtained by the incubation of rat plasma with trypsin (Okamoto & Greenbaum, 1983a), the classical method for obtaining Bk from human plasma. T-kinin is thus a candidate for the role of endogenous kinin in the rat.

Central Bk receptors have still to be classified. The objective of this study was to characterize the receptors which mediate the central pressor response to Bk, by using analogues which interact with different subpopulations of receptors.

Methods

The peptides used in this study (Table 1) were synthetic products made in this laboratory, with the exception of D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-Bk which was kindly supplied by Dr J.M. Stewart from the University of Colorado (Denver, CO, U.S.A.).

Four month old female normotensive Wistar rats (NR), weighing approximately 200 g, with a mean arterial pressure of 111 ± 3 mmHg, were used. The animals were anaesthetized with a mixture of pentobarbitone and chloral hydrate and permanent cannulae were placed in the posterior portion of the fourth ventricle (−11.4 mm antero-posterior, 2.4 mm vertical and 0.0 mm lateral in stereotaxic coordinates, Paxinos & Watson, 1986). The cannulae were anchored to the skull by jeweller's screws embedded in dental acrylic cement. Following the intracerebroventricular surgery a polyethelene catheter (PE10 connected to PE50), filled with heparinized saline, was placed in the abdominal aorta, through the left femoral artery. The other end of the cannula was slipped beneath the skin and exteriorized on the back of the animal. After surgery, the rats were individually housed in plastic cages (30 × 20 × 10 cm) which also served as recording chambers. Two days after implantation surgery the effect of centrally administered Bk and analogues on the mean arterial blood pressure was recorded, in the unanaesthetized and unrestrained animals, with a Narco P-1000B pressure transducer and a DMP-4B physiograph (Narco Biosystems, Houston, TX, U.S.A.). Dose-response curves for Bk, TK and LLBk were obtained by injecting 1 μl saline containing various concentrations of the peptides at 30 min intervals, and no more than three doses were administered to each animal. Proper cannula placement was verified histologically in all subjects by an injection of dye at the end of the experiment. The rat brains were excised and placed in 10% formaldehyde for two weeks before histology. The lack of diffusion of the dye into the expected ventricular space was the criterion for excluding an animal.

Regression lines obtained for the linear parts of the dose-response curves were compared for critical differences following covariance analyses and tests for regression, linearity and parallelism (Snedecor & Cochran, 1967). For differences between independent means, Student's *t* test was used.

Table 2 Hypertensive effect of kinins injected into the posterior region of the fourth ventricle

	ED_{50} (pmol)	*Latency* (s)	*Duration* (s)	*Maximal effect* (mmHg)
Bk	32	16 ± 10	91 ± 36	31 ± 15
TK	51	24 ± 12	155 ± 40*	30 ± 11
LLBk	6*	18 ± 11	175 ± 26*	31 ± 7
DABk	(>11,000)	—	—	No effect

Values are the mean ± s.d. of at least 10 animals.
* Significantly different from Bk ($P < 0.05$). For key to abbreviations used see Table 1.

Results

Effect of kinin agonists on arterial pressure

The kinin agonists, Bk, DABk, TK and LLBk were injected into the posterior region of the fourth ventricle of normotensive Wistar rats. Bradykinin, TK or LLBk, in the pmol range, produced increases in the mean arterial pressure which in some animals reached 60 mmHg above baseline. The response to Bk occurred with a mean latency of 16 s and lasted an average of 90 s. T-kinin and LLBk exhibited similar latencies but the effects of both peptides were of longer duration (Table 2). No effect was observed with DABk, an agonist of the smooth muscle B_1-receptor, although it was injected in doses from 8 to 11,000 pmol.

The dose-response curves for the three peptides which presented pressor activity are shown in Figure 1. The kinins had the same maximal effect with an average value of 30 mmHg. From Figure 1, it can be seen that LLBk (ED_{50} = 6 pmol) is approximately 5 times more potent than Bk. The regression lines corresponding to the linear portion of the dose-response curve to Bk and TK did not differ significantly from each other.

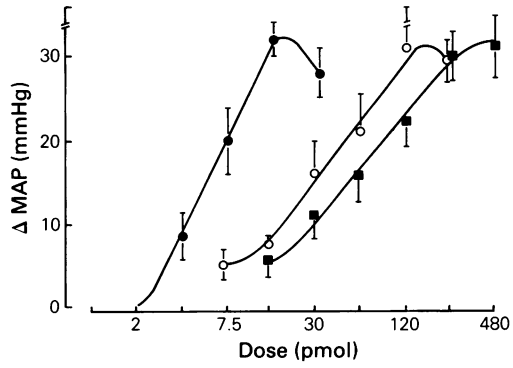

Figure 1 Log dose-response curves for the systemic pressor effects, measured as changes in the mean arterial pressure (ΔMAP), of bradykinin (○),Lys-Lys-bradykinin (●) and T-kinin (■) injected into the fourth ventricle of normotensive Wistar rats. The injection volume was 1 μl in all cases. The regression lines corresponding to the linear portion of the curves were homogeneous regarding parallelism. The curve for Lys-Lys-bradykinin differed significantly from the others ($P < 0.05$, analyses of covariance). Each point represents the mean obtained from 8 to 10 rats. Vertical lines show s.e.mean.

Effect of antagonists on the central kinin pressor response

For the antagonism studies, the analogues were mixed with Bk in the appropriate concentrations so that 1 μl contained the desired amount of agonist and antagonist.

Preliminary experiments showed that the inhibitor DAHTDBk rapidly blocks the Bk receptors. The intracerebroventricular injection of the antagonists five or ten min before administration of Bk did not enhance inhibition. Thirty min after DAHTDBk no inhibition of the pressor effect could be observed.

The Bk antagonists DALBk (400–11,500 pmol) and DAHTDBk (200–1800 pmol), when injected into the fourth ventricle alone, did not cause alterations in the mean arterial pressure (n = 10).

The B_1-receptor antagonist DALBk (10–6000 pmol) did not alter the hypertensive response to Bk (Figure 2). However, DAHTDBk (600 pmol) blocked the responses to Bk, TK and LLBk (Figure 3). The pressor effect of Bk was inhibited in a dose-dependent manner by 200–1200 pmol of DAHTDBk, which caused parallel displacements of the Bk dose-response curves (Figure 4). With a higher dose of DAHTDBk (1800 pmol) it was impossible to obtain a full dose-response curve because of the appearance of a hypotensive response, in approximately 60% of the animals. In these animals the decrease in arterial pressure appeared alone or following a hypertensive

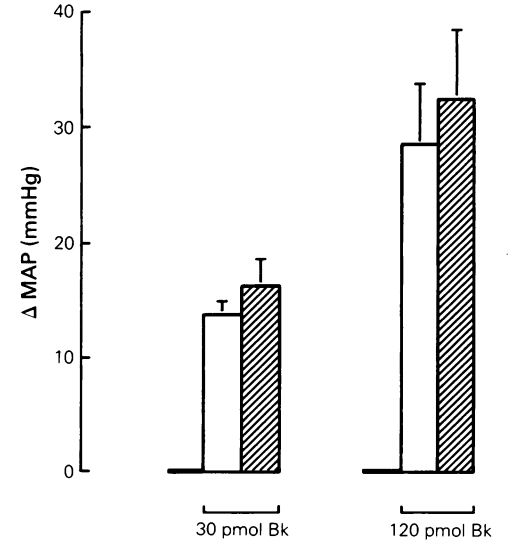

Figure 2 Effect of bradykinin (Bk 30 and 120 pmol) on the mean arterial pressure (ΔMAP) in the absence (open columns) and presence (hatched columns) of 6 nmol of the bradykinin B_1-antagonist, des-Arg^9-[Leu^8]-bradykinin (DALBk). The solid columns represent the effect of DALBk alone. Each column represents the mean obtained from 8 rats; vertical lines show s.e.mean.

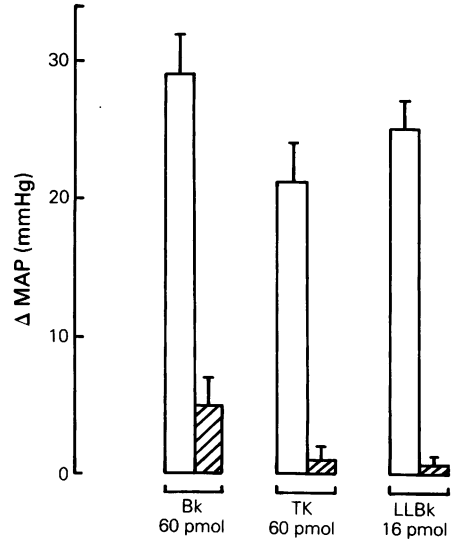

Figure 3 Inhibitory effect of 0.6 nmol D-Arg-[Hyp^3, Thi^{5,8},D-Phe^7]-Bk on the pressor effect of bradykinin (BK), T-kinin (TK) or Lys-Lys-Bk (LLBk). The responses to the agonists (open columns) were significantly ($P < 0.05$) reduced in the presence of the antagonist (hatched columns). Each column represents the mean obtained from 9 rats; vertical lines show s.e.mean.

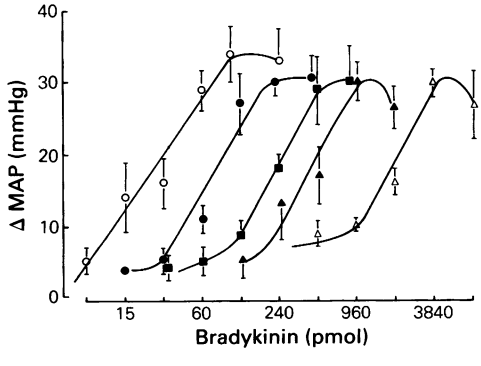

Figure 4 Log dose-response curves obtained for bradykinin (Bk) on the mean arterial pressure (ΔMAP) alone (○) and in the presence of different doses (●, 0.2 nmol; ■, 0.6 nmol; ▲, 0.8 nmol; △, 1.2 nmol) of D-Arg-[Hyp^3,Thi^{5,8}-D-Phe^7]-Bk. The regression lines were homogeneous as regards parallelism and differed significantly from each other ($P < 0.05$, analyses of covariance). Each point is the mean of determinations carried out in 15 animals. Vertical lines show s.e.mean.

response of small amplitude. The remaining animals presented blunted pressor responses to 7600 pmol of Bk. Neither the agonist alone nor the antagonist, at the highest doses employed, caused a hypotensive response.

Discussion

Bradykinin, TK or LLBk, when injected intracerebroventricularly into the posterior region of the fourth ventricle, consistently produced an increase in the mean arterial pressure. The response to TK was similar in pattern to that of Bk or LLBk. The query as to which kinin is the mediator of this effect is an open question since no kinin has been identified as a mediator of any physiological function in the central nervous system. Bradykinin, its higher homologues or novel kinins such as TK are possible candidates for this role in the rat. Since TK has been obtained from the incubation of rat plasma with trypsin (Okamoto & Greenbaum, 1983a), it has been postulated that the Bk analogue Ile-Ser-Bk or TK is one of the endogenous kinins in the rat. Apparently TK is a mediator of the inflammatory response in the rat (Barlas *et al.*, 1985; Sakamoto *et al.*, 1987).

The differences in the duration of the response to the three kinins, as well as the greater potency of LLBk, may be ascribed to pharmacokinetic properties of the Bk analogues. TK (Okamoto & Greenbaum, 1983b) and LLBk (Roblero *et al.*, 1973), respectively, are more resistant to kininase activity than Bk. TK has been shown to have a lower specific activity than Bk (Okamoto & Greenbaum, 1983b), thus the final biological activity of TK (or other peptides) results from an interplay of affinity, efficacy and resistance to degradation.

In contrast to the results obtained with Bk, TK and LLBk, no noteworthy cardiovascular effects were observed following intraventricular administration of the specific B_1-agonist DABk. This result is in agreement with a previous experiment, in which DABk was injected into the lateral ventricle of rats (Correa & Graeff, 1974), and indicates that the Bk receptors involved in the central pressor response are not of the B_1 subtype. This is also supported by the finding that the response to Bk was not antagonized by the B_1-antagonist DALBk. On the other hand, the responses to Bk, TK and LLBk were antagonized by the B_2-antagonist DAHTDBk, indicating that the receptors which mediate the central hypertensive response in normotensive rats are, in all probability, of the B_2 type. The parallel displacement to the right of the Bk dose-response curves in the presence of DAHTDBk indicates that this compound acts as a competitive antagonist in this system. Another antagonist [Thi^{5,8}-D-Phe^7]-Bk with

similar properties to those of DAHTDBk has been shown to inhibit the pressor action of Bk in spontaneously hypertensive rats (Lindsey *et al.*, 1988).

The hypotensive response that was observed following a high dose of Bk in conjunction with antagonist suggests the existence of a site where kinins exert a centrally mediated vasodilator response. This effect could occur in response to stimulation of systems with low sensitivity to Bk which are not blocked by the antagonist. Alternatively, the hypotensive effect could be exerted at a site which is relatively less accessible to the antagonist than to Bk. The increase in the net charge of DAHTDBk, in relation to that of Bk given by the extra D-Arg, could hinder diffusion of inhibitor in tissue.

Another aspect which was examined was whether the antagonists portray biological activity on the central nervous system receptors, as is the case in some other preparations (Llona *et al.*, 1987; Braas *et al.*, 1988). The B_1- and B_2-antagonists DALBk or DAHTDBk, administered over a wide dose range, were devoid of any effect on blood pressure.

The central nervous system receptors which mediate the pressor response to Bk are stimulated by analogues which interact with B_2 Bk receptors and are inhibited by B_2 competitive antagonists. No evidence was found that B_1-receptors are involved in this response, since B_1-agonists or antagonists did not have any effect on blood pressure or inhibitory action on the Bk pressor response.

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κ-Opioid-induced changes in renal water and electrolyte management and endocrine secretion

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1 Subcutaneous injection of the κ-opioid agonist U50,488 into conscious, saline-loaded rats was associated with a diuresis, antinatriuresis and antikaliuresis which lasted for up to 3 h. Plasma renin activity and corticosterone levels were elevated but plasma vasopressin (AVP) and aldosterone levels were unaltered in similarly treated rats.

2 U50,488 administration to adrenal demedulated rats was not associated with a diuresis but produced an antinatriuresis, though sodium excretion rates were higher in demedullated than in sham-operated animals. Plasma AVP and corticosterone levels were not affected by demedulation or subsequent U50,488 treatment. Sham-operated, U50,488-treated rats showed the expected increase in plasma corticosterone levels.

3 U50,488 administration resulted in an antidiuresis and an antinatriuresis in AVP-deficient Brattleboro DI rats.

4 When coupled with fasting stress U50,488 administration resulted in similar but attenuated renal responses compared with those observed in unfasted rats. Basal plasma corticosterone levels were elevated in fasted animals and were further increased by U50,488.

5 Both water and electrolyte handling by the kidney are altered by U50,488. The diuretic effects of U50,488 were reversed by adrenal demedullation and in the absence of endogenous AVP, but the antinatriuretic actions were not altered, suggesting that the effects upon renal water and electrolyte excretion may be mediated by separate mechanisms.

Introduction

κ-Opioid agonists have been shown to produce a profound diuresis in the rat (Slizgi & Ludens, 1982; Leander, 1983a,b; VonVoigtlander *et al.*, 1983) which was initially attributed to inhibition of vasopressin (AVP) secretion (Miller, 1975). More recently, however, the κ-agonist bremazocine has been shown to increase circulating plasma AVP levels yet still cause a diuresis (Huidobro-Toro & Parada, 1985), suggesting that other factors acting in the periphery may be involved. Removal of the adrenal medulla has been shown to attenuate the diuretic actions of the κ-agonist U50,488 (Blackburn *et al.*, 1986), although the reasons for this effect were not clear. Adrenal medullary AVP (Ang & Jenkins, 1984) did not appear to exert a direct influence on renal water handling, since untreated demedullated rats maintained stable water and salt balances (Blackburn *et al.*, 1986), suggesting that some other medullary factor may interact with κ-opioids.

Although the diuretic response to κ-agonists has been well documented, potential effects on renal electrolyte handling have not been fully investigated. Previously described protocols involving long (5 h), single sample urine collections may have masked short term responses. Accordingly, we have used a more frequent sampling protocol in order to investigate the time course of effects of U50,488 on renal water and electrolyte handling. In addition, the influence of the adrenal medulla in κ-opioid-induced diuresis and the role of AVP and other hormones have been further examined. A preliminary account of some of these data was presented to the Physiological Society (Ashton *et al.*, 1987).

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Methods

Animals

Adult male Alderley Park Wistar (APW, 180–210 g) and Brattleboro rats homozygous for diabetes insipidus (DI, 220–320 g) were obtained from colonies maintained at ICI Pharmaceuticals and the University of Manchester, respectively. A 12 h light:12 h dark photoperiod was maintained and animals had free access to food (Labsure PMD diet) and water. APW rats were routinely fasted overnight before experimentation.

Time course responses to U50,488

Pairs of APW rats ($n = 16$ pairs) were placed in metabolism cages (Jencons, Hertfordshire), 1 h before stomach loading with saline (0.154 M NaCl at 4 ml $100\,g^{-1}$, orally). Immediately following the stomach load, half of the animals were treated with U50,488, and the remaining animals received vehicle alone. Urine was collected at hourly intervals for 6 h. Sodium and potassium concentrations were measured by flame photometry (Model IL-943, International Laboratory apparatus, Warrington) and urine osmolality by freezing point depression (Camlab, Roebling Osmometer, Cambridge).

A second group of APW rats ($n = 47$) were similarly prepared and were killed by decapitation at 0.5 ($n = 15$), 1 ($n = 16$) or 2 h ($n = 16$) after administration of the agonist or vehicle. The separated plasma from collected trunk blood was stored at −20°C before radioimmunoassay for arginine vasopressin and corticosterone concentrations. Plasma samples obtained from animals killed 2 h after injection were also subjected to radioimmunoassay for plasma renin activity and aldosterone.

Adrenal demedullation

Bilateral adrenal demedullation and sham operations were performed on groups of APW rats ($n = 16$ in each group) under Saffan ($12\,mg\,kg^{-1}$ i.v. Alphaxalone/Alphadolone acetate, Glaxovet) anaesthesia, according to the method described by Borkowski & Quinn (1983). All animals were used not less than seven days after surgery. Demedullated ($n = 8$ pairs) and sham-operated animals ($n = 8$ pairs) were housed in pairs in metabolism cages and received a saline stomach load, as described above. Animals received injections of either U50,488 or vehicle alone and urine was collected for 0.5, 1 or 2 h. Analyses of sodium and potassium content and osmolality were as described above.

Further groups of demedullated ($n = 46$) and sham-operated ($n = 40$) animals were prepared in a similar manner and were killed by decapitation at either 0.5, 1 or 2 h after injection of U50,488 or vehicle. The separated plasma was stored at −20°C prior to radioimmunoassay for arginine vasopressin and corticosterone concentrations.

Absence of arginine vasopressin

Groups of DI rats were housed individually in metabolism cages before saline loading (0.154 M NaCl, 2.5 ml $100\,g^{-1}$ orally) and s.c. injection of U50,488 ($n = 7$) or vehicle ($n = 8$). Urine was collected over the following 2 h. Sodium and potassium concentrations were determined by flame photometry (Corning 455 flame photometer) and osmolality by freezing point depression (Roebling Automatik Osmometer, L.H. Roebling, Berlin, W. Germany).

Effects of fasting

Groups of APW rats were either fasted overnight ($n = 4$ pairs) as usual or allowed free access to food ($n = 4$ pairs) before the normal stomach loading procedure and administration of U50,488 or vehicle, as described above. Urine was collected over a 2 h period and sodium and potassium concentrations and osmolality were determined. Animals were killed by decapitation 2 h after injection and the separated plasma was stored at −20°C prior to radioimmunoassay for corticosterone concentration.

Radioimmunoassays

Plasma arginine vasopressin was measured by specific radioimmunoassay following extraction with acetone and petroleum ether using the method described by Ashton & Balment (1988). Inter- and intra-assay coefficients of variation were 14.6% ($n = 12$) and 17.1% ($n = 10$) respectively. The minimum detectable plasma vasopressin concentration was $0.1\,\mu u\,ml^{-1}$ ($1\,\mu u = 2.2$ pg).

Plasma corticosterone was measured in ethanol extracted samples according to the method of Kime (1977). Inter- and intra-assay coefficients of variation were 10.1% ($n = 30$) and 13.6% ($n = 10$) respectively. The minimum detectable plasma corticosterone concentration was $400\,pg\,100\,ml^{-1}$.

Plasma aldosterone was measured following initial separation from other steroids by LH20 chromatography by the method described by Milne et al. (1982). Inter- and intra-assay coefficients of variation were 12.7% ($n = 42$) and 14.4% ($n = 10$) respectively. The minimum detectable plasma aldosterone concentration was $2\,pg\,100\,ml^{-1}$.

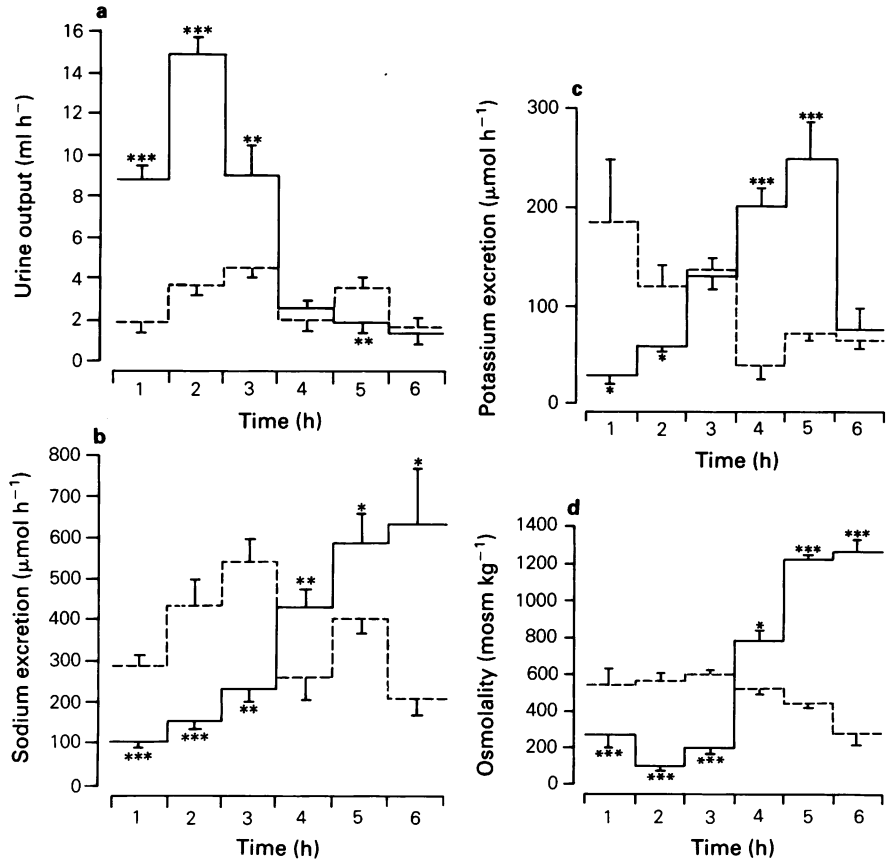

Figure 1 Effect of U50,488 (10 mg kg^{-1} s.c., $n = 8$ pairs, solid line) and vehicle administration (0.154 M NaCl, $n = 8$ pairs, dashed line) on urine output (a), sodium (b) and potassium excretion (c) and urine osmolality (d) in conscious saline loaded (4 ml 100 g^{-1}, orally) APW rats. Statistical comparisons between vehicle- and U50,488-treated groups are indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Plasma renin activity, expressed as the rate of angiotensin I generation, was measured by the method of Oldham *et al.* (1984).

Drugs

The κ-opioid agonist U50,488 (*trans*-3,-4-dichloro-N-methyl-N[2-(1-pyrrolidinyl)cyclohexyl]benzene-acetamine methane sulphate, synthesized at ICI Pharmaceuticals, Macclesfield) was administered, by subcutaneous injection, at a dose rate of 10 mg kg^{-1}. Control animals received vehicle only injections (0.154 M NaCl) at a rate of 1 ml kg^{-1}.

Statistical analysis

All values are presented as the mean ± s.e.mean. Statistical comparisons were by Student's *t* test for unpaired data.

Results

Time course responses to U50,488

κ-Agonist administration, at the submaximal dose of 10 mg kg^{-1} (Blackburn *et al.*, 1986), was associated with a diuresis which reached a peak at 2 h post injection (Figure 1). Urine output had fallen to levels comparable with those of the control, vehicle-injected animals by the fourth hour and gradually declined thereafter. Cumulative urine output over the 6 h study period was significantly higher in agonist-treated animals than in vehicle-injected rats (vehicle, 17.1 ± 2.6 vs U50,488, 38.3 ± 3.9 ml 6 h^{-1}, $P < 0.001$). Sodium and potassium excretion rates (Figure 1) were lower in U50,488- than in vehicle-treated rats over the first 3 and 2 h post injection respectively. This retention of sodium and potassium

Table 1 Plasma vasopressin (AVP) and corticosterone concentrations in intact, sham-operated and adrenal-demedulated conscious, saline-loaded (4 ml 100 g^{-1}, orally) APW rats killed at 0.5, 1 or 2 h after administration of U50,488 (10 mg kg^{-1} s.c., $n = 64$) or vehicle (0.154 M NaCl, $n = 69$)

Plasma AVP ($\mu u\, ml^{-1}$)

	Intact		*Sham*		*Demedullated*	
Time (h)	Vehicle	U50,488	Vehicle	U50,488	Vehicle	U50,488
0.5	1.9 ± 0.5	2.7 ± 0.5	3.4 ± 0.5	2.9 ± 0.4	3.3 ± 0.4	3.9 ± 0.9
1	1.3 ± 0.2	1.8 ± 0.5	1.6 ± 0.4	1.7 ± 0.7	2.7 ± 0.8	2.2 ± 0.7
2	3.2 ± 0.5	2.8 ± 0.3	2.5 ± 0.6	2.7 ± 0.3	3.0 ± 0.6	3.9 ± 0.6

Plasma corticosterone ($\mu g\, 100\, ml^{-1}$)

	Intact			*Sham*			*Demedullated*	
Time (h)	Vehicle		U50,488	Vehicle		U50,488	Vehicle	U50,488
0.5	9.9 ± 1.5	*	13.0 ± 0.4	9.7 ± 1.1	***	14.7 ± 0.8	10.8 ± 0.8	10.6 ± 0.8
1	6.2 ± 1.1	***	13.4 ± 0.7	5.7 ± 1.0	**	10.7 ± 0.7	5.4 ± 0.6	7.1 ± 1.0
2	10.5 ± 1.1	*	14.3 ± 0.9	8.4 ± 1.3	***	16.7 ± 0.8	8.6 ± 0.9	10.1 ± 0.7

Statistical comparisons between vehicle and U50,488-treated animals in each of the three groups are indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

was associated with a subsequent rebound natriuresis and kaliuresis over the final 2–3 h of the experimental period, such that cumulative excretion rates over 6 h did not differ between U50,488- and vehicle-injected animals (Na^+ vehicle, 2130.0 ± 270.0 vs. U50,488, 2124.0 ± 306.0 μmol $6\,h^{-1}$ NS; K^+ vehicle, 624.0 ± 114.0 vs. U50,488, 756.0 ± 102.0 μmol $6\,h^{-1}$, NS). In accordance with the retention of electrolytes and the diuresis shown by U50,488-treated rats, urine osmolality (Figure 1) was lower than that seen in control animals over the initial 3 h, and subsequently increased during the last 3 h of observation.

Over the first 2 h following κ-agonist injection, when the diuretic effects were maximal, plasma AVP levels (Table 1) did not differ from those measured in control animals. However, U50,488-treated rats had elevated plasma corticosterone (Table 1) but not aldosterone concentrations (2 h post-injection control, 156.6 ± 23.7 vs. U50,488, 153.6 ± 23.3 ng $100\,ml^{-1}$, NS). Plasma renin activity was also elevated 2 h after U50,488 injection compared with vehicle injected controls (control, 2.86 ± 0.59 vs. U50,488, 4.96 ± 0.40 ng angiotensin $I\,ml^{-1}\,h^{-1}$, $P < 0.05$).

Adrenal demedullation

Adrenal demedullation attenuated the renal effects of U50,488. κ-Agonist administration in demedullated animals was associated with a much lower rate of urine production than in agonist-treated, sham-operated rats (Figure 2) or vehicle injected, demedullated rats (2 h, $P < 0.001$). Cumulative sodium excretion was elevated in U50,488-treated, demedullated animals compared with the similarly treated sham-operated group (Figure 2), though this remained lower than that shown by vehicle-injected, demedullated animals (2 h, $P < 0.05$). Prior adrenal demedullation did not influence the antikaliuretic action of U50,488 previously observed in intact animals. Cumulative potassium loss was reduced in U50,488-treated, demedullated animals compared with vehicle-injected, demedullated rats by 2 h (vehicle, 408.0 ± 24.0 vs. U50,488, 252.0 ± 24.0 μmol, $P < 0.001$). In accordance with these effects on water and electrolyte excretion, urine osmolality was higher following agonist treatment in demedullated animals than in the sham groups (2 h sham, 389.0 ± 111.0 vs. demedullated, 783.0 ± 36.0 mosm kg^{-1}, $P < 0.01$). However, there were no significant differences between U50,488 and vehicle-injected, demedullated animals.

Plasma AVP levels were not altered by adrenal demedullation nor U50,488 injection (Table 1). Plasma corticosterone, however, was significantly increased ($P < 0.01$) in U50,488-treated, sham-operated animals by comparison with the level in the other three treatment groups at each time point (Table 1). This elevation in plasma corticosterone was in accordance with the increase in corticosterone noted in intact rats in response to U50,488 (Table 1) and was notably absent following U50,488 administration in demedullated animals.

Absence of arginine vasopressin

The effects of U50,488 on renal water handling were altered in rats congenitally lacking AVP. In contrast to the diuretic response shown by AVP-replete animals (Figure 1) over the first 2 h following agonist administration there was a reduction in urine output

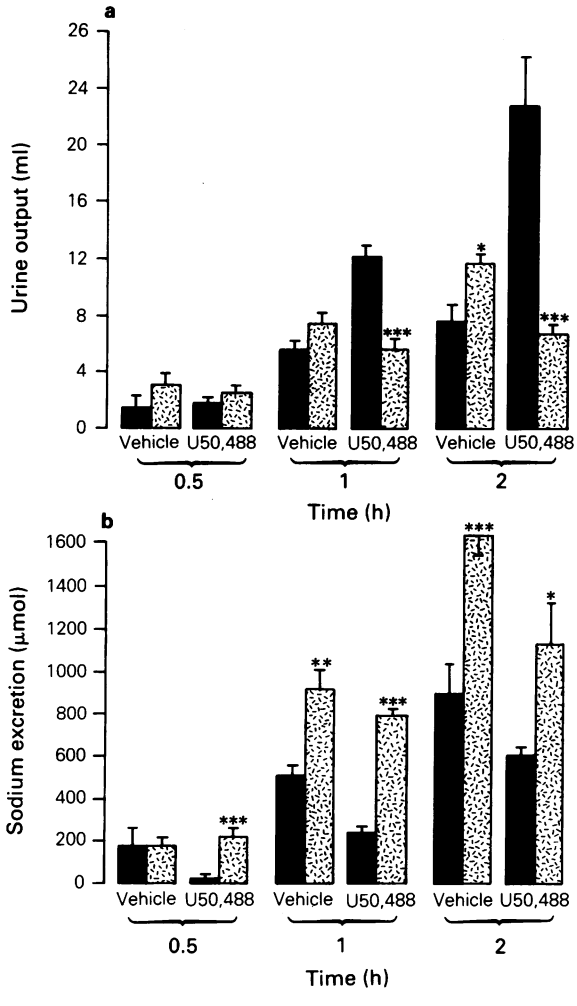

Figure 2 Effect of adrenal demedullation (stippled bars) and sham-operation (solid bars) on changes in urine output (a) and sodium excretion (b) in response to U50,488 (10 mg kg^{-1} s.c., $n = 8$ pairs) and vehicle administration (0.154 M NaCl, $n = 8$ pairs) in conscious, saline-loaded (4 ml 100 g^{-1}, orally) APW rats. Statistical comparisons between sham and demedullated animals receiving either U50,488 or vehicle are indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

in DI rats compared with vehicle-injected animals (Table 2). However, the retention of sodium and potassium observed in AVP-replete animals (Figure 1) upon U50,488 injection was also observed in AVP-deficient DI rats (Table 2). This retention of electrolytes in DI rats followed a similar time course to that observed in APW rats (Figure 1).

Table 2 Effect of U50,488 (10 mg kg^{-1} s.c., $n = 7$) and vehicle administration (0.154 M NaCl, $n = 8$) on the initial 2 h urine output and sodium and potassium excretion in conscious, saline-loaded (2.5 ml 100 g^{-1}, orally) vasopressin-deficient Brattleboro DI rats

	Vehicle		*U50,488*
Urine output (ml)	15.1 ± 2.1	**	6.5 ± 1.2
Sodium excretion (μmol)	323.0 ± 50.9	***	61.4 ± 24.1
Potassium excretion (μmol)	101.0 ± 14.8	*	57.3 ± 10.1

Statistical comparisons between vehicle and U50,488 treated animals are indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Effects of fasting

Overnight fasting before U50,488 administration was associated with similar, but attenuated, renal responses to the agonist compared with those shown by unfasted animals. Urine output was lower in fasted animals receiving either the vehicle or U50,488 compared with similarly treated, unfasted rats (Figure 3). Sodium excretion was also lower in vehicle-injected, fasted rats than in unfasted rats (Figure 3). Potassium excretion was reduced in fasted rats compared with unfasted rats in both vehicle (105.0 ± 19.9 vs. 547.8 ± 147.7 μmol, $P < 0.05$) and U50,488 (54.0 ± 6.9 vs. 261.0 ± 53.2 μmol, $P < 0.01$) treated groups. In contrast, plasma corticosterone concentrations were greater in both groups of fasted animals than in unfasted rats (Figure 3). A further increase in corticosterone associated with U50,488 injection (Table 1) was however, still observed in the fasted animals (Figure 3).

Discussion

It has been proposed that the diuretic effect of κ-agonists is due entirely to a suppression of vasopressin release, since this effect is absent in Brattleboro rats which are homozygous for diabetes insipidus (Leander, 1983b), and is abolished by administration of the synthetic vasopressin analogue, desmopressin (Leander *et al.*, 1985). However, in the present studies no changes were apparent in vasopressin levels in normal, sham-operated or demedullated saline-loaded rats following a challenge with a submaximal standard diuretic dose of the κ-agonist, U50,488. Thus, contrary to previous studies, suppression of neurohypophysial vasopressin secretion

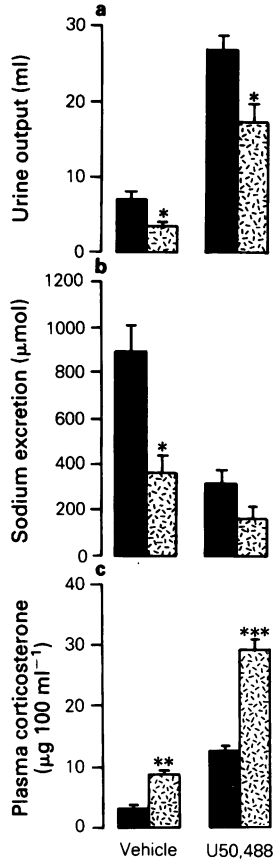

Figure 3 Effect of U50,488 ($10\,mg\,kg^{-1}$ s.c., $n = 4$ pairs) and vehicle administration (0.154 M NaCl, $n = 4$ pairs) on the initial 2 h urine output (a) and sodium excretion (b) and on plasma corticosterone concentration (c) in conscious, saline loaded ($4\,ml\,100\,g^{-1}$, orally) fasted (stippled bars) and unfasted (solid bars) APW rats. Statistical comparisons between fasted and unfasted animals receiving either U50,488 or vehicle are indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

does not appear to be primarily involved in κ-agonist-induced diuresis in the rat and that additional mechanisms may contribute to the diuretic effect.

Although AVP appears to be involved in the diuretic effects of U50,488, this clearly does not involve suppression of plasma levels. In the Brattleboro DI rat, which congenitally lacks AVP, U50,488 administration was associated with an antidiuresis, a response which is normally associated with μ-receptor agonists such as morphine (Huidobro-Toro, 1980), rather than κ-agonists. There is no evidence to suggest that U50,488 acts at μ-receptors, however there are several reports which suggest that κ-agonists may interact with renal AVP receptors. Gavend *et al.* (1978) showed that cyclazocine, which is structurally similar to the κ-agonist ethylketocyclazocine (EKC), attenuated the antidiuretic response to exogenous AVP in water-loaded rats and suggested that cyclazocine antagonized the renal effects of AVP. Slizgi & Ludens (1982) showed that although EKC reduced plasma AVP levels to a similar extent as water loading ($10\,mg\,kg^{-1}$), urine formation was disproportionately greater in EKC-treated rats than in the hydrated rats. These authors also suggested additional mechanisms were contributing to the diuretic effect possibly by attenuating AVP action in the kidney. To support this concept they showed that EKC inhibited AVP-stimulated water flow across toad bladder, which is a model of the late distal tubule and collecting duct (Slizgi & Ludens, 1982). More recently these workers and others have shown the presence of renal κ-opioid binding sites which indicates that κ-opioids may have some direct action at the kidney as well as a central site of action (Quirion *et al.*, 1983; Slizgi & Ludens, 1985). Certainly AVP appears to be required to facilitate the diuretic actions of U50,488 but further investigations are required to define the precise relationship between AVP and the κ-agonists.

This study thus provides further evidence for a peripheral site of action of κ-agonist-induced diuresis in the rat. We have previously reported that following adrenal demedullation in the rat, κ-opioid agonists were no longer capable of inducing diuresis (Blackburn *et al.*, 1986), though the renal diuretic response to furosemide and clonidine and the antidiuretic response to morphine and buprenorphine were not impaired.

Adrenal demedullation did not appear to compromise adrenocortical function, since comparable plasma corticosterone levels were evident in vehicle-injected sham and demedullated rats in this and the previous study (Blackburn *et al.*, 1986). The consistent increase in plasma corticosterone in response to U50,488 observed here and in previous studies (Lahti & Collins, 1982; VonVoitlander *et al.*, 1983; Eisenberg, 1985; Iyengar *et al.*, 1986), which can be blocked by naloxone (Iyengar *et al.*, 1985), has been considered part of the stress response evoked by κ-opiates (Iyengar *et al.*, 1986). Undoubtedly the stress of fasting in this study would appear to have exaggerated the steroid response to U50,488, while clearly the response was attenuated in demedullated animals. The great reduction in adrenal and plasma catecholamine concentrations (92–99%) in demedullated animals (Blackburn *et al.*, 1986) might be expected to influence the renal responses; however, it appears that κ-agonists do not induce diuresis via action on medullary catecholamine release, since

pretreatment with the β-adrenoceptor antagonist propranolol and the α_1-adrenoceptor antagonist prazosin had no effect on tifluadom-induced diuresis (Blackburn *et al.*, 1986). Although Birch & Hayes (1989) reported that the α_2-adrenoceptor antagonist idazoxan ($1\,mg\,kg^{-1}$) attenuated a κ-opioid induced diuresis, we did not observe such an effect at doses of $0.5\,mg\,kg^{-1}$ (Blackburn *et al.*, 1986) or $2\,mg\,kg^{-1}$ (unpublished observations). Whilst the adrenal medulla has been shown to contain AVP (Ang & Jenkins, 1984), it seems unlikely that this plays a major role in the κ-agonist-induced diuresis either, since demedullated rats maintained normal water and salt balance (Blackburn *et al.*, 1986). Indeed, plasma AVP levels in sham and demedullated groups were not different. Thus, the mechanisms involved in the attenuation of a κ-agonist induced diuresis by adrenal demedullation are not readily apparent, but the absence of the normally associated increase in plasma corticosterone might be important.

An important contribution of this study is the detailed description of the time course of U50,488's renal effects, which has revealed an action on both urinary water and electrolyte excretion. Previous studies have examined urine output data over a single 5 h collection period (e.g. Slizgi & Ludens, 1982; VonVoigtlander *et al.*, 1983; Huidobro-Toro & Parada, 1985) or expressed urine output as a cumulative value over 2 and 5 h (e.g. Leander, 1983a; Leander *et al.*, 1985). The data described in the present study have shown that the diuretic activity of s.c. administered U50,488 is limited to the first 3 h post-injection, and that this is associated with an antinatriuresis and antikaliuresis. We have previously demonstrated that both the diuretic and antinatriuretic actions of U50,488 are blocked by a high, but not a low, dose of naloxone in conscious (Blackburn *et al.*, 1986) and anaesthetized preparations (Balment *et al.*, 1986), which suggest that these effects are mediated through the κ-receptor. Slizgi *et al.* (1984) found that U50,488 caused a slight decrease in sodium excretion and had no effect on potassium in the anaesthetized dog, but the dose employed was half that used in this study ($5\,mg\,kg^{-1}$ cf. $10\,mg\,kg^{-1}$). Huidobro-Toro & Parada (1985) noted a similar reduction in electrolyte excretion using the same dose of U50,488 ($10\,mg\,kg^{-1}$) and proposed a role for endogenous κ-opioids in the regulation of both fluid and electrolytes. The observation that U50,488 administration was associated with raised renin activity, that dynorphin A-(1-10) amide stimulates secretion of atrial natriuretic peptide (ANP) (Xie *et al.*, 1988) and that other κ-agonists have been shown to stimulate corticosterone release (VonVoigtlander *et al.*, 1983, Fuller & Leander, 1984; Eisenberg, 1985; Iyengar *et al.*, 1986) suggests that changes in renal haemodynamics or glomerular filtration rate contribute to these observed changes in urinary sodium and water loss. The renal retention of sodium did not appear to be associated with major changes in plasma aldosterone levels.

Slizgi *et al.* (1984) suggested that the U50,488-induced diuresis may reflect a decrease in tubular reabsorption of water, but the associated increase in tubular fluid flow rate might be expected to result in a natriuresis rather than the observed retention of sodium, indicating perhaps that the effects of U50,488 on sodium excretion are mediated by another mechanism.

Whilst adrenal demedullation attenuated the diuretic effects of U50,488, it did not alter the antinatriuretic response. Sodium excretion rates were higher in demedullated groups, but the percentage fall in the excretion rate between vehicle and U50,488 groups was similar in sham (32%) and demedullated (30%) animals, suggesting that the factor(s) affected by demedullation may only influence the renal diuretic response to U50,488. Further support for the concept that the diuretic and antinatriuretic actions of U50,488 may be mediated separately comes from the observation that DI rats responded in a similar manner to AVP-replete APW rats by showing a fall in sodium excretion in response to U50,488, whilst undergoing an antidiuresis rather than a diuresis.

In summary, we have shown by using a protocol of hourly urine sampling that the κ-agonist U50,488 exerts a diuretic effect in the hydrated rat for 3–4 h post-injection. This diuresis did not appear to be mediated by inhibition of AVP secretion, but could be attenuated by prior adrenal demedullation. In addition U50,488 administration was associated with an antinatriuresis and antikaliuresis, which appeared to be mediated via different mechanisms from those involved in the diuretic response.

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Comparison of the survival of endothelium-derived relaxing factor and nitric oxide within the isolated perfused mesenteric arterial bed of the rat

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1 The survival of the endothelium-derived relaxing factor (EDRF) released by acetylcholine (ACh) in the rat isolated perfused mesenteric vascular bed was compared to that of exogenously applied nitric oxide (NO) by direct bioassay of the effluent from the mesenteric bed on rabbit aortic strips (RbAs).

2 NO (0.1–10 nmol) produced dose-related vasodilatations in the mesenteric vascular bed and also survived in the effluent in concentrations sufficient to provoke relaxations of the RbAs. ACh (1.7 pmol–300 nmol) caused dose-related vasodilatations in the mesenteric bed but no EDRF was detected in the effluent. This was true even when ACh provoked much larger vasodilatations in the mesenteric bed than those to doses of NO that survived passage through the bed.

3 Removal of the endothelial cells in the mesenteric vascular bed abolished vasodilatations in response to ACh but did not significantly affect those to NO. Survival of NO through the mesenteric bed was not increased.

4 Superoxide dismutase (SOD, 10 u ml^{-1}) did not significantly affect the vasodilator effects of either ACh or NO. It did increase the survival of NO through the mesenteric vascular bed but native EDRF was still not detected in the effluent.

5 The absence of bioassayable EDRF in the effluent following ACh-induced vasodilatations might be explained by the volume into which EDRF is released abluminally being much smaller than that into which it is released luminally.

Introduction

Endothelium-derived relaxing factor (EDRF, Furchgott & Zawadzki, 1980) is an unstable endogenous vasodilator, now identified as nitric oxide (NO, Palmer *et al.*, 1987; Kelm *et al.*, 1988). EDRF can be detected in the effluent coming from perfused main arteries and veins (Griffith *et al.*, 1984; Ignarro *et al.*, 1987) as well as that from cultures of endothelial cells held within artificial columns (Cocks *et al.*, 1985; Gryglewski *et al.*, 1986a; de Nucci *et al.*, 1988a). Although perfused vascular beds do demonstrate vasodilatations dependent on the release of EDRF (Furchgott *et al.*, 1987), EDRF has not been detected in the effluent coming from them. We have directly addressed this apparent contradiction by recording the endothelium-dependent responses within the rat isolated perfused mesenteric bed while simultaneously bioassaying for the presence of EDRF in the bed effluent. Furthermore, we have assessed the survival of EDRF within the mesenteric bed by the injection of exogenous NO, and compared the endothelium-dependent responses of the bed to its endothelium-independent responses to doses of NO that survived to the assay tissues.

Some of these results were presented to the British Pharmacological Society (de Nucci *et al.*, 1988b; Warner *et al.*, 1988).

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Methods

Rat isolated perfused mesenteric vascular bed

The isolated perfused mesenteric vascular bed was prepared according to the method of McGregor (1965) from male Wistar rats (200–300 g) pretreated with heparin (1000 u kg^{-1}, i.p.). The mesenteric bed was perfused at a constant rate of 5 ml min^{-1} with warmed (37.5°C) and gassed (95% O_2: 5% CO_2) Krebs solution which contained indomethacin (5 μM)

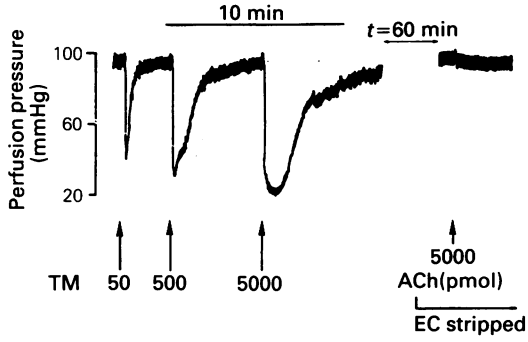

Figure 1 Effect of removal of the endothelial cells (EC) on the responses of the rat isolated perfused mesenteric vascular bed to acetylcholine (ACh). Tone was introduced into the mesenteric vasculature by an infusion of methoxamine (100 μM). ACh injected through the mesenteric bed (TM) produced dose-related vasodilatations. After the endothelial cells were removed by perfusing sodium deoxycholate (7 mM) for 30 s no vasodilatation was observed in response to ACh.

and sometimes superoxide dismutase (SOD, 10 u ml^{-1}) and/or albumin (0.5% w/v). Vasoconstriction was induced by an infusion of noradrenaline (NA, 0.5–500 μM), U46619 (11-dideoxy-9α-methano epoxyprostaglandin $F_{2\alpha}$, 0.03–1.5 μM), or methoxamine (30–100 μM) which produced a rise in the perfusion pressure from a basal pressure of 15–22 mmHg to an induced pressure of 50–130 mmHg.

Bioassay cascade

In some experiments the effluent from the mesenteric bed superfused in cascade (Vane, 1964) three spirally cut rabbit aortic strips (RbAs) with the endothelium removed which were also contracted by NA, U46619, or methoxamine. An infusion of hyoscine (0.3 μM) was added over the tissues throughout the experiments to abolish any direct effects of ACh on the assay tissues. Effluent from the mesenteric bed reached the consecutive RbAs after 1, 4, and 7 s. Drugs were injected either over the assay tissues (OT) as a control or through the mesenteric bed (TM). The assay tissues were calibrated by the relaxant effects of glyceryl trinitrate, and the auxotonic recordings of the three RbAs were adjusted electronically to be roughly equal.

Removal of the endothelial cells

The endothelium was removed from some mesenteric preparations by perfusing them with sodium deoxycholate (7 mM) for 30 s (Byfield *et al.*, 1986). The direct vasodilator responses of the mesenteric bed were reliably retained after stripping the endothelium when albumin (0.5% w/v) was present in the Krebs solution throughout the experiment. In experiments where the mesenteric effluent superfused a bioassay cascade, the mesenteric outflow was temporarily diverted during sodium deoxycholate perfusion to avoid damage to the RbAs.

Survival of nitric oxide in Krebs solution

To assess the half-life of NO in Krebs solution the mesenteric vascular bed was replaced with a delay coil (20–70 s) of silastic rubber tubing. Responses of the assay tissues to doses of NO injected through the delay coil were then bracketed by doses injected directly over the tissues to allow an estimate of the survival of NO after mixing in Krebs solution for different times.

Materials

The Krebs buffer had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.17, $CaCl_2 \cdot 6H_2O$ 2.5, $NaHCO_3$ 25 and glucose 5.6. Acetylcholine chloride (ACh), histamine dihydrochloride, noradrenaline bitartrate (NA), methoxamine hydrochloride, indomethacin, superoxide dismutase (SOD, from bovine erythrocytes), albumin (bovine fraction V), and methylene blue were purchased from Sigma Chemical Co. (Poole, U.K.). Glyceryl trinitrate (GTN) was obtained from Lipha. U46619 was a generous gift of J. Pike (Upjohn, Kalamazoo, MI). Helium was obtained from B.O.C. Medical Gases (Middlesex, U.K.), and nitric oxide (NO) from BDH (Dagenham, U.K.); NO solutions were prepared by injection of 0.1–1 ml of NO gas at atmospheric pressure into 40 ml of helium-deoxygenated water kept in a Wheaton flask (de Nucci *et al.*, 1988b).

Statistics

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

Results

Comparison of the survival of endothelium-derived relaxing factor and nitric oxide

When injected intra-arterially ACh (1.7 pmol–300 nmol), NO (0.1–30 nmol) or GTN (40–400 pmol) produced dose-related vasodilatations of the mesenteric bed ($n = 22$). The vasodilatations induced by ACh were due to EDRF release because they were not blocked by indomethacin ($n > 40$), but were

abolished by removal of the endothelial cells ($n = 12$, Figure 1), and inhibited by infusion of methylene blue (100 μM, $n = 8$), or by infusion of oxyhaemoglobin (30 μM, $n = 5$).

ACh caused vasodilatations of the mesenteric vascular bed equivalent to or greater than those produced by doses of NO that survived to relax the assay tissues. Nevertheless, the EDRF released was not detectable in the effluent by the RbAs in the absence or presence of infusions of SOD (10 u ml^{-1}). Indeed, a dose of ACh, equivalent to 10 nmol of NO in terms of the vasodilatation induced in the mesenteric bed, did not cause relaxation of the RbAs whereas 1 nmol NO through the mesenteric bed did (Figure 2).

The effect of removal of the endothelium on survival of nitric oxide and glyceryl trinitrate through the mesenteric vascular bed

Removal of the mesenteric endothelium by a brief infusion of sodium deoxycholate did not affect the survival through the mesenteric vasculature of either NO or GTN ($n = 3$). Vasodilatations to GTN ($n = 5$) or NO ($n = 3$) were not significantly increased.

Effects of superoxide dismutase on responses of the mesenteric vascular bed

SOD (10 u ml^{-1}, TM) neither affected the basal perfusion pressure ($n = 8$) nor the vasodilatations produced by ACh (1.7–550 pmol, $n = 3$), NO or GTN (Table 1).

Effects of superoxide dismutase on the survival of nitric oxide

GTN (40–400 pmol) injected into the mesenteric bed caused dose-related relaxations of the assay tissues to the effluent which were similar to those produced by the same doses given directly to the assay tissues either in the absence or presence of SOD ($n = 10$). In contrast, NO injected into the mesenteric bed largely disappeared before reaching the assay tissues

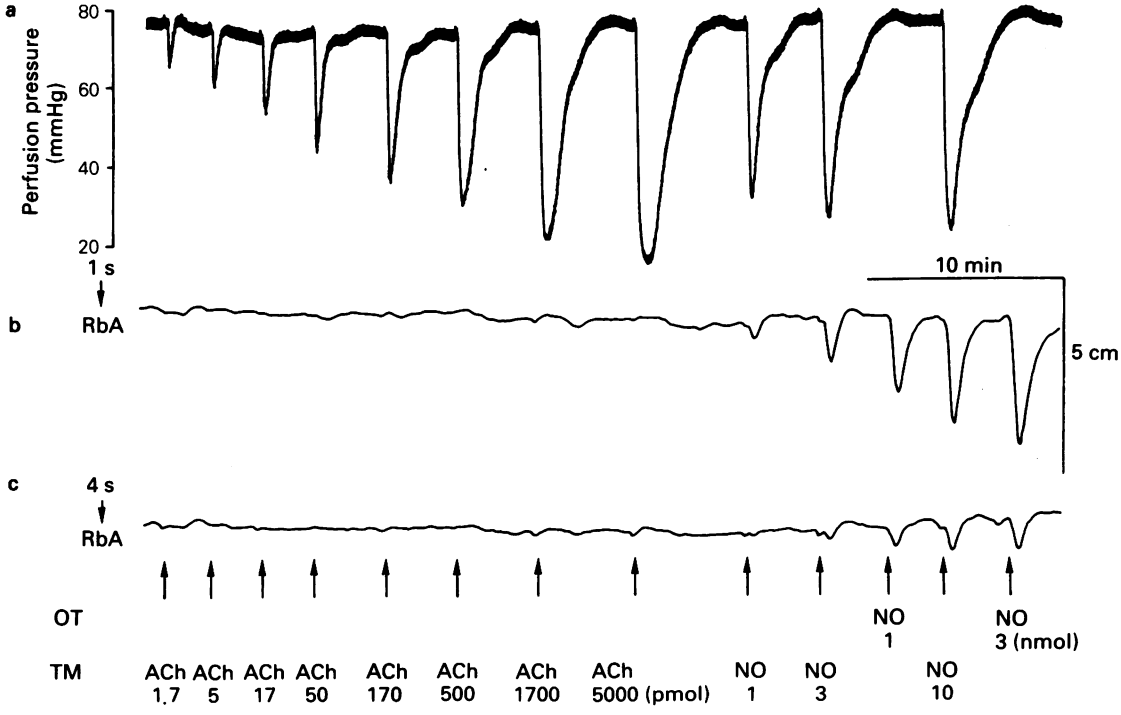

Figure 2 Comparison of the survival of endothelium-derived relaxing factor (EDRF) and exogenous nitric oxide (NO) through the rat isolated perfused mesenteric vascular bed in the presence of superoxide dismutase. Tone was introduced into the mesenteric vasculature and the assay tissues by infusion of U46619 (300 nM). EDRF released by injections of acetylcholine (ACh) through the mesenteric bed (TM) caused vasodilatations in the mesenteric vasculature (a) but was not detectable by the rabbit aortae (RbAs, b and c) bathed in the effluent. NO when injected TM produced both vasodilatations in the mesenteric bed and relaxations of the RbAs. The survival of NO through the mesenteric bed was assessed by bracketing doses of NO injected directly over the tissues (OT).

Table 1 Effect of superoxide dismutase (SOD, 10 u ml^{-1}) on the responses of the isolated perfused mesenteric vasculature of the rat to acetylcholine (ACh, 0.0055–0.55 nmol), nitric oxide (NO, 0.1–10 nmol) and glyceryl trinitrate (GTN, 0.22 nmol)

Drug (nmol)	*Vasodilatation* (% induced tone) −SOD	+SOD
ACh (0.0055)	20 ± 3.5	24 ± 3
ACh (0.55)	52 ± 3.5	48 ± 8
NO (0.1)	27 ± 3.7	33 ± 3.5
NO (1)	50 ± 3.5	63 ± 6.5
NO (10)	67 ± 7.5	71 ± 6.3
GTN (0.2)	60 ± 0	63 ± 2.6

and doses 5.0 ± 0.7 ($n = 9$) times higher than those given directly over the tissues were required to give similar relaxation of the RbAs, indicating removal of 80%. Infusions of SOD intra-arterially significantly ($P < 0.05$) increased the survival of NO in the mesenteric bed so that doses only 2.9 ± 0.5 times higher than those given directly over the tissues were necessary to produce similar relaxation of RbAs indicating a removal of 65% (Figure 3).

Survival of nitric oxide in Krebs solution

The $t_{1/2}$ of NO in the delay coil in the absence of SOD was 25 ± 3 s ($n = 8$). Infusion of SOD through the delay coil enhanced the survival, as detected by the first tissue, of NO injected through the coil

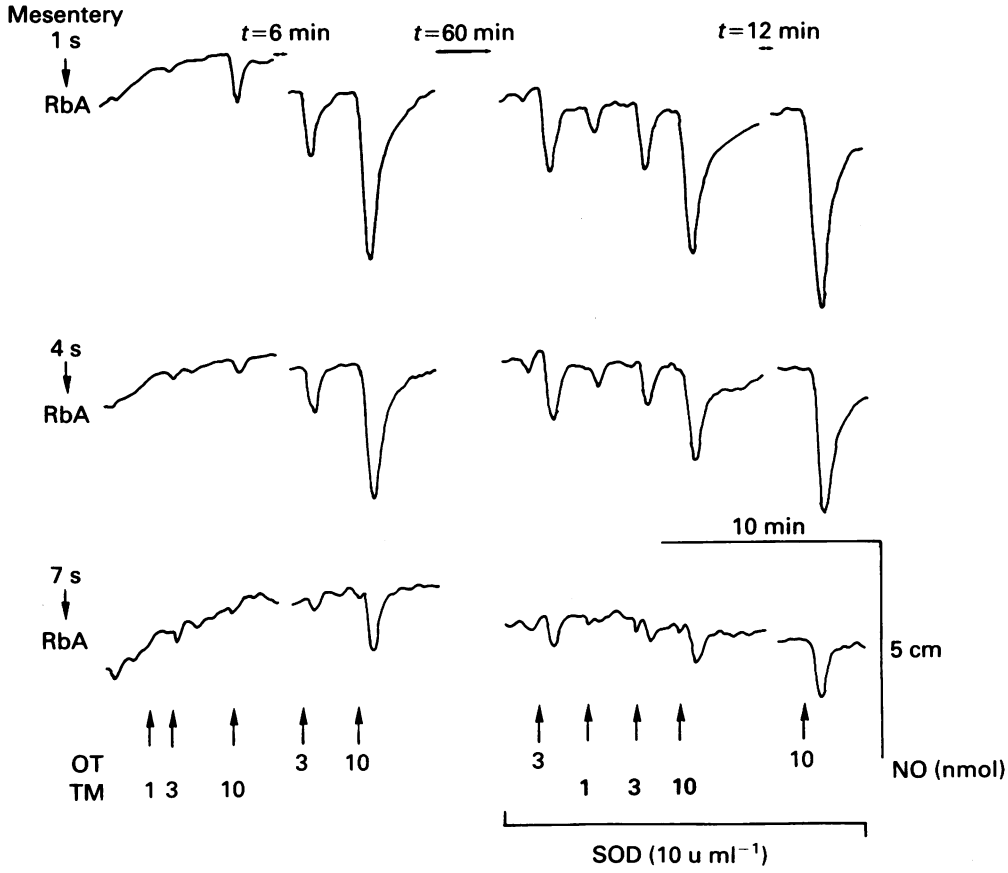

Figure 3 Effect of superoxide dismutase (SOD) on the survival of nitric oxide (NO) through the rat isolated perfused mesenteric vascular bed. The effluent from the mesenteric bed was superfused over strips of rabbit aorta (RbA) precontracted by U46619 (1.5 μM). When 10 nmol NO was injected through the mesenteric bed (TM) in the absence of SOD it produced relaxations of the assay tissues smaller than those produced by 3 nmol NO injected over the tissues (OT). In the presence of SOD (TM), 10 nmol NO (TM) produced relaxations of the assay tissues greater than those produced by 3 nmol NO over the tissues. The SOD infusion was started from the point indicated.

($t_{1/2} = 61 \pm 13$ s, $n = 8$; $P < 0.025$). Infusion of SOD either over the tissues or through the delay coil had no effect on the responses of the first tissue to NO injected directly over the tissues.

Discussion

Our results clearly demonstrate that there is superoxide anion (O_2^-) in the Krebs solution because SOD increased the $t_{1/2}$ of NO from 25 to 61 s in the buffer. The finding that SOD increased the survival of NO in the mesenteric circulation by a greater amount than can be accounted for by O_2^- in the Krebs solution (because of the short (5–8 s) transit time) suggests that there is additional production of O_2^- by the mesenteric vascular bed. This may be a consequence of tissue damage rather than a physiological release, for although endothelial cells may be stimulated to produce O_2^- (Matsubara & Ziff, 1986) such a release would only serve actively to destroy EDRF (Gryglewski *et al.*, 1986b).

Exogenous NO at doses that produced smaller responses within the mesenteric bed than endogenously generated EDRF survived to the assay tissues whereas EDRF did not. A possible explanation for this is that EDRF and NO are different. However, this is unlikely since both substances are similarly inhibited by methylene blue and haemoglobin as has been shown before (Martin *et al.*, 1985; Furchgott *et al.*, 1987) and as we confirm here. Furthermore, Kelm *et al.* (1988) have demonstrated that endothelial cells release NO without it being attached to a carrier molecule. A second interpretation is that the endothelial cells form a significant diffusion barrier to EDRF, limiting its access to the lumen. This is not supported by our results showing that the responses of the vasculature to exogenous NO were not potentiated by removal of the endothelial cells. Furthermore, there is no evidence that the endothelial cells are specialised in removing EDRF since detergent treatment did not increase the survival of NO through the mesenteric vascular bed.

Alternatively, the endothelial cells lining these resistance vessels may preferentially release EDRF in the direction of the vascular smooth muscle, as has been suggested by Bassenge *et al.* (1987). However, the most direct explanation for our results is that the endothelial cells release EDRF in equal amounts luminally and abluminally. The smooth muscle cells will experience a higher concentration of EDRF than is present in the lumen of the blood vessel because the fluid volume into which EDRF is released between the endothelium and the smooth muscle is markedly smaller than that within and flowing through the blood vessel lumen. Therefore, when injecting exogenous NO to match the concentration of endogenous EDRF experienced by the smooth muscle the luminal concentration of NO must be the same as the interstitial concentration of EDRF, even if NO is freely diffusible across the endothelium. This would explain why NO but not EDRF survived in detectable concentrations to the assay tissues.

It has been suggested recently that the metabolism, synthesis or release of EDRF or NO may be different in the venous side of the circulation (Moncada *et al.*, 1988) because although EDRF can be detected coming from arterial tissues it cannot be detected from venous tissues or intact perfused vascular beds. However, Ignarro *et al.* (1987) have shown EDRF can be detected in the effluent from perfused main bovine intrapulmonary vein. Further, the results presented here in which only the arterial side of a vascular bed was used show that the difference may not lie between arterial and venous tissues but between large conduit vessels, either arterial or venous, and the remainder of the vasculature.

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GR32191, a highly potent and specific thromboxane A_2 receptor blocking drug on platelets and vascular and airways smooth muscle *in vitro*

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1 The thromboxane A_2 (TP)-receptor blocking activity and specificity of action of GR32191 ([1*R*-[1α(*Z*),2β,3β,5α]]-(+)-7-[5-([1,1'-biphenyl]-4-ylmethoxy)-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptonoic acid has been evaluated in human platelets and various smooth muscle preparations, both vascular and non-vascular, from a range of species including man.

2 Utilising a platelet counting method to assess aggregation the drug was found to antagonise, in a surmountable manner, human platelet aggregation produced by the TP-receptor agonists, U-46619, EP171 and SQ26655, in whole blood and physiological buffer, with pA_2 values of approximately 8.3 and 8.7 in the two media respectively. In the presence of GR32191 the rate of aggregation induced by U-46619 was slowed.

3 The effect of GR32191 upon U-46619-induced platelet shape change and aggregation in platelet-rich plasma was evaluated utilising a turbidometric technique. Both shape change and aggregation were antagonised by GR32191. At relatively high concentrations of the drug a slowing of aggregation and shape change to U-44619 was seen and an unsurmountable antagonism became apparent.

4 The action of GR32191 upon human platelets was specific with platelet aggregation induced by adenosine 5'-diphosphate, platelet activating factor, vasopressin and adrenaline and the inhibitory effects of prostacylin (PGI_2), prostaglandin D_2 (PGD_2) and N-ethylcarboxamide-adenosine (NECA) being unaffected by concentrations of the drug as high as 10 μM. Furthermore, at concentrations of up to 100 μM, the drug itself produced no shape change or aggregation, of human platelets.

5 GR32191 also specifically and potently antagonised in a competitive, surmountable manner the contractile actions of U-46619 upon human vascular smooth muscle and antagonised U-46619-induced contractions of vascular and airways smooth muscle preparations from rat, dog, guinea-pig and rabbit with varying potency. This is discussed in terms of possible heterogeneity of TP-receptors.

6 GR32191 therefore represents a highly potent and specific TP-receptor blocking drug. This profile of action, coupled to its long duration of effect in man described elsewhere, make it an ideal drug tool for elucidating the physiological and pathophysiological role of thromboxane A_2.

Introduction

With the discovery of thromboxane A_2 (TXA_2, Hamberg *et al.*, 1975), and identification of its potent platelet aggregatory and vasoconstrictor actions, attempts were soon in progress to synthesize structural analogues of these compounds as stable mimetics and antagonists. Compounds, such as U-46619 (Bundy, 1975), were very quickly discovered which appeared to mimic the actions of TXA_2 and in some situations those of prostaglandin H_2 (PGH_2). The action of such compounds suggested the existence of a specific receptor through which TXA_2 exerts its biological effects (see MacIntyre, 1981) and this prostanoid receptor type has been termed a TP-receptor (Kennedy *et al.*, 1982).

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Many of the early attempts to synthesize TP-receptor blocking drugs yielded partial agonists or compounds which also possessed TXA_2 synthase inhibiting activity. The first compound which appeared to meet the criteria of a specific TP-receptor blocking drug was 13-azaprostanoic acid (13-APA) (Le Breton *et al.*, 1979) which specifically antagonised human platelet aggregation to arachidonic acid, PGH_2 and U-46619 but did not affect TXA_2 synthesis. However the compound was very weak and not suitable for *in vivo* studies. Nicolaou *et al.* (1979) originally observed a similar profile to that of 13-APA for pinane thromboxane A_2 (PTA_2). This compound, however, also possessed thromboxane synthase inhibitory activity and has subsequently been shown to exhibit weak partial TP-receptor agonist activity on human platelets and in addition to elevate cyclic AMP (Armstrong *et al.*, 1985).

It was at about this time that we described the pharmacology of the prostanoid, AH19437 (Coleman *et al.*, 1981) (Figure 1). The compound, although being an advance over 13-APA in terms of potency, was still relatively weak with a pA_2 against U-46619-induced aggregation of human platelets and contraction of rat isolated aorta of approximately 6.0. It was, however, specific being without effect up to relatively high concentrations upon all other prostanoid and non-prostanoid receptor types studied. Subsequent chemical modification of AH19437 (Collington *et al.*, 1983) led to the discovery of AH23848, a TP-receptor blocking drug almost two orders of magnitude more potent than the parent drug (Brittain *et al.*, 1985). However, AH23848 also displayed some TP-receptor partial agonist activity and for this and other reasons its development was halted (Lumley, 1986).

GR32191 (Figure 1) is a close analogue of AH23848 in which the TP-receptor partial agonist activity is absent and the TP-receptor blocking potency is enhanced. We describe here in detail the *in vitro* pharmacology of GR32191 upon human platelets and vascular and non-vascular smooth muscle from a range of species.

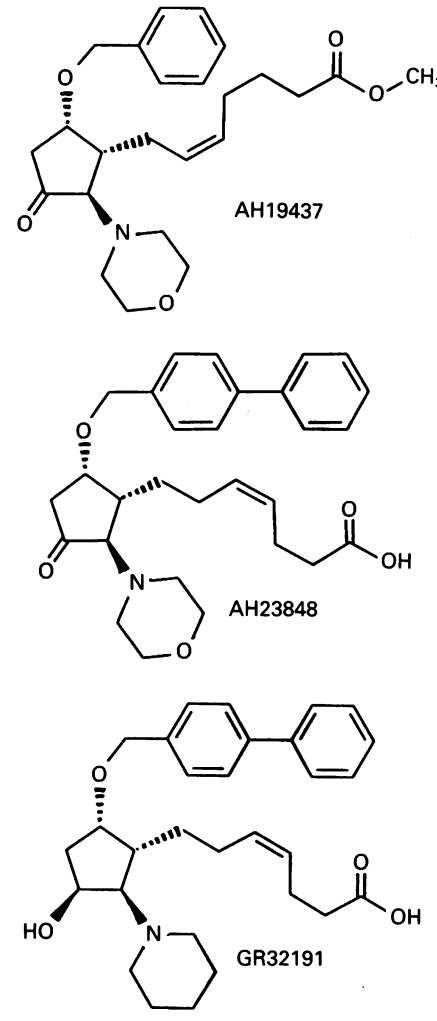


Figure 1 The development of TP-receptor blocking drugs; the chemical structures of AH19437, AH23848 and GR32191.

Methods

Preparation of platelets

Human blood was collected from healthy male volunteers and anticoagulated with trisodium citrate as described elsewhere (Keery & Lumley, 1988). Platelet-rich plasma (PRP) was prepared by centrifugation (Mistral 3000, MSE) of citrated whole blood (1340 *g* for 3 min at 25°C) and kept at room temperature until used. Human resuspended platelets were prepared from PRP (Keery & Lumley, 1988).

Platelet aggregation: counting method

Platelet aggregation was quantified by monitoring the fall in single platelet count produced by aggregatory agents in aspirin-treated aliquots of either whole blood or platelets resuspended in modified Krebs solution, using an Ultra-Flo 100 whole blood platelet counter (Becton-Dickinson) (Lumley & Humphrey, 1981; Keery & Lumley, 1988). Duplicate platelet counts were recorded before and at intervals following addition of an aggregating agent until a nadir in the platelet count was achieved. The intervals employed, which were based upon detailed time-

course studies were, 1 and 3 min (adenosine 5'-diphosphate (ADP), platelet-activating factor (Paf), vasopressin and adrenaline), 5 min (U-46619 and SQ26655), and 10 min (EP171). Percentage platelet aggregation and agonist EC_{50} values were calculated as described previously (Keery & Lumley, 1988).

To quantify the potency of TP-receptor blocking drugs such as GR32191, a control concentration-effect curve was first constructed to the TP-receptor agonist, e.g. U-46619. Concentration-effect curves, randomised over the course of a series of experiments, were then repeated each in the presence of one of three concentrations of receptor blocker (10 min pre-incubation). Finally a second control curve was constructed to allow correction for any spontaneous change in platelet sensitivity. In specificity experiments, aggregation concentration-effect curves to the agonist under study were constructed in the absence (control) and presence of GR32191, usually 10 μM (10 min incubation), followed by construction of a second control curve. To assess the effect of GR32191 upon the antiaggregatory effects of prostacyclin (PGI_2), PGD_2 and N-ethylcarboxamide adenosine (NECA), ADP was utilised as the agonist. Aggregation concentration-effect curves were constructed to ADP in the absence and presence of up to two concentrations of the anti-aggregatory agent (pre-incubated with whole blood for 5 min) which were then repeated in the additional presence of GR32191 (10 μM, 10 min pre-incubation). Finally at the end of the experiment a second ADP control curve was constructed.

Data from these experiments were expressed in the form of concentration-ratios (CR) as described previously (Keery & Lumley, 1988).

Platelet aggregation: optical aggregometer

The effect of GR32191 upon platelet aggregation and shape change induced by both U-46619 and ADP in human PRP was assessed in a six-channel Born-type aggregometer (Glaxo Bioengineering Department). Platelet aggregation was measured as an increase in light transmission and minimum and maximum light transmission set on a chart recorder (Ormed, MX6) using PRP and platelet-poor plasma, respectively. Platelet shape change was recorded as a decrease in light transmission, full scale deflection being calibrated using a sample of PRP which had undergone maximum shape change, but not aggregation, to U-46619 (usually 100 nM). Aliquots (0.3 ml) of aspirin-treated (2 mM) PRP in glass tubes (1.5 ml; Payton Scientific Inc) were allowed to equilibrate at 37°C for 10 min without stirring. After this time stirring was initiated at 900 r.p.m. by means of a metal flea (Payton Scientific Inc) for 1–2 min, at which time the aggregating agent was added. For shape change measurements, EGTA (4 mM) was added 1 min before the addition of the aggregating agonist to prevent aggregation occurring. The effect of GR32191 upon U-46619- and ADP-induced aggregation and shape change was assessed as described above for the platelet counting technique. Each aggregation and shape change response was allowed to proceed to its maximum and, the change in light transmission measured in arbitrary units at this point, expressed as a percentage of the largest change achieved in the first control curve. Results were again expressed as CR values (see above) using the mean EC_{50} value for the duplicate control concentration-effect curves.

Vascular and airways smooth muscle

Human pulmonary arteries were obtained from macroscopically normal lung segments removed by surgical resection approximately 24 h earlier from patients with lung carcinoma. Thoracic aortae were obtained from male Wistar rats (200–300 g), male Dunkin Hartley guinea-pigs (300–500 g) and male New Zealand white rabbits (1.5–2.5 kg). Dog saphenous veins were obtained from barbitone anaesthetized beagle dogs (7–10 kg, see Coleman, 1987). A perspex rod of suitable diameter was inserted into the vessels which, after clearing away non-vascular tissue, were cut spirally (Coleman, 1987). Hearts were removed from beagle dogs killed by an overdose of sodium pentobarbitone. The proximal 20 mm portion of the left anterior descending coronary artery was removed, cleared of cardiac muscle and connective tissue and divided into 3 mm rings. Guinea-pig and rat lung strips were dissected as described for guinea-pig (Coleman, 1987). Four vascular or lung strips from each animal were suspended by cotton threads and rings between wire hooks for isometric recording of tension changes, in 10 ml organ baths containing a modified Krebs solution (Keery & Lumley, 1988) at 37 $\pm$ 0.5°C and bubbled with a 95% O_2 plus 5% CO_2 mixture. The Krebs solution contained atropine (0.8 μM), phenoxybenzamine (0.7 μM) and indomethacin (2.8 μM) unless otherwise stated. Vascular preparations were subjected to initial resting tensions of 0.3–0.5 g and lung strips to a tension of 0.2 g.

An equilibration period of 20–30 min was followed by a particular priming sequence, involving one or two sequential exposures to KCl (30 mM) and then U-46619 (100 nM), which for each preparation enabled two reproducible U-46619 concentration-effect curves to be obtained subsequently. The bathing solution was repeatedly changed between each exposure to re-establish initial resting tension and then a cumulative control concentration-effect curve to U-46619 constructed on each preparation. In some cases (guinea-pig and rat lung strip) repro-

ducible responses to U-46619 could only be achieved by repeating up to three concentration-effect curves to the agonist, the final curve being used as the 'control'. Following washout of U-46619, three preparations were each exposed for one hour to a different concentration of GR32191 (0.03, 0.3 or 3.0 μM) and the fourth to its vehicle and a final U-46619 concentration-effect curve constructed. On each preparation the increase in developed tension (g) was expressed as a percentage of the maximum obtained in the 'control' U-46619 concentration-effect curve. Concentration-ratio values were obtained as previously described and corrected for spontaneous changes in sensitivity to the agonist by dividing the CR value from the test preparations by the CR value obtained from the vehicle-treated preparation (see Humphrey *et al.*, 1988). If this latter value did not lie between 0.5 and 2.0 the data for the whole experiment were discarded.

The specificity of action of GR32191 (3–100 μM, one hour contact) was tested against 5-hydroxytryptamine (5-HT), KCl or histamine. Phenoxybenzamine was omitted from the Krebs solution bathing tissues exposed to 5-HT. Pairs of preparations from the same animal were studied for each agonist. A priming sequence of two KCl (30 mM) responses, only, was adopted on all preparations which was followed by construction of two agonist concentration-effect curves, the first in the absence, the second in the presence of GR32191 or its vehicle. The CR value obtained from the GR32191-treated preparation was corrected as described above using the CR from the vehicle-treated preparation.

Effects at other prostanoid and non-prostanoid receptors

In addition to testing its effects upon IP- and DP-receptors on human platelets, GR32191 (10 μM) was also tested for agonist and antagonist effects upon EP_1-, EP_2- and EP_3-receptors (guinea-pig fundus, cat trachea, guinea-pig vas deferens, prostaglandin E_2 (PGE_2) standard agonist) and FP-receptors (dog and cat iris, $PGF_{2\alpha}$ standard agonist) using methods previously described (Coleman *et al.*, 1987; Coleman, 1987). The compound (10 μM) was also tested (preparation; standard agonist; methodology reference) for agonist and antagonist effects upon α_1- (rabbit aorta; noradrenaline; Furchgott & Bhadrakom, 1953), β_1- (rat spontaneously beating right atrium and electrically stimulated left atrium; isoprenaline; Blinks, 1965; 1967) and β_2-adrenoceptors (electrically stimulated guinea-pig trachea; isoprenaline; Coleman & Nials, 1986), upon 5-HT_1- and 5-HT_2-receptors (dog saphenous vein and rabbit aorta respectively; 5-HT; Humphrey *et al.*, 1988), D_2-dopamine receptors (rabbit ear artery; quinpirole; Steinsland & Hieble, 1978) and M_1- (rat cortex; pirenzepine; Hammer *et al.*, 1986) and M_2-muscarinic receptors (rat salivary gland and left atrium; N-methylscopolamine; Hammer *et al.*, 1986).

Expression of results

Data were expressed as arithmetic or geometric mean values with 95% confidence intervals. Individual data were compared by means of paired or unpaired Student's *t* test. Data from interaction studies between TP-receptor agonists and antagonists were analysed by the method of Arunlakshana & Schild (1959).

Drugs used

GR32191 (hydrochloride salt, Glaxo Group Research Ltd) was prepared as a 1 mM stock solution by dissolving in distilled water. Tri-sodium citrate (129 mM; BDH), (−)-adrenaline (10 mM; Sigma), 5-hydroxytryptamine creatinine sulphate (10 mM; Sigma), potassium chloride (4 M; BDH) were all dissolved in distilled water at the concentration shown in parentheses; subsequent dilutions were made in 150 mM NaCl solution (saline). In the case of adrenaline, ascorbic acid was included in the stock solution and dilutions at a final concentration of 1 mM. Atropine sulphate (4 mM; Sigma), phenoxybenzamine hydrochloride (1 mM, Smith Kline and French), histamine acid phosphate (1 mM; BDH), vasopressin (1 mM; Sigma), EP171 ([1*R*-[1α,2β(*Z*),3α(1*E*.3R*),4α]]-7-[4-[(4-fluorophenoxy)-3-hydroxy-1-butenyl]-7-oxabicyclo [2.2.1] hept-2-yl]-5-heptenoic acid, 0.1 mM; gift), EGTA (ethyleneglycol-bis-(β-aminoethyl ether) N,N,N′,N′-tetracetic acid, 400 mM; Sigma) were dissolved in saline; dilutions were also prepared in saline. The EGTA solution also contained 20 mM HEPES (N-2-hydroxyethyl piperazine-N′-2-ethanesulphonic acid; Sigma) and the pH of the solution adjusted to 7.4 with 5 N NaOH. Prostaglandin E_2 (10 mM; Ono), SQ26655 ([1S-(1α,2α(*Z*),3β(1E, 3R*),4α]]-7-[3-(3-hydroxy-1-octenyl)-7-oxabicyclo [2.2.1]hept-2-yl]-5-heptenoic acid, 10 mM; gift), BM 13.505 (4-[2-[(phenylsulphonyl)amino]ethyl]phenoxyacetic acid, 10 mM; Glaxo Group Research Ltd) and BM 13.177 (4-[2-[[(4-chlorophenyl)sulphonyl] amino]ethyl]benzeneacetic acid, 10 mM; Glaxo Group Research Ltd) and indomethacin (10 mM; Sigma) were dissolved in 10% w/v sodium bicarbonate solution and diluted with saline to give a final bicarbonate concentration of 1% w/v. Dilutions were prepared in saline. Prostaglandin $F_{2\alpha}$ (dinoprost trimethamine, Upjohn) was supplied as a 5 mg ml^{-1} solution and dilutions were again prepared in saline. AH19437 and 13-azaprostanoic acid (10 mM, Glaxo

Group Ltd), EP092 ([1α,2β(Z),3α,4α]-(±)-7-[3-[1-[[(phenylamino)thioxomethyl]hydrazono]ethyl] bicyclo[2.2.1] hept-2-yl]-5-heptenoic acid, 1 mM; gift), SQ29548 ([1R-[1α,2β(Z),3β,4α]]-7-[3-[[2-[phenylamino) carbonyl] hydrazino]methyl]-7-oxabicyclo [2.2.1]hept-2-yl]-5-heptenoic acid, 1 mM; gift) and SQ28668 ([1α,2α(Z)3α(1E,3S*4R*)4α]-7-[3-(3-hydroxy-4-phenyl-1-pentenyl)-7-oxabicyclo[2.2.1]-hept-2-yl]-5-heptenoic acid, 1 mM; gift) were dissolved in absolute ethanol and saline added to give a final concentration of ethanol of 6% v/v; dilutions were prepared in saline. Details of the preparation of U46619, AH23848, aspirin, NECA (5'-N-ethylcarboxamide adenosine), PGI_2, PGD_2, ADP (adenosine-5'-diphosphate) and Paf DL-α-phosphatidylcholine, β-acetyl-γ-O-hexadecyl) are given elsewhere (Keery & Lumley, 1988). All other reagents used were Analar grade. In the text, concentrations of drugs used in whole blood experiments are expressed as the concentrations in whole blood with no correction for haematocrit. All drugs were kept on ice during an experiment.

Results

Activity of GR32191 on human platelets in whole blood and resuspended in physiological buffer

In human whole blood, U-46619 (0.03–1.0 μM) induced aggregation of platelets, as measured by the disappearance of single platelets, with a mean (95% confidence interval, n = number of experiments) EC_{50} value of 0.21 (0.11–0.42) μM ($n = 6$). GR32191 10–100 nM produced a progressive rightward displacement of the U-46619 concentration-effect curve for platelet aggregation. Concentration-ratios for U-46619 of 3.2 (1.9–5.5), 9.4 (4.0–22.2) and 34.7 (16.8–71.5) were obtained at GR32191 concentrations of 10, 30 and 100 nM, respectively. No depression of the maximum of the U-46619 concentration-effect curve was observed at the highest concentration of GR32191 used in these experiments, although a lengthening of the time to achieve peak aggregation was seen (Figure 2). Analysis of the data yielded a mean pA_2 value of 8.2 (7.9–8.5) ($n = 6$) and a slope of the regression of 1.29 (1.13–1.45) which was significantly greater than unity. To determine whether equilibrium had been achieved with GR32191 (30 nM) under these experimental conditions a comparison was made of 10 and 30 min incubations of the drug upon U-46619-induced platelet aggregation. No significant difference was observed with the two incubation times (mean ($n = 5$) with U-46619 CR values of 15.2 and 14.0, respectively).

GR32191 (10–100 nM) also antagonised platelet aggregation in whole blood induced by two other potent TP-receptor agonists, namely SQ26655 and EP171, with a similar potency to that seen against U-46619 (Table 1). No depression of the maximum response to EP171 was observed in these experiments but some slight depression, at the highest concentration of GR32191 used, was observed with SQ26655. In addition, at this concentration of GR32191 a slowing of the aggregatory response to SQ26655 was also observed. However, no such effect was observed when EP171 was used as the agonist.

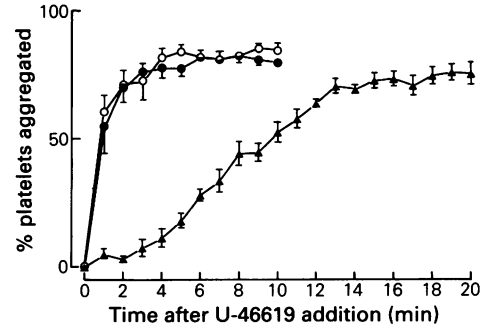


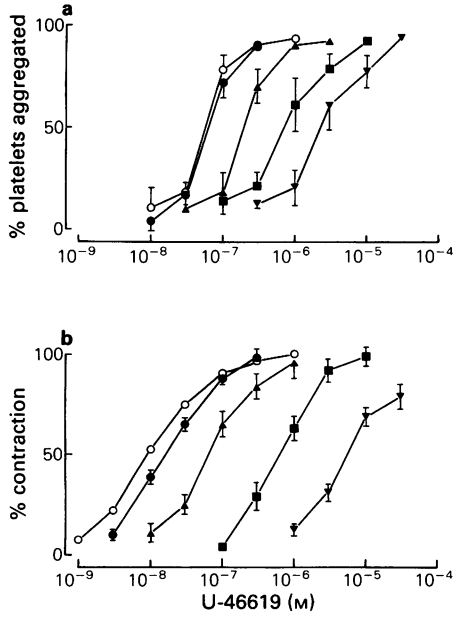
Figure 2 The effect of GR32191 (30 nM) upon the rate of U-46619-induced platelet aggregation in human whole blood. Curves are the mean from 4 experiments with vertical lines indicating s.e.mean. Control U-46619 (1 μM) response (○); response to U-46619 (30 μM) in the presence of GR32191 (30 nM) (▲); response to U-46619 (1 μM) in the presence of GR32191 vehicle (●).

The estimation of receptor blocking potency is complicated in whole blood by the presence of plasma proteins. The interaction of GR32191 with U-46619, SQ 26655 and EP171 was therefore investigated in a preparation of human platelets resuspended in modified Krebs solution. In this medium there was an enhancement in the potency of U-46619 for inducing platelet aggregation but, interestingly, not in that of the other two agonists, SQ26655 or EP171 (Table 1). As in whole blood, GR32191 (3–30 nM) produced a progressive rightward displacement of the U-46619 aggregation concentration-effect curve (Figure 3a). Mean U-46619 CRs of 3.1 (2.2–4.3), 13.7 (4.5–41.7) and 42.7 (15.5–117.2) were obtained with 3, 10 and 30 nM GR32191, respectively. These experiments yielded an apparent mean pA_2 value of 8.79 with a slope of the Schild regression of 1.31 (Table 1). When compared with the value obtained in whole blood this shows an approximate 2–3 fold increase in potency of GR32191 in this medium, indicating that the drug is susceptible to binding by plasma proteins. A similar enhancement of potency of GR32191 was obtained in resuspended platelets using SQ26655 and EP171 as the agonist; these results are summarised in Table 1.

Table 1 The potency of GR32191 upon human platelet aggregation induced by various TP- and non-TP-receptor agonists

Medium	Agonist	Agonist EC_{50} (nM)	GR32191 pA_2	GR32191 Slope	(n)
Whole blood[a]	U-46619	230 (110–470)	8.23 (7.90–8.56)	1.27 (1.09–1.44)	(6)
	SQ26655	32 (24–43)	8.32 (8.15–8.50)	1.61 (1.28–1.93)	(9)
	EP171	1.9 (1.5–2.3)	8.30 (8.26–8.35)	1.13 (1.06–1.20)	(4)
	ADP	900 (690–1170)	<5.0[b]	—	(9)
	PAF	57 (45–71)	<5.0[b]	—	(4)
	Adrenaline	1460 (950–2250)	<5.0[b]	—	(8)
	Vasopressin	117 (80–169)	<5.0[b]	—	(4)
Resuspended platelets[a]	U-46619	57 (41–80)	8.79 (8.63–8.94)	1.31 (1.0–1.58)	(4)
	SQ26655	27 (2.5–281)	8.64 (8.06–9.22)	1.55 (1.22–1.88)	(4)
	EP171	1.8 (1.6–2.0)	8.77 (8.69–8.86)	1.25 (1.14–1.35)	(4)
	ADP	1115 (870–1430)	<5.0[b]	—	(4)

Values are the mean (95% confidence interval) from n experiments.
[a] Platelet counting method.
[b] CR value of <2.0 obtained at 10 μM GR32191.



In both whole blood and in resuspended platelets GR32191 (10 μM) was without effect upon ADP-induced aggregation (Table 1). In addition in whole blood at this concentration the compound was also without effect upon platelet aggregation induced by adrenaline (0.1–3.0 μM), Paf (10–300 nM) or vasopressin (0.01–1.0 μM) (Table 1). The antiaggregatory effects of PGI_2, PGD_2 and NECA assessed against

Figure 3 The effect of GR32191 upon (a) U-46619-induced aggregation of human resuspended platelets and (b) contraction of human isolated pulmonary artery strips. Curves are the mean from 4 and 10 experiments in (a) and (b) respectively; vertical lines show s.e.mean. In (a) U-46619 control concentration-effect curves were performed at the beginning (○) and end (●) of the experiments and compared with those performed in the presence of 3 (▲), 10 (■) and 30 (▼) nM GR32191, respectively. In (b) in each experiment a control U-46619 concentration-effect curve was initially constructed on all four strips of pulmonary artery and the pooled data shown (○), $n = 40$; three of the four strips were then exposed to GR32191 30 (▲), 300 (■) and 3000 (▼) nM respectively, for one hour before repeating the U-46619 curves. The fourth preparation received GR32191 vehicle only (●).

Table 2 The antiaggregatory effects of prostacyclin (PGI_2), prostaglandin D_2 (PGD_2) and 5'-N-ethylcarboxamide adenosine (NECA) assessed against ADP-induced platelet aggregation in the absence and presence of GR32191 (10 μM)

Anti-aggregatory agent (μM)	*ADP concentration-ratio* absence of GR32191	presence of GR32191
PGI_2 (0.003)	14 (4.4–45)	9.4 (3.4–26)
(0.01)	118 (52–267)	147 (50–436)
PGD_2 (0.1)	5.6 (2.6–12)	4.6 (2.8–7.4)
(0.3)	71 (12–401)	64 (8.9–467)
NECA (1.0)	14 (6.9–28.4)	14 (7.1–27.4)

Values shown are geometric mean values (95% confidence intervals) of 4–6 determinations

ADP-induced platelet aggregation were also unaffected by GR32191 (10 μM, Table 2). Furthermore, GR32191 up to concentrations of 10 μM was without effect upon human single platelet count in either whole blood or resuspended platelets.

Activity in platelet-rich plasma

The profile of action of GR32191 was also assessed in human platelet-rich plasma (PRP) using a turbidometric technique. With this technique U-46619 induced platelet aggregation with a mean EC_{50} value of 0.5 (0.4–0.6) μM ($n = 7$). GR32191 (3–100 nM) antagonised U-46619-induced platelet aggregation. At the lower concentrations a parallel rightward displacement of the U-46619 concentration-effect curve was observed. For example at a concentration of 10 nM a U-46619 CR value of 5.7 (3.0–10.9) ($n = 6$) was obtained with no depression of the maximum response being observed. However, at a concentration of 100 nM a marked depression in the U-46619 maximum was seen which was also associated with a slowing of the aggregatory response.

U-46619-induced platelet shape change was monitored separately. The agonist potently induced a shape change response (mean EC_{50} value of 0.04 (0.01–0.09) μM ($n = 4$)) which was antagonised by GR32191. The profile of this antagonism appeared to be more like that seen using the platelet counting method, with GR32191 (3–100 nM) producing parallel rightward displacements of the agonist concentration-effect curve with no depression of the maximum effect. Interestingly, quantitative analysis of one such experiment yielded a pA_2 value of 8.78 and a slope of the Schild regression of 0.89. However, at higher concentrations of GR32191 (300 nM), a depression of the maximum of the shape change concentration-effect curve and slowing of the response was observed. In contrast to its effects upon U-46619, GR32191 (10 μM, $n = 2$) was without effect upon either aggregation or shape change induced by ADP in human PRP. In addition GR32191 itself, up to concentrations of 100 μM produced no shape change response or direct aggregatory effects in human PRP.

Vascular smooth muscle

U-46619 was a potent contractile agonist on all preparations of vascular smooth muscle studied (Table 3). GR32191 antagonised these U-46619-induced contractions in preparations from a variety of species including man (Table 3). The antagonism observed appeared to be of a competitive surmountable nature, except on the rabbit aorta where a slope of the Schild regression significantly less than unity was obtained. U-46619 concentration-effect curves upon human pulmonary artery strips in the absence and presence of GR32191 are shown in Figure 3b. The experiments, in which U-46619 mean ($n = 10$) CRs of 5.6 (3.4–9.3), 52 (27–98) and 521 (306–886) were obtained with 30, 300 and 3000 nM GR32191 respectively, yielded a mean pA_2 value of 8.18. GR32191 was of comparable potency to this upon vascular smooth muscle from rat, guinea-pig and dog, but appeared to be weaker upon rabbit aorta (Table 3).

The profile of action of GR32191 was examined further against a range of TP-receptor agonists upon the rat isolated aorta (Table 4). All of the agonists tested, namely SQ26655, EP171, PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 produced only contractions of the preparation and these contractions were antagonised by GR32191 yielding comparable pA_2 values of between 7.5 and 8.0 in all cases.

The compound was specific where tested, being without significant effect upon contractions of the preparations induced by 5-hydroxytrpytamine, potassium chloride or histamine at concentrations up to 10 (rat and guinea-pig) or 100 (rabbit) μM (Table 3). GR32191, up to concentrations as high as 10 μM, was also found to be without direct contractile effect upon any of the vascular smooth muscle preparations examined.

Airways smooth muscle

GR32191 also antagonised U-46619-induced contractions of airways smooth muscle from rat and guinea-pig (Table 3). As in vascular smooth muscle

Table 3 The potency and specificity of action of GR32191 upon isolated vascular and airways smooth muscle preparations from a range of species

Preparation	U-46619[a] EC_{50} (nM)	pA_2[a] vs U46619	Slope[a] vs U46619	(n)	pA2[b] vs 5-HT	pA2[b] vs KCl	pA2[b] vs Histamine
Human pulmonary artery	11.3 (8.8–14.5)	8.18 (7.81–8.55)	1.06 (0.92–1.20)	(10)	NT	NT	NT
Rat aorta	16.6 (12.7–21.7)	7.87 (7.62–8.13)	0.93 (0.83–1.04)	(8)	<5.5	<5.5	NT
Guinea-pig aorta	70 (32–152)	8.77 (8.10–9.44)	0.91 (0.77–1.06)	(6)	NT	<5.0	<5.0
Dog saphenous vein	5.2 (2.3–11.7)	8.72 (8.42–9.02)	0.90 (0.69–1.12)	(5)	NT	NT	NT
Dog coronary artery	15.9 (3.8–67)	8.26 (7.90–8.62	1.11 (0.73–1.48)	(3)	NT	NT	NT
Rabbit aorta	14.1 (8.5–23.5)	7.21 (6.82–7.60)	0.78 (0.68–0.88)	(6)	≃4.0	<4.0	NT
Rat lung	309 (246–388)	7.14 (6.85–7.44)	0.94 (0.80–1.08)	(6)	<5.0	<5.0	NT
Guinea-pig lung	50 (26.8–93)	8.21 (7.87–8.55)	1.05 (0.87–1.23)	(7)	NT	<5.0	<5.0

[a] Values are the means of n observations with 95% confidence intervals in parentheses.
[b] Values represent data from 2–5 experiments. NT = not tested.

the antagonism appeared to be of a competitive, surmountable type and was specific with contractions of the preparations to either 5-hydroxytryptamine, potassium chloride or histamine being unaffected by concentrations of GR32191 up to 10 μM (Table 3).

Actions at other prostanoid and non-prostanoid receptor types

GR32191 (10 μM), in contrast to PGE_2 the standard agonist, did not produce a contraction of the guinea-pig isolated fundus or relaxation of the cat isolated carbachol-contracted trachea, nor did it antagonise these actions of PGE_2, ruling out agonist or antagonist actions at either EP_1- or EP_2-prostaglandin receptors. Furthermore, at this concentration the compound neither affected contractions of the guinea-pig electrically stimulated isolated vas deferens, nor contracted the dog or cat isolated iris sphincter muscle, nor did it antagonise the actions of the standard agonist on each preparation, further ruling out actions at either EP_3 or FP-receptors, respectively. GR32191 was also tested at a single concentration of 10 μM in a range of tissues in experiments designed to detect agonist and antagonist activity at various autonomic receptor types (see Methods). With the exception of the rat isolated spontaneously beating right atrium, in which it produced bradycardia (mean 51 (range 51–52) beats min^{-1} ($n = 3$)), GR32191 was without direct effect upon any of the tissues studied. In addition it failed to alter significantly responses to the standard agonist on any of the test systems examined so ruling out an action at α_1-, β_1- and β_2-adrenoceptors, 5-HT_1- or 5-HT_2-, dopamine D_2- or muscarinic M_1- or M_2-receptor types.

Table 4 The potency of GR32191 against contractions of the rat isolated aorta induced by various natural and synthetic prostanoids

Agonist	pA_2	Slope	(n)
U-46619	7.9 (7.6–8.1)	0.9 (0.8–1.0)	(8)
EP-171	7.6 (7.2–7.9)	0.9 (0.7–1.1)	(6)
SQ26655	8.0 (7.8–8.2)	1.0 (0.9–1.1)	(6)
PGD_2	7.8 (7.5–8.1)	1.1 (0.9–1.4)	(6)
PGE_2	7.9 (7.7–8.1)	1.1 (1.0–1.2)	(6)
$PGF_{2\alpha}$	8.1 (7.7–8.4)	1.0 (0.8–1.1)	(6)
PGI_2	7.9 (7.6–8.2)	1.1 (0.8–1.3)	(6)

Values are arithmetic means (95% confidence interval) from n experiments.

Discussion

The present study has shown GR32191 to be a potent and specific TP-receptor blocking drug *in vitro* on platelets and vascular as well as airways smooth muscle preparations from a variety of species including man. The drug, at concentrations up to at least three orders of magnitude greater than those which produce a threshold blockade of the TP-receptor, does not interact with any other receptor types examined, prostanoid or non-prostanoid, nor

does it have any action upon cyclo-oxygenase, thromboxane synthase or prostacyclin synthase (this study; Hornby *et al.*, 1989). The compound is orally active and well tolerated in man, producing a long lasting antagonism of U-46619-induced platelet aggregation *ex vivo* (Thomas *et al.*, 1987). This profile of high potency, specificity and duration of action make GR32191 an ideal drug tool to elucidate the pathophysiological role of TXA_2 in human thromboembolic disease.

The profile of action of GR32191 is consistent with a blocking action at TP-receptors. Thus the drug antagonises platelet aggregation and vascular and airways smooth muscle contraction induced by agents such as U-46619, SQ26655 and EP171, which have been characterised as TP-receptor agonists (Kattleman *et al.*, 1986; Harris *et al.*, 1987; Lawrence *et al.*, 1988). In addition GR32191 antagonises platelet aggregation to agents which act indirectly via endogenously formed TXA_2 such as collagen and arachidonic acid (Hornby *et al.*, 1989). The drug also antagonises platelet aggregation induced by the prostaglandin endoperoxide PGH_2 (Hornby *et al.*, 1989). implying, as others have suggested (Armstrong *et al.*, 1983) that PGH_2 and TXA_2 interact with the same receptor to induce platelet aggregation. However, in addition to this indirect evidence, recent radioligand binding studies have directly shown that GR32191 interacts with the platelet TP-receptor (Lawrence *et al.*, 1988). Thus, GR32191 was found to displace the TP-receptor ligand [^{125}I]-PTA-OH (Narumiya *et al.*, 1986) from human platelets with an estimated mean ($\pm$s.e.mean) K_D value of 4.6 ± 0.7 nM. This value compares closely with the K_D value of 1.7 ± 0.2 nM for GR32191, derived from the pA_2 for U-46619 in human resuspended platelets (present study).

The profile of action of GR32191 upon vascular and airways smooth muscle appears to be of a competitive, surmountable type. Thus the lack of any depression of the maximum response to TP-receptor agonists and slopes of the Schild regression not significantly different from unity (the only exception being the rabbit aorta) are both observations which support this conclusion. In contrast, on human platelets the drug slows the rate of response to U-46619, whether it is aggregation or shape change, and can lead to a suppression of the agonist maximum response. This is particularly noticeable when using the turbidometric technique for assessing platelet aggregation (this study; Hornby *et al.*, 1989). In addition, slopes of the Schild regression significantly greater than unity for antagonism of platelet aggregation were observed whether using U-46619 or other TP-receptor agonists such as SQ26655 and EP171. It may be, however, that the slowing of the aggregatory response can be separated from the mechanism producing the high slope of the Schild regression, since with the TP-receptor agonist EP-171 only the latter effect was observed with GR32191. The slowing of the aggregatory response and suppression of the maximum response have also been observed with other TP-receptor blocking drugs of similar structure to GR32191 (e.g. AH23848, Brittain *et al.*, 1985) as well as with several other antagonists of widely differing chemical structure such as EP092 (Armstrong *et al.*, 1985), SQ29548 (Ogletree *et al.*, 1985) and ICI 192,605 (Jessup *et al.*, 1988). One common feature of most of these studies is the use of the TP-receptor agonist U-46619 to induce platelet aggregation. It has been shown that at high concentrations this agonist can elevate intra-platelet cyclic AMP (Best *et al.*, 1979), possibly by stimulation of the platelet IP-receptor (Stratton & Hornby, 1987). Therefore at the high concentrations of U-46619 needed to induce aggregation in the presence of TP-receptor blockade, this effect may influence the profile of antagonism of platelet aggregation observed. However, this is unlikely to be the sole explanation for the effects seen with drugs like GR32191, since experiments with other TP-receptor blocking drugs such as EP045 (Armstrong *et al.*, 1985), BM 13.177 (Patscheke & Stegmeier, 1985) and BM 13.505 (Patscheke *et al.*, 1987), in which profiles consistent with surmountable competitive antagonism were obtained, also utilised U-46619 as the agonist.

An alternative explanation for the antagonist profile observed with GR32191 on platelets may be related to its rate of dissociation from the platelet TP-receptor. Preliminary experiments with [^{3}H]-GR32191 have shown that it exhibits a relatively slow dissociation rate ($t_{1/2}$ of approximately 60 min) from TP-receptors on human platelets (Armstrong, personal communication). Slow dissociation of an antagonist coupled with the use of a weak agonist would be expected to produce a hemi-equilibrium state, such that full equilibrium between agonist and receptors in the presence of antagonist does not occur (see Kenakin, 1984). Since U-46619 appears to be a low efficacy agonist, which needs to occupy about 40% of the receptor population to elicit full aggregation (Armstrong *et al.*, 1985), this would predictably lead to the reduced maximal response observed to U-46619 in the presence of high concentrations of GR32191 and/or slopes of Schild regressions greater than unity. Interestingly, the receptor occupancy required by U-46619 to induce shape change is much less than for aggregation (approximately 5%). It is perhaps not surprising therefore that, under experimental conditions (turbidometric measurement) in which the maximum aggregatory response to U-46619 was markedly suppressed in the presence of GR32191, a similar suppression of the shape change response maximum was not observed.

A further important feature of GR32191 is its ability to block TP-receptors in human platelets and vascular smooth muscle over a similar concentration range. This profile of blockade of TP-receptors in both tissue types is desirable in a drug of this type since it is likely that, in addition to its platelet aggregating activity, TXA_2-induced vasoconstriction may also play a key role in the clinical pathology of ischaemic vascular disease. GR32191 also antagonised U-46619-induced contraction in preparations of vascular and airways smooth muscle from other species. However, differences in the potency of the drug were apparent. One of the preparations where the drug appeared to be weak (low pA_2 and low slope) was the rabbit aorta. We have previously observed this profile with AH23848 (unpublished data) and it has been found for other TP-receptor blocking drugs (Jones *et al.*, 1982; 1987; Narumiya *et al.*, 1986). Another preparation in which GR32191 appeared to be weak (low pA_2) was the rat lung. The data in rat lung contrasts with that in the rat aorta which in our experience has been predictive for the potency of compounds such as GR32191 and AH23848 (Brittain *et al.*, 1985) upon human vascular smooth muscle. This preparation is a useful one for studying the effects of TXA_2 and its mimetics since, from the evidence presented in the present study, it appears that the only prostanoid receptors it contains are of the TP-type (Table 4).

The differences in the antagonist potency of GR32191 and other TP-receptor blocking drugs in various tissues discussed above could be taken as evidence for TP-receptor heterogeneity. However, there appears to be no obvious pattern to the differences. It is therefore conceivable that drugs such as GR32191, which are structurally quite different from the natural ligand TXA_2, may not be the ideal drug tools to investigate the possible sub-division of receptors. For example, such drugs may interact with binding sites which do not form part of the receptor itself and it is these exoreceptor sites which are poorly conserved between species. An analogous situation has been claimed for antagonists of the $5\text{-}HT_2$ receptor in smooth muscle (Leff & Martin, 1986).

GR32191 is one of several currently available and structurally diverse TP-receptor blocking drugs. The drug represents a progression, in terms of potency and profile, from our previously published results with compounds AH19437 and AH23848 (see Introduction). Thus the compound is more potent than AH23848 upon human platelets and vascular smooth muscle *in vitro* but more importantly lacks partial agonist activity at any TP-receptor examined to date (this study; Lumley *et al.*, 1988). Other TP-receptor blocking drugs of note include SQ29548 (Ogletree *et al.*, 1985), BM 13.177 and BM 13.505 (Patscheke & Stegmeier, 1985; Patscheke *et al.*, 1987), ICI 192,605 (Jessup *et al.*, 1988), EP092 (Armstrong *et al.*, 1985) and ONO 3708 (Kondo *et al.*, 1987). With the possible exception of the Boerhinger Mannheim compounds, these TP-receptor blocking drugs are highly potent *in vitro*. We have determined the potencies of several of these compounds, under identical conditions to those described in this study for GR32191 and the results are compared in Table 5. As can be seen GR32191 represents one of the most potent TP-receptor blocking drugs available. The other compounds which are potent *in vitro* and, in some cases *in vivo* in experimental animals are SQ29548, EP092 and ICI 192,605. However, none of these compounds appears yet to have been tested in man. Of those which have, namely BM 13.177, BM 13.505 and SQ28668 (Patscheke *et al.*, 1986; 1987; Friedhoff *et al.*, 1986), all are, in contrast to GR32191, relatively weak and of short duration of action.

Table 5 A comparison of the TP-receptor blocking activity of a range of compounds against U-46619-induced aggregation of human platelets and contraction of rat isolated aortic strip

	pA_2		
	Human platelets		
Compound	*Whole blood*	*Resuspended*	*Rat aorta*
AH19437	6.1 (5.8–6.3)	NT	5.9 (5.2–6.6)
AH23848	7.8 (7.6–8.0)	8.3 (8.0–8.7)	7.9 (7.5–8.4)
GR32191	8.2 (7.9–8.6)	8.8 (8.6–8.9)	7.9 (7.6–8.1)
SQ29548	7.8 (7.6–8.1)	8.5–8.9[c]	8.9 (8.4–9.3)
BM 13.177	6.0 (5.6–6.4)	5.4–6.2[c]	6.2 (5.4–7.0)
BM 13.505	6.7 (6.5–6.9)	7.6–8.1[c]	7.9 (7.4–8.4)
SQ28668	5.5 (5.0–6.0)	5.7–5.8[c]	6.0 (5.6–6.3)
EP092	7.2 (6.8–7.6)	NT	8.0–8.1[c]
13-APA	5.3 (5.1–5.4)	NT	4.8 (4.5–5.1)

Values are arithmetic means (95% confidence intervals) from at least 4 experiments.
[c] Range, $n = 2$–3.
NT = not tested.

In summary, GR32191 possesses a profile of action consistent with it being a TP-receptor blocking drug on platelets and vascular and airways smooth muscle from man and a range of other species. The high degree of specificity of GR32191 for TP-receptors over both prostanoid and non-prostanoid receptors, its lack of effect upon a range of enzyme systems and lack of direct effects upon platelets or smooth muscle demonstrate its value in defining the actions of drugs at TP-receptors. In addition, this profile of action of GR32191, coupled with its high oral potency and duration of action in man, make it an exciting drug for the clinical elucidation of the pathophysiological role of TXA_2.

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The effects of drugs interacting with opioid receptors on the early ventricular arrhythmias arising from myocardial ischaemia

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1 The effects of a range of opioid receptor agonists and antagonists with differing opioid receptor selectivities on ischaemia-induced arrhythmias in anaesthetised rats was investigated.

2 Naloxone was antiarrhythmic only at doses expected to antagonise κ- and δ-receptors in addition to μ-receptors.

3 The opioid receptor antagonist Mr 2266, which is twice as potent at κ-receptors as at μ-receptors dose-dependently reduced the incidence and severity of the arrhythmias resulting from coronary artery occlusion.

4 The opioid receptor antagonist M 8008 (1 mg kg^{-1}), which is twice as potent at δ-receptors as at μ-receptors but has very little affinity for the κ-receptor, did not exhibit any beneficial anti-arrhythmic properties.

5 MrZ 2593, a quarternary complex of naloxone which does not readily cross the blood brain barrier, was antiarrhythmic which implies that the antiarrhythmic actions of opioid receptor antagonists may be mediated via peripheral opioid receptors.

6 The agonists, diamorphine, [Leu] enkephalin and U-50,488H exhibited no significant arrhythmogenic effects under the present experimental conditions.

7 It is tentatively suggested that blockade of peripheral κ-receptors during acute myocardial ischaemia may result in an antiarrhythmic effect.

Introduction

It has previously been shown that naloxone (Fagbemi *et al.*, 1982) and meptazinol (Fagbemi *et al.*, 1983) reduce the incidence and severity of ventricular arrhythmias resulting from acute coronary artery occlusion in both conscious and anaesthetised rats. To explore the possibility that this action was receptor-mediated, the effects of two opioid receptor antagonists ((−)-WIN, 44,441-3 and (−)-Mr 1452) and their stereoisomers ((+)-WIN, 44,441-2 and (+)-Mr 1453) on ischaemia-induced arrhythmias in anaesthetised rats were investigated (Parratt & Sitsapesan, 1986). (−)-Mr 1452 but not (+)-Mr 1453 reduced, in a dose-dependent manner, the number of ventricular ectopic beats and the incidence of ventricular tachycardia (VT) and ventricular fibrillation (VF). A similar protective effect was observed with (−)-WIN 44,441,3 but not with (+)-WIN 44,441-2. Thus, these results indicated that a specific receptor-mediated effect was responsible for the beneficial effects of opioid receptor antagonists under conditions of myocardial ischaemia.

If specific opioid receptors are indeed involved to which opioid receptor subtypes do they belong? The present study was designed to attempt to answer this question by investigating the antiarrhythmic properties of a range of opioid antagonists with varying opioid receptor selectivities. The ligands used for this purpose were naloxone (most potent at μ-receptors, Paterson *et al.*, 1983), Mr 2266 (mainly μ and κ, Paterson *et al.*, 1983; Smith *et al.*, 1984) and M 8008 (μ and δ, Smith, 1987). The effects of Mr Z 2593, a quarternary complex of naloxone which does not readily enter the central nervous system (Tavani *et al.*, 1979; Bianchi *et al.*, 1982), were also investigated in an attempt to assess the importance of peripherally located opioid receptors.

Table 1 The effects of naloxone on the severity of the ventricular arrhythmias that occurred over the 0–30 min post-occlusion period

Group	n	*Ventricular ectopic count*	*Duration of VT* (s)	*Duration of VF* (s)	% *VT*	% *VF*	% *mortality*
Controls	11	583 ± 238	27.2 ± 12	5.4 ± 3.7	82	36	0
Naloxone 50 $\mu g kg^{-1}$ + 0.25 $\mu g kg^{-1} min^{-1}$	9	582 ± 163	29.6 ± 7.7	9.4 ± 9.1	89	44	11
Controls	7	1087 ± 305	64.9 ± 26	8.9 ± 4.7	100	57	0
Naloxone 0.5 $mg kg^{-1}$ + 0.25 $\mu g kg^{-1} min^{-1}$	11	*213 ± 70	*4.5 ± 1.4	5.8 ± 3.2	64	27	0
Controls	10	1307 ± 346	60.4 ± 23	21.8 ± 10.9	100	90	20
Naloxone 2.0 $mg kg^{-1}$ + 1 $\mu g kg^{-1} min^{-1}$	10	*463 ± 144	15.7 ± 5.6	7.7 ± 6.1	60	*30	0
Controls	15	1305 ± 320	70.6 ± 21.9	49.1 ± 20	100	67	13
Naloxone 5.0 $mg kg^{-1}$ + 2.5 $\mu g kg^{-1} min^{-1}$	13	591 ± 152	26.8 ± 9.2	6.0 ± 3.1	92	42	0
Controls	9	1258 ± 428	70.6 ± 21.9	0	100	0	0
MrZ 2593 1 $mg kg^{-1}$	10	*270 ± 69	*10.8 ± 4.6	0	100	0	0

The mean values ± s.e.mean for the ventricular ectopic count, the incidence and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF) and mortality are also shown. Values for the ventricular ectopic count and the duration of VT and VF are taken only from rats that survived beyond 30 min. n = number of animals.
* $P < 0.05$.

If opioid receptor antagonists are antiarrhythmic as a result of their antagonistic effects on endogenously released opioid peptides, it would seem appropriate to investigate the possibility that opioid agonists may be arrhythmogenic under similar conditions of ischaemia. This was examined using diamorphine (μ-agonist, Smith, 1984) U-50,488H (κ-agonist, Von Voightlander *et al.*, 1983) and [Leu] enkephalin which has δ-agonist properties (Paterson *et al.*, 1983). Preliminary accounts of some of these findings has been given to a meeting of the British Pharmacology Society (Mackenzie *et al.*, 1986a,b).

Methods

The method used was basically that described by Clark *et al.* (1980). Male Sprague-Dawley rats weighing between 250 and 350 g were anaesthetised with pentobarbitone sodium (6 mg 100 g^{-1} i.p.) and artificially ventilated with room air (54 strokes min^{-1}; stroke volume 2 ml 100 g^{-1}). Catheters were placed in a carotid artery (for pressure measurement) and in a femoral vein (for drug injection) and the electrocardiogram was recorded from standard limb leads. Rectal temperature was maintained at approximately 38°C. The chest was opened between the fourth and fifth ribs, approximately 2 mm to the left of the sternum. After opening the pericardium the heart was exteriorized and a 6/0 silk suture was placed under the left coronary artery. The heart was repositioned in the thoracic cavity and a 15 min stabilisation period was allowed. Drugs or vehicle were administered 15 min before coronary artery occlusion. The electrocardiogram and blood pressure were recorded for 30 min after occlusion of the coronary artery. The severity of the arrhythmias was assessed by counting the total number of ventricular extrasystoles occurring in the 30 min post-occlusion period and by measuring the duration and incidence of ventricular fibrillation (VF), ventricular tachycardia (VT) and mortality.

Statistics

Statistical analysis of differences in the incidence of arrhythmias and in mortality was carried out by use of the Fisher Exact Probability Test. For analysis of the difference between means Student's t test was used when one control group was compared with one test group, when more than one test group was compared with a single control group, analysis of variance plus Bartlet's test, followed by Dunnett's test was used. For non-parametric data, the Kruskal-Wallis test followed by the Mann-Witney-U test was used.

Drugs

The following drugs were used: naloxone hydrochloride (Salas, Italy), Mr 2266 (5,9-diethyl-2-(3-oxazolylmethyl)benzomorphan), (Dr H. Merz, Boehringer Ingelheim), M 8008 (N-cyclopropylmethyl-16(**R**)-methylmethylorvinol), (Reckitt & Colman, Hull), MrZ 2593 (1-N-allyl-N-methyl-7,8-dihydro-14-hydroxynormorphinone bromide), (Dr H Merz, Boehringer, Ingelheim) diamorphine hydro-

chloride (Reckitt & Colman, Hull), [Leu]enkephalin (Cambridge Research Biochemicals), U-50,488H (N-methyl - N' - (pyrrolydinylcyclohexyl) - 3,4 - dichloro - benzylamide), (Upjohn).

Results

The effects of opioid receptor antagonists on the consequences of coronary artery occlusion

Table 1 shows the effects of naloxone on the severity of ventricular arrhythmias, and on mortality, resulting from coronary artery occlusion. The lowest dose of naloxone used ($50\,\mu g\,kg^{-1}$ given 15 min before ligation together with an infusion of $0.25\,\mu g\,kg^{-1}\,min^{-1}$ commencing at this time and continuing throughout the occlusion period) had no significant effect on ventricular arrhythmias. Doses of $2\,mg\,kg^{-1}$ then $1\,\mu g\,kg^{-1}\,min^{-1}$ and $0.5\,mg\,kg^{-1}$ then $0.25\,\mu g\,kg^{-1}\,min^{-1}$ naloxone reduced the number of ventricular extrasystoles from 1307 ± 346 to 463 ± 144 ($P < 0.05$) and from 1078 ± 305 to 213 ± 70 ($P < 0.05$) respectively. Naloxone ($0.5\,mg\,kg^{-1}$ then $0.25\,\mu g\,kg^{-1}\,min^{-1}$) also reduced the duration of VT from 65 ± 26 to 4.5 ± 1.4 s ($P < 0.05$) and, in a higher dose ($2\,mg\,kg^{-1}$ then $1\,\mu g\,kg^{-1}\,min^{-1}$) reduced the incidence of VF from 90% to 30%. The quarternary derivative of naloxone, Mr Z 2593, at a dose of $1\,mg\,kg^{-1}$, was also antiarrhythmic (Table 1). It reduced significantly the ventricular ectopic count from 1258 ± 428 to 270 ± 69 ($P < 0.05$) and the duration of VT from 117.9 ± 42.8 to 10.8 ± 4.6 s. Neither naloxone nor Mr Z 2593 significantly modified heart rate or mean arterial blood pressure (MABP) before or after coronary artery occlusion. For example, in control animals blood pressure fell 1 min after occlusion from 106 ± 6 mmHg to 79 ± 4 mmHg, with recovery to 103 ± 5 mmHg at 15 min whereas in those rats pretreated with naloxone ($2\,mg\,kg^{-1}$ then $1\,\mu g\,kg^{-1}\,min^{-1}$) blood pressure fell on occlusion from 89 ± 5 mmHg to 68 ± 4 mmHg at 1 min with recovery to 82 ± 7 mmHg at 15 min.

Like naloxone, Mr 2266 also caused a dose-dependent reduction in the number of ventricular extrasystoles and in the duration of VT (Figure 1). For example, Mr 2266 at a dose of $4\,mg\,kg^{-1}$ reduced the number of ventricular ectopic beats from 1111 ± 238 to 246 ± 74 ($P < 0.01$) and the duration of ventricular tachycardia from 69 ± 23 to 7 ± 3.5 s. Mr 2266 had little effect on arterial pressure but did transiently reduce heart rate (464 ± 23 beats min^{-1} to 437 ± 20 beats min^{-1} with a dose of $2\,mg\,kg^{-1}$ and 435 ± 20 beats min^{-1} to 331 ± 12 beats min^{-1} with a $4\,mg\,kg^{-1}$ dose).

M8008 ($1\,mg\,kg^{-1}$) did not modify the total number of ventricular extrasystoles (754 ± 206 ($n = 11$) in the controls; 608 ± 129 ($n = 10$) in the treated group), the duration and incidence of either VT (59 ± 19 vs 46 ± 12 s (NS)) or VF (8.4 ± 8.0 vs 0 s (NS)), blood pressure (79 ± 5 mmHg before administration and 85 ± 4 mmHg after) and heart rate (from 376 ± 17 beats min^{-1} to 370 ± 16 beats min^{-1}).

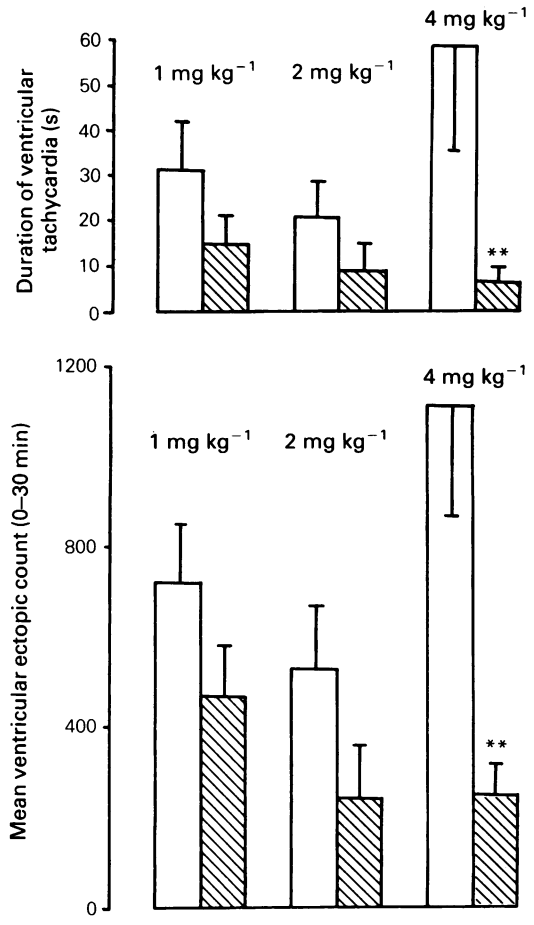

Figure 1 The mean ventricular ectopic count (b) and duration of ventricular tachycardia (a) in control and Mr 2266-treated rats. The mean values are shown and vertical lines indicate s.e.mean. $^{**}P < 0.01$. Open columns, controls; hatched columns, Mr 2266-treated rats.

The effects of opioid receptor agonists on the consequences of coronary artery occlusion

In the doses used, diamorphine, U-50,488H and [Leu] enkephalin had no significant effect on the early ventricular arrhythmias resulting from coronary artery occlusion (Table 2).

Table 2 The effects of diamorphine, [Leu] enkephalin and U-50,488H on the severity of the ventricular arrhythmias that occurred over the 0–30 min post-ligation period

Group	n	*Ventricular ectopic count*	*Duration of VT* (s)	*Duration of VF* (s)	*% VT*	*% VF*	*% mortality*
Controls	10	1088 ± 328	65.6 ± 26.0	4.7 ± 2.7	100	40	0
Diamorphine (0.05 mg kg^{-1})	10	983 ± 474	54.5 ± 27.0	1.7 ± 1.3	100	50	20
Diamorphine (0.20 mg kg^{-1})	10	814 ± 276	50.1 ± 21.8	7.0 ± 5.7	90	20	0
Diamorphine (0.50 mg kg^{-1})	10	622 ± 163	32.2 ± 11.6	8.1 ± 8.1	100	30	20
Controls	10	1325 ± 347	86.9 ± 27.0	22.6 ± 12.9	100	50	10
[Leu] enkephalin							
0.1 μg kg^{-1} min^{-1}	10	644 ± 109	33.8 ± 9.4	15.8 ± 8.1	100	50	0
0.5 μg kg^{-1} min^{-1}	9	760 ± 234	36.9 ± 14.9	18.9 ± 14.5	100	56	11
2 μg kg^{-1} min^{-1}	9	1430 ± 349	83.2 ± 25.3	37.6 ± 16.9	100	67	0
10 μg kg^{-1} min^{-1}	8	1187 ± 371	63.9 ± 24.1	29.3 ± 11.2	100	75	0
Controls	10	1284 ± 385	88.9 ± 34.1	20.9 ± 8.3	100	70	10
U-50,488H (1 mg kg^{-1})	10	924 ± 342	54.4 ± 23.9	7.6 ± 3.9	100	30	0
Infusions							
Controls	10	994 ± 331	57.0 ± 22.4	16.6 ± 8.3	80	50	0
U-50,488H (0.1 mg kg^{-1})	10	881 ± 201	53.4 ± 15.4	12.4 ± 6.9	100	50	0
U-50,488H (0.3 mg kg^{-1})	9	779 ± 281	40.0 ± 17.6	5.8 ± 4.2	100	22	0

Mean values ± s.e.mean are shown. n = number of animals.

Table 3 summarises the effects of diamorphine and U-50,488H on blood pressure and heart rate. [Leu] enkephalin was without significant effects on these parameters in doses up to 2 μg kg^{-1} min^{-1}; at the highest dose used (10 μg kg^{-1} min^{-1}) there was a transient, slight increase in arterial pressure (from 100 ± 4 to 109 ± 8 mmHg; $P < 0.05$). The administration of diamorphine resulted in gradual and sustained increases in both blood pressure and heart rate such that just before coronary artery occlusion these were significantly higher than those in the controls. Diamorphine also attenuated the decrease in pressure which normally resulted from occlusion (Table 3). U-50,488H (1 mg kg^{-1}) given as a bolus dose 15 min before coronary artery occlusion markedly decreased arterial blood pressure and heart rate. Smaller doses given by infusion also tended to decrease rate.

Discussion

In a previous study by Parratt & Sitsapesan (1986), WIN,44,441-3 (an antagonist with a similar profile to naloxone, Ward *et al.*, 1983) and Mr 1452 (a relatively non-selective opioid antagonist, Smith *et al.*, 1984) were shown to be antiarrhythmic in anaesthetised rats. In the present study, antagonists which act preferentially at μ- or κ-receptors (i.e. naloxone, Mr 2266) were antiarrhythmic whereas M 8008 which is twice as potent at δ-receptors than at μ-receptors and which has little affinity for κ-receptors (Smith, 1987) was without effect. These results suggest that δ-receptors are unlikely to be involved in the antiarrhythmic actions of opioid antagonists. At least ten times more naloxone is required to antagonise κ- or δ-receptors than is required to antagonise μ-receptors (Lord *et al.*, 1977; Robson *et al.*, 1983). Work by other investigators (e.g. Leander, 1983) would suggest that, at the doses of naloxone which were antiarrhythmic in this model, naloxone was antagonising δ- and κ-receptors in addition to μ-receptors.

When a lower dose (50 μg kg^{-1} + 0.25 μg kg^{-1} min^{-1}), calculated to antagonise μ-receptors only, was used, naloxone was not antiarrhythmic. On the basis of these results it is suggested that κ-receptors may be important in mediating the antiarrhythmic actions of opioid receptor antagonists. A peripheral site of action of these antagonists is suggested, but certainly not proved, by the observation that Mr Z 2593, a quarternary naloxone derivative that does not easily cross the blood brain barrier, was also antiarrhythmic. Furthermore, Zhan *et al.* (1985) have demonstrated an antiarrhythmic action of naloxone in the rat isolated heart.

The characterisation of the opioid receptor subtype(s) mediating the antiarrhythmic effect of these antagonists has important clinical implications. An opioid analgesic acting via stimulation of μ-receptors is usually administered during acute myocardial infarction. Thus, on the basis of these experimental results it may be of more benefit to administer a drug possessing both μ-receptor agonist

Table 3 The effect of diamorphine and U-50,488H on heart rate and mean arterial blood pressure before and at various times after coronary artery ligation in anaesthetised rats

Heart rate (beats min^{-1})

		Time post-ligation (min)				
Group	n	−15	−10	0	1	15
Controls	10	398 ± 24	375 ± 22	395 ± 13	414 ± 22	414 ± 15
Diamorphine (0.05 mg kg^{-1})	10	375 ± 19	430 ± 16	450 ± 9*	428 ± 15	441 ± 9
Diamorphine (0.2 mg kg^{-1})	10	400 ± 12	447 ± 12*	468 ± 6*	463 ± 7	455 ± 10
Diamorphine (0.5 mg kg^{-1})	10	402 ± 16	413 ± 15	436 ± 11	442 ± 13	420 ± 16
Controls	10	461 ± 15	446 ± 17	461 ± 12	462 ± 12	460 ± 12
U-50,488H (0.1 mg kg^{-1} min^{-1})	10	450 ± 15	451 ± 14	437 ± 14	435 ± 16*	443 ± 12
U-50,488H (0.3 mg kg^{-1} min^{-1})	9	436 ± 12	440 ± 13	426 ± 11*	414 ± 15*	406 ± 13**
Controls	10	361 ± 7	377 ± 18	374 ± 14	366 ± 11	408 ± 17
U-50,488H (1 mg kg^{-1})	10	348 ± 14	308 ± 18**	314 ± 13**	301 ± 11*	323 ± 12**

Mean arterial blood pressure (mmHg)

		Time post-ligation (min)				
Group	n	−15	−10	0	1	15
Controls	10	94 ± 6	98 ± 6	101 ± 4	81 ± 7	110 ± 8
Diamorphine (0.05 mg kg^{-1})	10	88 ± 6	129 ± 8*	133 ± 4*	97 ± 4	117 ± 6
Diamorphine (0.2 mg kg^{-1})	10	74 ± 3	137 ± 4*	143 ± 4*	131 ± 5	131 ± 5
Diamorphine (0.5 mg kg^{-1})	10	86 ± 3	116 ± 7	120 ± 7	116 ± 8*	118 ± 10
Controls	10	90 ± 5	96 ± 6	113 ± 7	86 ± 4	88 ± 2
U-50,488H (0.1 mg kg^{-1} min^{-1})	10	90 ± 3	107 ± 4	104 ± 5	80 ± 3	91 ± 7
U-50,488H (0.3 mg kg^{-1} min^{-1})	9	100 ± 4	114 ± 6	110 ± 5	77 ± 3	91 ± 6
Controls	10	77 ± 1	95 ± 6	83 ± 6	69 ± 3	83 ± 8
U-50,488H (1 mg kg^{-1})	10	77 ± 2	64 ± 4**	70 ± 3*	47 ± 2**	53 ± 4**

The mean values ± s.e.mean are shown, n = number of animals. *$P < 0.05$; **$P < 0.01$.

and κ-receptor antagonist properties. Indeed, it has been shown that analgesics such as meptazinol and buprenorphine, which are partial agonists at μ-receptors also possess antiarrhythmic actions (Fagbemi *et al.*, 1983; Sitsapesan *et al.*, 1987).

The agonists used in this study, U-50,488H (κ-agonist, Von Voightlander *et al.*, 1983), [Leu] enkephalin (most potent at δ-receptors, Paterson *et al.*, 1983) and diamorphine (μ-agonist, Smith, 1984) were without effect on the arrhythmias resulting from coronary artery occlusion. One possible reason for this lack of effect may be that the opioid receptors concerned are maximally stimulated by endogenous opioids released during the period of ischaemia. It is of interest to note that in rat isolated perfused hearts, in which opioid release could be small, β-endorphin has been shown to cause arrhythmias (Lee *et al.*, 1984). Another possibility is that the reduction in heart rate observed with the κ-agonist, U-50,488H, or the ability of diamorphine to attenuate the fall in arterial blood pressure that occurs upon occlusion may have obscured any direct arrhythmogenic effect. It could also be argued that the antiarrhythmic action of these opioid receptor antagonists is not mediated via opioid receptors but is due to a direct electrophysiological effect. Recent work by Brasch (1986) has demonstrated that both (+)- and (−)-isomers of naloxone may prolong the action potential duration of guinea-pig papillary muscle, an effect which is considered to be antiarrhythmic. On the other hand, this explanation does not take account of the observed stereospecificity of the antiarrhythmic action of the opioid receptor antagonists (Parratt & Sitsapesan, 1986). Further work is obviously required in this area to assess the relative importance of opioid receptor mediated and direct membranal effects of these drugs underlying their antiarrhythmic properties.

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The antiarrhythmic and cardiac electrophysiological effects of buprenorphine

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1 The effects of buprenorphine, given intravenously, on the incidence and severity of early acute coronary artery occlusion-induced arrhythmias were examined in anaesthetised rats. The electrophysiological effects of buprenorphine were also examined in sheep Purkinje fibres and rat papillary muscles, superfused *in vitro* with either a normal or a hypoxic, hyperkalaemic and acidotic physiological salt solution (PSS).

2 In anaesthetised rats subjected to acute coronary artery occlusion, pretreatment with buprenorphine (1 mg kg^{-1} i.v.) markedly reduced the incidence of ventricular extra-systoles during the initial 30 min post-occlusion period. The incidence of ventricular fibrillation (VF) was also significantly reduced from 56% to 10%.

3 At the antiarrhythmic dose (1 mg kg^{-1}), buprenorphine also attenuated the sudden fall in systemic arterial blood pressure induced by acute coronary artery ligation.

4 In normal sheep Purkinje fibres and rat papillary muscles, buprenorphine (10^{-6}–10^{-5} M) significantly reduced the action potential height and maximum rate of depolarisation of phase zero (MRD) and prolonged the duration of the action potential.

5 Superfusion of sheep Purkinje fibres and rat papillary muscles with a hypoxic, hyperkalaemic and acidotic PSS resulted in marked reductions in resting membrane potential, upstroke and duration of the action potential.

6 In the presence of the modified compared with normal PSS, buprenorphine reduced the action potential height and MRD of both sheep Purkinje fibres and rat papillary muscles to a greater extent, although its ability to prolong the action potential duration was attenuated.

7 The antiarrhythmic effects of buprenorphine observed *in vivo* may be explained by its direct cardiac electrophysiological effects. Buprenorphine might be useful in relieving pain and in reducing the severity of arrhythmias in the early stages of acute myocardial infarction.

Introduction

Since acute myocardial infarction is usually associated with severe pain it is common practice clinically to administer a narcotic analgesic. However, the opioid antagonist, naloxone, has been shown to reduce the incidence and severity of arrhythmias resulting from acute coronary artery occlusion in experimental animals (Fagbemi *et al.*, 1982; Huang *et al.*, 1986). This protective effect has also been observed with a number of other opioid antagonists. Further, this effect is stereospecific (Parratt & Sitsapesan, 1986), which suggests that the antiarrhythmic action is mediated through antagonism of the effects of endogenous opioid peptides at specific receptors. Of the three generally accepted opioid receptor subtypes (μ, κ and δ), it appears that only antagonism at the κ-receptor is associated with antiarrhythmic activity (Mackenzie *et al.*, 1986; Sitsapesan & Parratt, 1989). It has thus been inferred that it may be more beneficial to administer an opioid with μ-agonist activity (to provide analgesia) and κ-antagonist activity (antiarrhythmic) in the early stages of acute myocardial infarction (Sitsapesan & Parratt, 1989).

The present studies were designed to evaluate the effect of buprenorphine, a compound with partial μ-agonist/κ-antagonist activity (Richards & Sadee, 1985; Takemori *et al.*, 1986) on the incidence and severity of early arrhythmias associated with acute coronary artery occlusion in anaesthetised rats. In order to elucidate further the possible mechanisms involved, the cardiac electrophysiological effects of buprenorphine were also studied *in vitro* in normal

sheep Purkinje fibres and rat ventricular muscles and in similar preparations exposed to conditions that mimic mild myocardial ischaemia (Boachie-Ansah *et al.*, 1989).

Methods

Anaesthetised rats

Male Sprague-Dawley rats (250–400 g body weight) were anaesthetised with pentobarbitone sodium (60 mg kg^{-1}, i.p.). The trachea and femoral vein were cannulated to allow artificial respiration and drug administration, respectively. Systemic arterial blood pressure was monitored via a catheter placed in the carotid artery, and recorded, together with a standard lead 1 ECG, by use of a Mingograf 82 ink-jet recorder (Elema Schonander). Rectal temperature was maintained at approximately 38°C.

The chest was opened between the fourth and fifth ribs, approximately 2 mm to the left of the sternum. Artificial ventilation with room air (stroke volume 1.5 ml 100 g^{-1}; rate 54 beats min^{-1}) was commenced immediately to maintain normal arterial Po_2, Pco_2, and pH parameters. After the pericardium had been incised, the heart was exteriorized and a 6/0 braided silk suture was placed under the left main coronary artery. The heart was repositioned in the thoracic cavity and the animal left to recover for 15 min.

Buprenorphine (0.01, 0.1 and 1.0 mg kg^{-1}) or saline was administered intravenously and after another 15 min the ligature was tied. The severity of the resulting arrhythmias was assessed by counting the total number of ventricular extrasystoles occurring during the 0–30 min postligation period and by computing the mortality rate as well as the incidence and duration of ventricular fibrillation (VF) and ventricular tachycardia (VT, defined as any run of six or more consecutive ventricular extrasystoles), as described by Clark *et al.* (1980).

Electrophysiological studies

Experiments were performed on both sheep cardiac Purkinje fibres and rat papillary muscles superfused *in vitro* with either a normal physiological salt solution (PSS) or a modified PSS (i.e., a hypoxic, hyperkalaemic, and acidotic solution).

Sheep Purkinje fibres Sheep hearts were collected from a local abattoir and delivered to the laboratory in cooled normal PSS within 30 min of excision. Free-running Purkinje fibres were dissected and pinned to the silastic base of a recording chamber and superfused at a rate of 5 ml min^{-1} with normal PSS equilibrated with 95% O_2/5% CO_2 at a temperature of 36.5 ± 0.5°C. The composition of the normal PSS was as follows (mM): NaCl 125, $NaHCO_3$ 25, NaH_2PO_4 1.2, $MgCl_2$ 1.0, KCl 5.4, $CaCl_2$ 1.8, glucose 5.5. That of the modified PSS was as follows (mM): NaCl 141.5, $NaHCO_3$ 8.5, NaH_2PO_4 1.2, $MgCl_2$ 1.0, KCl 8.0, $CaCl_2$ 1.8, glucose 5.5. The modified solution was equilibrated with 95% N_2/5% CO_2 to yield a Po_2 and a pH in the organ bath of 34.1 ± 0.6 mmHg and 6.8 ± 0.01 units, respectively, compared with values in normal PSS of 419 ± 31 mmHg and 7.31 ± 0.02 units.

Rat papillary muscles Male Sprague-Dawley rats (250–350 g body weight) were stunned and their hearts quickly removed and placed in cold normal PSS. Left papillary muscles were excised and mounted in a recording chamber as above and superfused at a rate of 10 ml min^{-1} with normal PSS equilibrated with 95% O_2/5% CO_2 at a temperature of 34.5 ± 0.5°C. The compositions of the normal and modified PSS used were the same as above except for (mM): $CaCl_2$ 2.5, glucose 11.0. The modified PSS was gassed with 95% N_2/5% CO_2 to yield a bath Po_2 and pH in the ranges described above for sheep Purkinje fibres.

Rectangular pulses 1 ms in duration and twice threshold voltage, delivered through a bipolar silver electrode, were used to stimulate both preparations at a rate of 1.5 Hz. Transmembrane action potentials were recorded using conventional microelectrode techniques. The variables measured were resting membrane potential (RMP); action potential amplitude (APA); the maximum rate of depolarization of phase zero (MRD), which was determined by an electronic differentiating circuit; the action potential duration at 50 and 90% repolarisation levels (APD_{50} and APD_{90}). In all cases, the preparations were allowed to equilibrate in normal PSS for 1–2 h before exposure to the modified PSS or drug.

Experimental protocol

To observe drug effects on normal preparations, 6–10 action potentials were recorded before and 30–40 min after cumulative addition of buprenorphine, which was dissolved in reservoirs of gassed normal PSS to obtain final bath concentrations detailed below. In a different set of preparations, the effects of the modified PSS alone were studied by recording multiple action potentials before and at 30, 60, 90, and 120 min following exposure to the modified PSS. To observe drug-induced effects in the presence of the modified PSS, other preparations were used; action potentials were recorded before and 30 min

Table 1 Effect of buprenorphine on the incidence and severity of early arrhythmias resulting from acute coronary artery occlusion in anaesthetized rats

			Ventricular tachycardia (VT)		*Ventricular fibrillation (VF)*		
Treatment	n	*Ventricular ectopic beats*	*Duration* (s)	*Incidence* (%)	*Duration* (s)	*Incidence* (%)	*Mortality* (%)
Control	27	1518 ± 190	102.1 ± 17.8	100	22.8 ± 18.2	56	22
Buprenorphine							
0.01 mg kg^{-1}	10	1617 ± 398	96.1 ± 24.7	100	2.2	30	20
0.1 mg kg^{-1}	19	1049 ± 189	74.6 ± 18.5	100	37.6 ± 15.9	21	0
1.0 mg kg^{-1}	20	692 ± 128**	43.9 ± 11	85	34.2 ± 33.2	10*	5

Values are expressed as means ± s.e.mean.
* $P < 0.025$, ** $P < 0.005$, significantly different from value in control group. Ventricular ectopic beats and the durations of VT and VF were counted in survivors only.

after superfusion with the modified solution alone, and subsequently after the administration of two cumulative concentrations of buprenorphine dissolved in modified PSS.

Analysis of data

Data from the electrophysiological studies are expressed either as mean values ± s.e.mean or mean percentage change from control values ± s.e.mean. In both the anaesthetised rat and electrophysiological studies, multiple treatment and control means were analysed by use of a one-way analysis of variance and, where the F-value permitted further analysis, individual treatment means were compared with respective control values by a Modified *t* test. A Chi-squared test was used to compare the % incidences of ventricular tachycardia (VT) and fibrillation (VF), and mortality in control and treatment groups. In all other comparisons an unpaired or paired Student's *t* test (two-tailed) was employed as appropriate. $P < 0.05$ was considered to be statistically significant.

Results

Anaesthetised rats

In control rats, acute occlusion of the left main coronary artery resulted in marked ectopic activity in the initial 30 min post-ligation period. As shown in Table 1, 56% of control animals had ventricular fibrillation (VF); only 22% died as a result of VF because of the ability of the rat heart to revert spontaneously to normal sinus rhythm. Intravenous administration of buprenorphine, 1 mg kg^{-1}, resulted in significant reductions in both the total number of extrasystoles and the percentage incidence of VF,

Table 2 Effects of buprenorphine on mean arterial blood pressure in anaesthetized rats subjected to acute coronary artery occlusion

				Post-ligation (min)			
Treatment	n	*Pre-drug*	*Pre-ligation*	1	5	15	30
Control	21	86 ± 3	94 ± 3†	68 ± 3	77 ± 3	87 ± 5	82 ± 4
Buprenorphine							
0.01 mg kg^{-1}	8	83 ± 2	104 ± 6†	66 ± 6	78 ± 10	78 ± 9	83 ± 9
0.1 mg kg^{-1}	19	86 ± 3	101 ± 4†	89 ± 5*	89 ± 6	84 ± 4	78 ± 5
1.0 mg kg^{-1}	19	88 ± 4	107 ± 6†	97 ± 4*	92 ± 6*	92 ± 5	87 ± 5

Values are expressed as means ± s.e.mean.
† $P < 0.05$ significantly different from pre-drug value.
* $P < 0.05$ significantly different from value in control group.

and tended also to reduce the duration of ventricular tachycardia (VT) (Table 1). The two lower doses of buprenorphine, 0.01 and 0.1 mg kg^{-1}, did not cause statistically significant differences in any of the arrhythmic indices, although the latter dose did tend to reduce the total ectopic count and the incidence of VF.

The effects of buprenorphine and of coronary artery occlusion on mean arterial blood pressure are summarised in Table 2. In the doses used, buprenorphine only tended to elevate systemic arterial blood pressure during the pre-ligation period. In control rats, acute coronary artery occlusion caused an immediate fall in blood pressure followed by a slow recovery towards pre-ligation levels. Buprenorphine, 0.1 and 1.0 mg kg^{-1}, attenuated this immediate fall in blood pressure (reductions of 12% and 9%, respectively, compared with 28% in controls). Heart rate was not significantly changed from the control value of 411 ± 16 beats min^{-1} by coronary artery occlusion or by buprenorphine in the concentrations used.

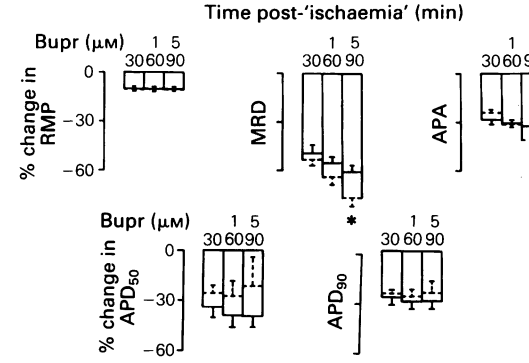

Figure 1 Percentage changes (from control values) in sheep Purkinje action potential characteristics induced by modified PSS alone (unbroken line) and modified PSS plus buprenorphine (Bupr), 10^{-6} and 5×10^{-6} M (broken line). *Significantly different from value in modified PSS alone. $n = 5$–8. RMP = resting membrane potential, MRD = maximum rate of depolarisation of phase zero, APA = action potential amplitude, APD_{50} and APD_{90} = action potential duration at 50% and 90% repolarisation, respectively.

Electrophysiological studies

Sheep Purkinje fibres The effects of buprenorphine (10^{-8}–10^{-5} M) on normal sheep Purkinje action potentials are summarised in Table 3. Buprenorphine (10^{-6}–10^{-5} M) caused significant concentration-dependent reductions in the maximum rate of depolarisation of phase zero (MRD) without modifying the resting membrane potential. At the highest concentration used (10^{-5} M), buprenorphine also reduced the amplitude of the action potential (APA) and prolonged the action potential duration at 90% repolarisation (APD_{90}).

Figure 1 shows the effects of superfusing sheep Purkinje fibres either with the modified PSS alone or that containing buprenorphine (10^{-6} and 5×10^{-6} M). Within 30 min of exposure, the hypoxic, hyperkalaemic and acidotic PSS had reduced significantly all of the measured variables. There were no further significant reductions in these variables during the 30–120 min exposure period to the modified PSS alone. When given after the first 30 min of exposure to the modified PSS, buprenorphine (10^{-6} and 5×10^{-6} M) reduced both the MRD and APA of the depressed action potentials without affecting the resting membrane potential or action potential duration (Figure 1). Thus, as illustrated in Figure 2, under ischaemic compared to normal conditions, buprenorphine had a greater depressant effect on the

Table 3 The % changes induced by buprenorphine (10^{-8} to 10^{-5} M) in action potential characteristics of normal sheep Purkinje fibres

		% *change after buprenorphine* (M)				
	Control	10^{-8}	10^{-7}	10^{-6}	5×10^{-6}	10^{-5}
RMP (mV)	−83.3 ± 1.6	−0.2 ± 0.6	−0.4 ± 0.9	−0.2 ± 0.5	−2.4 ± 0.7	−3.2 ± 0.7
MRD (V s^{-1})	487 ± 16	−1.0 ± 1.6	−4.3 ± 2.7	−10.6 ± 4.1*	−20.8 ± 3.2*	−60.2 ± 7.8*
APA (mV)	114.4 ± 2.2	−0.3 ± 0.8	−0.6 ± 1.1	−1.9 ± 0.5	−6.2 ± 1.5	−25.0 ± 2.9*
APD_{50} (ms)	140.4 ± 13.6	−1.3 ± 2.4	−2.9 ± 2.2	+1.0 ± 3.1	−0.1 ± 2.6	+6.6 ± 6.7
APD_{90} (ms)	231.4 ± 14.2	+1.0 ± 2.2	+1.4 ± 2.2	+6.2 ± 3.2	+10.9 ± 4.0	+25.0 ± 6.8*

Values shown are mean ± s.e.mean, $n = 6$.
* $P < 0.05$ significantly different from zero. Abbreviations are: RMP = resting membrane potential; MRD = maximum rate of depolarisation of phase zero; APA = action potential amplitude; APD_{50} and APD_{90} = action potential duration at 50% and 90% repolarisation, respectively.

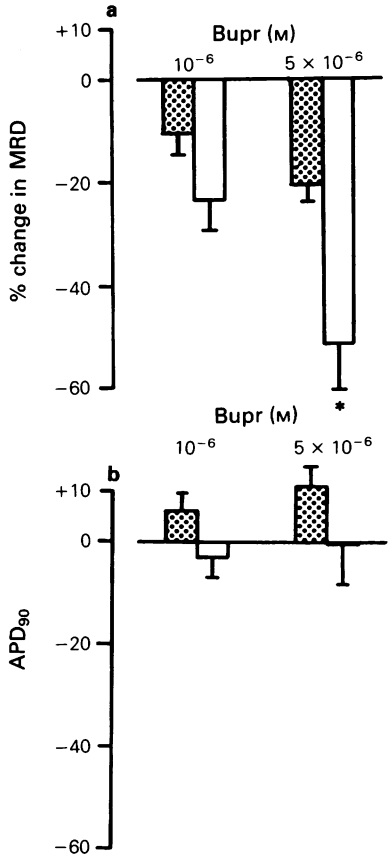

Figure 2 The percentage reduction in (a) maximum rate of depolarisation (MRD) and in (b) action potential duration (APD_{90}) of sheep Purkinje fibres caused by buprenorphine (Bupr), 10^{-6} and 5×10^{-6} M, dissolved either in normal (stippled columns) or modified (open columns PSS). $n = 5$–6. *$P < 0.05$, significantly different from effect in normal PSS.

upstroke of the action potential but failed to prolong APD_{90}.

Rat ventricular muscle On normal rat papillary muscle, the most marked effect of buprenorphine (10^{-6}–10^{-5} M) was a concentration-dependent prolongation of APD_{90}. Increases in APD_{90} of 8 ± 3%, 11 ± 2% ($P < 0.05$) and 22 ± 3% ($P < 0.05$) above the control value of 94.8 ± 4.1 ms were observed following superfusion with 10^{-6}, 5×10^{-6} and 10^{-5} M buprenorphine, respectively. In these concentrations, buprenorphine also reduced significantly the action potential amplitude (from 100.7 ± 1.2 to 94.6 ± 0.9 mV) and tended to reduce MRD (from 117.5 ± 5 to 109 ± 2 $V s^{-1}$).

Exposure of rat papillary muscle to the modified PSS alone had similar effects to those observed in the sheep Purkinje fibres. However, as can be seen in Table 4, although the changes in RMP, MRD and APA were maximal by 30 min, those in the APD were not. Both APD_{50} and APD_{90} of the rat ventricular muscle continued to decline throughout the 2 h of exposure to the 'ischaemic' solution. Buprenorphine, 10^{-5} M, prevented this decline in APD_{50} and APD_{90}, the respective values in the presence of the drug being 21.8 ± 1.3 and 45.6 ± 2.4 ms. Under ischaemic conditions, buprenorphine (5×10^{-6} and 10^{-5} M) also decreased the MRD and APA without modifying the resting membrane potential. As was observed in Purkinje tissue, this effect of buprenorphine was more marked in the presence of the modified PSS (Figure 3).

Discussion

The results of these studies demonstrate that pretreatment of anaesthetized rats with buprenorphine reduces the incidence and severity of the early

Table 4 The effects of the 'ischaemic' (i.e. hypoxic, hyperkalaemic, and acidotic) physiological salt solution on action potential characteristics of rat ventricular muscle paced at 1.5 Hz

		Time 'post-ischaemia' (min)			
	Control	30	60	90	120
RMP (mV)	−78.9 ± 0.8	−67.6 ± 0.6*	−69.5 ± 1.2*	−68.2 ± 0.8*	−68.8 ± 0.7*
MRD ($V s^{-1}$)	165 ± 8	99 ± 10*	101 ± 7*	98 ± 9*	96 ± 10*
APA (mV)	105.1 ± 1.8	88.6 ± 1.6*	88.2 ± 2.3*	85.7 ± 2.4*	85.0 ± 2.6*
APD_{50} (ms)	21.1 ± 1.1	21.8 ± 0.8	19.0 ± 1.7	17.9 ± 1.6	16.6 ± 1.5
APD_{90} (ms)	70.3 ± 3.5	49.9 ± 3.4*	41.3 ± 3.4*,†	38.6 ± 3.6*,†	35.6 ± 3.1*,†

Values shown are mean ± s.e.mean, $n = 6$.
* $P < 0.05$ significantly different from control value.
† $P < 0.05$ significantly different value at 30 min post-ischaemia. For abbreviations used see Table 3.

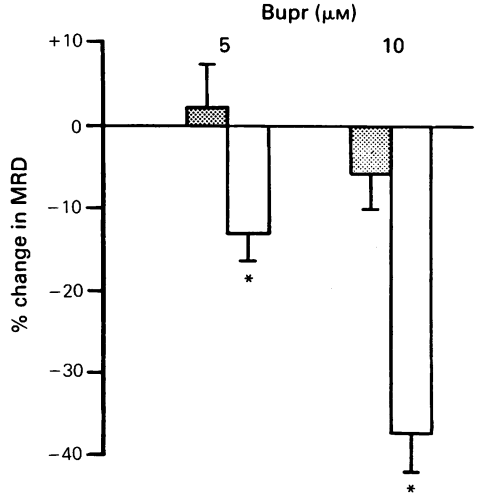

Figure 3 The percentage reduction in the maximum rate of depolarisation (MRD) of rat papillary muscle caused by buprenorphine (Bupr), 5×10^{-6} and 10^{-5} M, dissolved in normal (stippled columns) or modified (open columns) PSS. $n = 5$–6. $^*P < 0.05$, significantly different from effect in normal PSS.

arrhythmias resulting from acute myocardial ischaemia. Further, buprenorphine, in micromolar concentrations, exerts direct effects on the electrophysiological characteristics of normal cardiac tissues and of cardiac tissues exposed *in vitro* to conditions that mimic mild myocardial ischaemia.

The protective effects of buprenorphine against these early ischaemia-induced arrhythmias are similar to those observed for the opioid receptor antagonist, naloxone, and the opioid agonist/antagonist, meptazinol (Fagbemi *et al.*, 1982; 1983). Recent binding studies in the rat indicate that buprenorphine achieves about 50% occupancy of both μ- and κ-opioid receptor sites at doses around $30\,\mu g\,kg^{-1}$, and saturates both sites *in vivo* at 250–$500\,\mu g\,kg^{-1}$ (Sadee *et al.*, 1983). Therefore, the present finding that buprenorphine, at a dose of $1\,mg\,kg^{-1}$, suppresses early ischaemic arrhythmias raises the possibility that this antiarrhythmic effect may be related or secondary to its opioid receptor occupancy. These antiarrhythmic effects were not associated with marked haemodynamic effects, apart from a slight attenuation of the acute ligation-induced fall in arterial blood pressure. The mechanism underlying this effect is not known, although the finding that diamorphine produces a similar effect in anesthetized rats (R. Sitsapesan, unpublished observations) would tend to suggest μ-receptor activation as a possible basis. Unlike buprenorphine, however, the diamorphine effect was not associated with any protection against early ischaemic arrhythmias (R. Sitsapesan, unpublished observations). Thus, it is unlikely that this effect on blood pressure is responsible for the antiarrhythmic effects of buprenorphine.

Buprenorphine also exerted effects on the electrophysiological characteristics of normal sheep Purkinje and rat ventricular tissues *in vitro*, reducing both the maximum rate of depolarization and action potential height and prolonging the action potential duration. The depressant effects on MRD and action potential amplitude may be due to blockade of fast Na^+ channels, the opening of which are responsible for the upstroke of action potential in Purkinje and ventricular tissue. The mechanism underlying the action potential prolongation, however, remains unknown. The fact that this effect was more pronounced on terminal repolarisation suggests a possible block of delayed outward K^+ conductance, but further studies are required to provide a definitive answer. The above effects of buprenorphine are qualitatively similar to those obtained for naloxone and meptazinol on guinea-pig and rat ventricular muscles *in vitro* (Fagbemi *et al.*, 1983; Brasch, 1986).

The changes induced by the modified PSS in sheep Purkinje and rat ventricular action potential characteristics, namely, marked reductions in resting membrane potential, maximum rate of depolarization of phase 0, action potential amplitude and action potential duration, are similar to those described previously in isolated cardiac tissues (Gilmour & Zipes, 1980; Evans *et al.*, 1984) and during mild myocardial ischaemia *in vivo* (Downar *et al.*, 1977). Evidence suggests that these changes are due largely to the elevated extracellular K^+ concentration, with some less major contribution from the concomitant hypoxia and acidosis (Boachie-Ansah *et al.*, 1989). The changes remained relatively stable during the 30–120 min exposure period, thus allowing evaluation of drug action on depressed fast responses to be carried out. The continued, steady reductions in rat ventricular action potential duration, induced by the prolonged exposure to the modified PSS, have also been observed in rabbit ventricular muscles (G. Boachie-Ansah, unpublished observations) and may be due to the greater susceptibility of ventricular tissue to prolonged hypoxia (Fozzard, 1975; Wit & Bigger, 1975).

In the presence of the modified PSS, buprenorphine caused more marked reductions in the MRD and action potential amplitude in both sheep Purkinje and rat ventricular tissue and prevented the steady decline in rat ventricular action potential duration. The ability to prolong sheep Purkinje action potentials was, however, virtually lost under

simulated ischaemic conditions. The potentiation of the depressant effect of buprenorphine on phase zero of the action potential may be due to the membrane depolarization induced by the modified PSS and suggests that buprenorphine, like other antiarrhythmic drugs such as lignocaine, preferentially blocks the inactivated form of the fast Na^+ channel (Hondeghem & Katzung, 1984). The mechanism(s) underlying the apparent loss or attenuation of the ability of buprenorphine to prolong sheep Purkinje action potential under conditions that mimicked myocardial ischaemia is not clear. If prolongation of the action potential duration by buprenorphine is mediated via blockade of the outward K^+ current as suggested, then it is possible that this effect was swamped by the opposing actions of hypoxia and elevated K^+ to increase K^+ conductance.

It is possible that the cardiac electrophysiological effects of buprenorphine described in these studies (depression of the MRD and action potential height and prolongation of the action potential duration) are responsible for the observed protective effect of buprenorphine *in vivo*. The ability to block fast Na^+ channels (leading to depression of MRD), especially in depolarized tissue, is thought to be responsible for the antiarrhythmic actions of the Class I antiarrhythmic drugs such as lignocaine (Vaughan Williams, 1980; Carmeliet, 1984). Similarly, prolongation of the duration of the cardiac action potential is thought to be the basis of the antiarrhythmic actions of the Class III antiarrhythmic drugs like amiodarone (Vaughan Williams, 1980). Furthermore, drugs possessing either of these properties have been shown to suppress occlusion-induced arrhythmias in anaesthetised rats (Botting *et al.*, 1985).

It remains to be resolved, however, whether the observed antiarrhythmic and cardiac electrophysiological effects of buprenorphine are, indeed, mediated via specific opioid receptor activation. The rather high concentration ($1\,mg\,kg^{-1}$) required to confer protection against ischaemic arrhythmias would tend to rule out the possible involvement of specific opioid receptor activation. However, there is increasing evidence that, unlike the antagonism of exogenously administered opioid receptor agonists, effective antagonism of endogenous opioid systems may require much higher concentrations of opioid receptor antagonists (i.e., in the $mg\,kg^{-1}$ range; Cohen *et al.*, 1982). Furthermore, the antiarrhythmic activity of several opioid receptor antagonists has been shown to be stereospecific in the rat model, notwithstanding the fact that high concentrations ($1–3\,mg\,kg^{-1}$) of drug were required to confer protection (Parratt & Sitsapesan, 1986). Thus, the possibility still exists that the antiarrhythmic actions of buprenorphine were mediated via opioid receptors, although it is not possible to specify which receptor subtype(s) are involved from the present study.

The equally high concentrations (10^{-6}–10^{-5} M) of buprenorphine required to modify cardiac action potential characteristics could also be used to argue against the possible involvement of opioid receptor activation. In this respect, it is of interest to note that even higher concentrations ($3–120 \times 10^{-6}$ M) of naloxone were required to produce similar effects on guinea-pig atrial ventricular action potentials and that the effects of naloxone were not stereospecific (Brasch, 1986), which suggests that they are not mediated via opioid receptor activation. It is not known, however, whether the buprenorphine effects are similarly non-stereospecific. Thus, further studies would be required to address the issue of the possible relationship of specific opioid receptor activation to the antiarrhythmic and cardiac electrophysiological effects of buprenorphine.

In summary, buprenorphine afforded protection against early ischaemia-induced arrhythmias in anaesthetised rats. In normally-polarized sheep Purkinje fibres and rat papillary muscles, buprenorphine reduced the action potential amplitude and maximum rate of depolarisation of phase zero and prolonged the action potential duration. In both sheep Purkinje fibres and rat papillary muscles exposed to combined hypoxia, hyperkalaemia and acidosis, the ability of buprenorphine to depress the action potential amplitude and maximum rate of depolarization of phase zero was enhanced, whereas its ability to prolong the action potential duration was attenuated. It is suggested that these cardiac electrophysiological actions may underlie the observed antiarrhythmic activity of buprenorphine *in vivo*. Further, buprenorphine might serve the useful dual purpose of relieving pain and reducing the severity of arrhythmias in the early stages of acute myocardial infarction.

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The involvement of lactate and calcium as mediators of the electrical and mechanical responses of the myocardium to conditions of simulated ischaemia

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1 Rat isolated and superfused atria were exposed to a lactate-containing solution simulating the composition of extracellular fluid during myocardial ischaemia (SI).

2 Atria subjected to SI showed a decreased adenosine 5'-triphosphate (ATP) content, a rise in diastolic tension, a diminished conduction velocity of action potentials and shortened refractory periods. All these changes were less pronounced during lactate-free SI.

3 Atria preloaded with calcium displayed exaggerated responses measured electrically and mechanically during exposure to SI, whereas atria previously depleted of calcium displayed diminished electrical and mechanical responses to SI. Neither calcium loading nor calcium depletion modified the SI-induced depletion of the atrial stores of ATP.

4 Sulphinpyrazone protected atria against all aspects of the response to SI, but failed to protect the muscle under conditions of lactate-free SI. It is concluded that during SI, sulphinpyrazone protects against a lactate-mediated inhibition of the glycolytic synthesis of ATP.

5 Flufenamate exaggerated all responses of the atria to SI. These deleterious actions were still observed during lactate-free SI. It is concluded that flufenamate inhibits the synthesis of ATP in the mitochondria.

Introduction

Simulated ischaemia (SI) is said to exist when a tissue is exposed *in vitro* to a solution the composition of which resembles that found extracellularly in regions of ischaemia. During SI in rat atrial muscle the conduction velocity (CV) of action potentials is reduced, as are the durations of both action potentials and refractory periods. These responses are accompanied by progressive contracture development and a characteristic pattern of structural changes, notably in the mitochondria (Northover & Northover, 1988). Return of the myocardium to a solution of normal composition gradually reverses all of these changes. Pretreatment of the atria with sulphinpyrazone protects the myocardium during SI, but flufenamate, another non-steroidal anti-inflammatory agent, has a deleterious action (Northover & Northover, 1988).

A rise in the diastolic concentration of Ca in the myoplasm is believed to represent an important stage in the series of events responsible for the structural and functional changes observed during SI (Northover & Northover, 1988). In some of the present experiments the diastolic Ca concentration in the myoplasm has been deliberately elevated before exposure to SI. By noting which components of the response to SI were enhanced under these circumstances evidence was obtained concerning the ways in which Ca is involved as a mediator.

Lactate is another substance known to accumulate in the ischaemic myocardium (Neely & Grotyohann, 1984), and is an ingredient of the fluid used to produce SI. Moreover, lactate is known to potentiate some of the functional changes in the atria produced by the other ingredients of this fluid (Northover, 1987). Under certain circumstances lactate is known to inhibit glycolysis (Rovetto *et al.*, 1973, 1975; Mochizuki & Neely, 1979; Neely & Grotyohann, 1984), to exert a negative inotropic action (Tennant, 1935), and to interfere with both mitochondrial structure (Armiger *et al.*, 1974) and the oxidation of

fatty acids (Bielefeld *et al.*, 1983). The present experiments have sought to identify the circumstances under which lactate inhibits ATP production, and the involvement of such inhibition in the overall deterioration produced by SI.

Finally, attempts have been made to identify the extent to which sulphinpyrazone and flufenamate alter responses to SI by modifying the involvement of lactate and calcium in this process.

Methods

Rats of the Sprague-Dawley strain weighing 340–410 g were killed by a blow to the head. The heart was removed quickly, the atria separated from the ventricles and the former attached with their endocardial surface upwards to the base of a superfusion trough maintained at 34°C. The muscle was exposed, unless specified otherwise, to a normal superfusate (NS) of the following composition (mM): NaCl 138, KCl 4.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, NaH_2PO_4 0.5, $NaHCO_3$ 10 and glucose 10, and gassed with a mixture of 95% O_2 plus 5% CO_2, giving a pH of 7.3. In experiments where the NaCl concentration was halved, sucrose (138 mM) was added to maintain osmolality. In experiments where lanthanum chloride was added to the superfusate, sodium phosphate and bicarbonate were replaced by 4 mM sodium *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonate as a pH buffer. Atria were stimulated throughout an experiment at 4 Hz via a pair of platinum wire electrodes placed on the right atrial appendage. Square wave pulses of current, each 2 ms in duration and isolated from earth, were used at a voltage of twice the prevailing diastolic threshold.

Electrical changes

Transmembrane potentials were recorded from subendocardial muscle fibres by means of glass microelectrodes filled with a 3M solution of KCl and having resistances of $1–2 \times 10^7$ ohms. One microelectrode was inserted intracellularly in the right atrium. Voltages detected by this electrode were passed by a single-ended high input-impedance coupler with facilities for capacitance neutralisation (type 8124, C.F. Palmer) to both an oscilloscope and a transient store microprocessor (type 140, Bioscience). Stored signals were able to be replayed from the latter device at speeds up to 2000 fold slower than those at which they were recorded. The effective refractory period (ERP) of the right atrial muscle was determined by paired stimuli, and was taken as the interval between the closest pair of stimuli both of which yielded action potentials. The CV of action potentials between the right and left atria was measured with the aid of a second microelectrode inserted intracellularly in the left atrium. Voltage signals from both microelectrodes were displayed on a dual channel oscilloscope. Knowing the distance between the 2 microelectrodes and the time interval between the upstrokes of the action potentials recorded from them, it was possible to calculate the apparent CV. Since action potentials may not have conducted via the most direct route between the 2 electrodes, however, the CV may have been underestimated.

Mechanical changes

The right atrial appendage was anchored to the base of the superfusion trough and the left atrial appendage was connected via a length of nylon suture to a Nihon Khoden strain gauge (Type SB-1T). Diastolic tension was adjusted at the start of each experiment to 100 mg. Tension records were made on slowly moving paper via a d.c. amplifier (Type 3552, Cardiovascular Instruments) coupled to a heated stylus recorder (Type 5041, Lectromed).

Simulated ischaemia

At the start of each experiment the atria were allowed to equilibrate in normal superfusate for 1 h, at which time control values for refractory periods and CV were determined. The superfusate was then changed to one of abnormal composition, simulating the extracellular fluid of ischaemic muscle, as suggested by Ferrier *et al.* (1985). Unless specified otherwise, this fluid contained racemic sodium lactate (20 mM), was made up without added glucose, and had a pH of 6.4 produced by reduction of the $NaHCO_3$ content to 4 mM. Hypoxia was produced by replacing O_2 by N_2. All gases used were supplied by British Oxygen Company. The O_2 was of Medishield purity. The other gases contained 0.002% or less of O_2. Po_2 of the superfusate during SI was 16–25 mmHg. A tissue was allowed to remain in SI for 15 min and thereafter returned to normal superfusate. Measurements given in this paper, unless otherwise specified, were made after 15 min in SI. Each tissue was exposed to SI on 4 occasions, each of 15 min. Preliminary experiments showed that there was no statistically significant difference between the responses to 4 consecutive exposures to SI. In some of the experiments SI was superimposed upon another intervention that was intended to raise the myoplasmic Ca concentration. For this purpose the tissue was exposed to the Ca-raising intervention for 30 min, during the last 15 min of which conditions of SI also prevailed.

Assay of ATP

Atria to be assayed for ATP were clamped between the jaws of a pair of metal tongs that had been chilled in liquid N_2. The frozen tissue was powdered in a steel percussion mortar, also cooled in liquid N_2. The powdered muscle was rapidly transferred to a preweighed glass vial containing ice-cold 10% aqueous perchloric acid solution, and the vial reweighed. The contents of the vial were then homogenized in a cold room, with a small hand-operated Dounce tissue grinder surrounded by melting ice. The homogenate was centrifuged at 2000 *g* for 15 min at 2°C and the supernatant collected. The solid residue was then re-extracted with further perchloric acid solution. The two supernatants were combined, neutralised with 10 M KOH solution, and then centrifuged at 2000 *g* for 15 min at 2°C. The neutralised supernatant was assayed for ATP by first reacting it with glucose in the presence of hexokinase. The glucose-6-phosphate produced was oxidised in the presence of nicotinamide adenine dinucleotide and glucose-6-phosphate dehydrogenase. The reaction was monitored in a spectrophotometer at 340 nm and the glycogen content expressed as μmol glucose per g wet weight of muscle.

Drugs and other agents

Sulphinpyrazone was a gift from Ciba-Geigy and flufenamate was a gift from Parke Davis and Company. They were dissolved in water with a small excess of Na_2CO_3 and the solution adjusted to neutrality with acetic acid. All other drugs and reagents were purchased from Sigma.

Statistics

Variables are expressed throughout as means ± s.e. Means for different treatment groups were compared by means of Student's *t* tests.

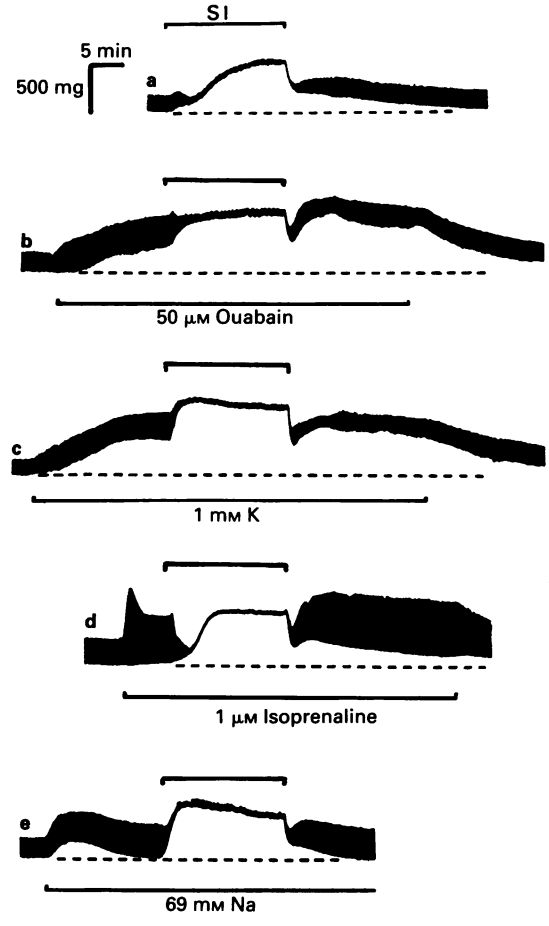

Figure 1 Atrial tension recorded during exposure to simulated ischaemia (SI) for the period indicated by the horizontal bar above each panel. In panels (b)–(e) there are also continuous horizontal bars beneath each record, and during the latter periods the superfusate contained 50 μM ouabain (b), 1 mM K (c), 1 μM isoprenaline (d), or 69 mM Na (e).

Results

Effects of elevating the myoplasmic Ca concentration

The Ca concentration in the myoplasm was elevated in the present experiments by means of several different pharmacological interventions, namely by exposure to NS containing ouabain (10–50 μM), by exposure to NS containing a reduced K concentration (1 or 2 mM) or Na concentration (69 mM), or a raised Ca concentration (10 mM), or by means of NS containing isoprenaline (0.1–1.0 μM). Each of these interventions caused the expected increase in systolic developed tension (Figure 1). Positive inotropism was well sustained, except with isoprenaline. Some, but not all of these interventions also caused a rise in diastolic tension. Any rise in diastolic tension, however, only began after systolic tension had already begun to rise. Moreover, a higher concentration of ouabain (> 10 μM) or a lower concentration of K (< 2 mM) was needed to produce contracture than was needed to cause detectable positive inotropism. Where diastolic tension did rise during one of these interventions, it was progressive with time, except in the case of the response to a low Na concentration, where only a temporary contracture was produced (Figure 1). Unlike SI, none of the above interventions caused a significant decline in the ATP

Table 1 Effects of various calcium-raising interventions on responses of atria to simulated ischaemia (SI)

Drug (μM)	*Na* (mM)	*K* (mM)	*Ca* (mM)	*ERP* (ms)	*CV* ($m\ s^{-1}$)	*DT* (mg)	*ATP* ($\mu mol\ g^{-1}$)
Control	138	5	2	16 ± 2	0.18 ± 0.06	424 ± 52	1.82 ± 0.21
	69	5	2	17 ± 3	0.19 ± 0.07	419 ± 75	1.86 ± 0.35
	138	2	2	16 ± 3	0.14 ± 0.06	488 ± 60	1.74 ± 0.20
	138	1	2	10 ± 2*	0.07 ± 0.03*	654 ± 62*	1.61 ± 0.19
	138	5	10	11 ± 2*	0.18 ± 0.03	570 ± 48*	1.70 ± 0.18
Ouabain (10)	138	5	2	15 ± 3	0.16 ± 0.05	466 ± 37	1.99 ± 0.25
Ouabain (50)	138	5	2	9 ± 2*	0.08 ± 0.02*	685 ± 40*	1.70 ± 0.16
Isopren (1)	138	5	2	17 ± 3	0.19 ± 0.07	302 ± 44*	2.05 ± 0.23

Tabulated values represent means of 6–18 observations. A significant difference exists ($P < 0.05$) between a value marked * and the corresponding value for control atria exposed to SI.
Isopren = isoprenaline; ERP = effective refractory period; CV = conduction velocity; DT = diastolic tension; ATP = adenosine triphosphate content.

content of the muscle within the first 30 min (cf. Tables 1 and 2). When SI was superimposed upon another intervention that had already raised diastolic tension, the final contracture tension reached after 15 min in SI was greater than that reached in the presence of SI alone (Table 1). Contracture tension was not augmented, however, when SI was superimposed upon an intervention that failed to raise diastolic tension, as in the case of isoprenaline (Table 1), or upon an intervention that raised diastolic tension only temporarily, as in the case of a low Na concentration (Table 1). Indeed, contracture tension during SI in the presence of isoprenaline was significantly less than in the absence of this catecholamine (Table 1).

Refractory periods and CV of action potentials declined in atria subjected to NS containing ouabain at a concentration sufficient to raise diastolic tension (20 or 50 μM in Table 2). When the K concentration of NS was lowered to 1 mM there was also a decline in ERP and CV values (Table 2). During exposure of atria to SI for 15 min in the presence of 50 μM ouabain or a lower K concentration (1 mM), the measured values of ERP and CV became significantly smaller than during SI in the absence of these interventions (Table 1).

Effects of non-steroidal anti-inflammatory agents

Sulphinpyrazone (5–50 μM) protected the myocardium against contracture development and the associated electrical and biochemical disturbances produced by exposure for 15 min to SI in the presence, but not in the absence of lactate. Flufenamate (1–5 μM), on the other hand, aggrevated all the disturbances produced by SI both in the presence of lactate and in its absence (Table 3). Neither sulphinpyrazone nor flufenamate in these ranges of con-

Table 2 Effects of composition of normal superfusate (NS) on responses of atria to various calcium-raising agents

Drug (μM)	*Na* (mM)	*K* (mM)	*Ca* (mM)	*ERP* (ms)	*CV* ($m\ s^{-1}$)	*DT* (mg)	*ATP* ($\mu mol\ g^{-1}$)
Control	138	5	2	33 ± 5	0.63 ± 0.11	100	3.71 ± 0.24
	69	5	2	32 ± 6	0.59 ± 0.16	98 ± 12	3.65 ± 0.27
	138	2	2	30 ± 5	0.55 ± 0.14	106 ± 15	3.70 ± 0.20
	138	1	2	20 ± 4*	0.26 ± 0.12*	419 ± 60*	3.39 ± 0.27
	138	5	10	25 ± 3*	0.45 ± 0.10*	148 ± 24	3.48 ± 0.30
Ouabain (10)	138	5	2	32 ± 6	0.57 ± 0.11	105 ± 23	3.66 ± 0.29
Ouabain (20)	138	5	2	30 ± 4*	0.48 ± 0.06*	125 ± 18	3.59 ± 0.25
Ouabain (50)	138	5	2	23 ± 3*	0.24 ± 0.10*	394 ± 50*	3.37 ± 0.22
Isopren (1)	138	5	2	20 ± 4*	0.55 ± 0.09	106 ± 9	3.54 ± 0.23

Tabulated values represent means of 6–36 observations. A significant difference exists ($P < 0.05$) between a value marked * and the corresponding value for control atria exposed to NS.
Isopren = isoprenaline; ERP = effective refractory period; CV = conduction velocity; DT = diastolic tension; ATP = adenosine triphosphate content.

Table 3 Influence of lactate on the effects of various drugs on the responses of atria to simulated ischaemia (SI)

Drug (μM)	*Lactate* (mM)	*ERP* (ms)	*CV* ($m\ s^{-1}$)	*DT* (mg)	*ATP* ($\mu mol\ g^{-1}$)
Control	20	16 ± 2	0.18 ± 0.06	424 ± 52	1.82 ± 0.21
Control	0	21 ± 3*	0.36 ± 0.07*	305 ± 29*	2.50 ± 0.27*
Sulphinpyrazone (50)	20	29 ± 4*	0.40 ± 0.10*	232 ± 25*	2.98 ± 0.22*
Sulphinpyrazone (50)	0	26 ± 4	0.41 ± 0.09	316 ± 41	2.94 ± 0.20
Flufenamate (5)	20	8 ± 2*	0.06 ± 0.05*	510 ± 57*	1.02 ± 0.13*
Flufenamate (5)	0	14 ± 2**	0.21 ± 0.07**	462 ± 43**	1.10 ± 0.11**
Dinitrophenol (3)	20	7 ± 2*	0.05 ± 0.04*	596 ± 60*	1.05 ± 0.17*
Dinitrophenol (3)	0	13 ± 2**	0.22 ± 0.04**	477 ± 36**	1.59 ± 0.26**
Lanthanum (1000)	20	26 ± 3*	0.38 ± 0.04*	211 ± 22*	1.94 ± 0.26
Lanthanum (1000)	0	29 ± 4*	0.50 ± 0.05**	155 ± 25**	2.58 ± 0.20**
Pyruvate (10,000)	20	17 ± 3	0.20 ± 0.08	394 ± 46	2.05 ± 0.23
Glucose (20,000)	20	30 ± 4*	0.50 ± 0.09*	123 ± 18*	3.57 ± 0.26*
Zero calcium	20	22 ± 3*	0.45 ± 0.08*	196 ± 25*	1.98 ± 0.19*
Zero calcium	0	29 ± 3**	0.57 ± 0.10**	181 ± 22**	2.62 ± 0.23**

Tabulated values represent means of 6–24 observations. A significant difference exists ($P < 0.05$) between a value marked * and the corresponding value for control atria exposed to lactate-containing SI, and between a value marked ** and the corresponding value for control atria exposed to lactate-free SI.
ERP = effective refractory period; CV = conduction velocity; DT = diastolic tension; ATP = adenosine triphosphate content.

centrations significantly altered diastolic tension or the systolic developed tension in NS, nor did either drug alter the loss of systolic developed tension during SI. Sulphinpyrazone failed to protect atria against the mechanical and electrical changes produced by exposure to NS containing ouabain (50 μM) or a low K concentration (1 mM). Flufenamate, however, enhanced all of the measured responses of the myocardium to both interventions.

Effects of glucose

Atria continued to behave quite normally, both mechanically and electrically, for 2–3 h in NS made up without glucose, despite a reduction in glycogen content from 12 $\mu mol\ g^{-1}$ in glucose-containing NS to only 2.6 $\mu mol\ g^{-1}$ after 1 h in glucose-free NS ($P < 0.05$). The ATP content of atria exposed to glucose-free NS for 1 h was almost identical to that

Table 4 Influence of lactate on the effects of various drugs on the responses of glycogen-depleted atria to simulated ischaemia (SI)

Drug (μM)	*Lactate* (mM)	*ERP* (ms)	*CV* ($m\ s^{-1}$)	*DT* (mg)	*ATP* ($\mu mol\ g^{-1}$)
Control	20	11 ± 2	0.12 ± 0.04	530 ± 49	1.02 ± 0.10
Control	0	12 ± 3	0.17 ± 0.06	541 ± 60	1.16 ± 0.14
Sulphinpyrazone (50)	20	12 ± 2	0.20 ± 0.07	496 ± 38	1.20 ± 0.15
Sulphinpyrazone (50)	0	14 ± 4	0.18 ± 0.05	490 ± 42	1.24 ± 0.16
Flufenamate (5)	20	6 ± 1*	0.07 ± 0.03	639 ± 57	0.39 ± 0.06*
Flufenamate (5)	0	7 ± 1**	0.05 ± 0.02**	615 ± 46	0.55 ± 0.09**
Dinitrophenol (3)	20	6 ± 2*	0.08 ± 0.04	701 ± 58*	0.36 ± 0.10*
Dinitrophenol (3)	0	6 ± 2**	0.06 ± 0.03**	690 ± 51**	0.67 ± 0.14**
Lanthanum (1000)	20	21 ± 4*	0.38 ± 0.12*	206 ± 29*	1.22 ± 0.19
Lanthanum (1000)	0	23 ± 5**	0.31 ± 0.10**	194 ± 16**	1.25 ± 0.22

Tabulated values represent means of 6–24 observations. A significant difference exists ($P < 0.05$) between a value marked * and the corresponding value for control atria exposed to lactate-containing SI, and between a value marked ** and the corresponding value for control atria exposed to lactate-free SI.
ERP = effective refractory period; CV = conduction velocity; DT = diastolic tension; ATP = adenosine triphosphate content.

of atria bathed with glucose-containing NS. Nevertheless, atrial fibrillation did eventually supervene in glycogen-depleted muscle, but this was not until the tissue had been exposed to glucose-free NS for at least 3 h. To avoid this rhythm disturbance all subsequent experiments of this type were performed after exposure to glucose-free NS for just 1 h. Responses to SI after prior glycogen-depletion were significantly greater than in atria which began a period of 15 min exposure to SI with a normal glycogen store (cf. Tables 1 and 4). More significantly, responses of the glycogen-depleted muscle to SI were no longer inhibited by sulphinpyrazone (Table 4), although still potentiated by flufenamate or dinitrophenol (Table 4). Glucose (20 mM) was protective against all aspects of the response to SI, an action not shared by pyruvate (Table 3). A supraphysiological concentration of glucose failed to protect atria, however, against any of the responses to ouabain or a low K concentration in NS. Unlike the situation during SI, the ATP content of the myocardium was not significantly depleted during the latter 2 interventions (Table 2).

Effects of lactate

Conditions of SI, in the absence of added lactate, elicited smaller electrical, mechanical and biochemical responses than in the presence of lactate (Table 3). The deleterious effects of lactate on responses to SI were no longer seen, however, in atria that had already been depleted of glycogen by prior treatment with glucose-free NS for 1 h (Table 4).

Lactate (20 mM) exerted a negative inotropic effect when added to NS. Despite this, no significant change in ATP content or in ERP and CV values was seen under these conditions. When lactate-containing NS was suddenly replaced by lactate-free NS, however, the muscle developed a brief but intense contracture (Figure 2) associated with depletion of ATP content, shortened ERP values and a diminished CV (Table 5). In some tissues there were also short self-terminating paroxysms of atrial tachycardia provoked by the paired stimuli used for measuring refractory periods. All these features were maximally developed 4–6 min after lactate was discontinued and had fully subsided within a further 15–20 min. Similar responses were observed when the lactate-containing NS was made up with the NaCl content reduced to preserve isotonicity throughout. A reciprocal relationship was found between the concentration of glucose in the NS and the severity of the responses to sudden discontinuation of lactate (Table 5). Sulphinpyrazone and

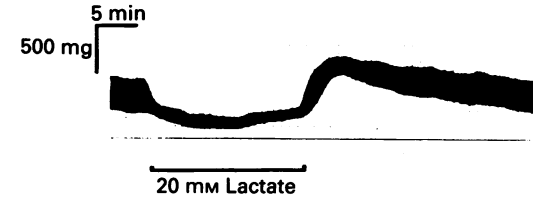

Figure 2 Atrial tension during exposure to normal superfusate. The superfusate contained sodium lactate (20 mM) during the 20 min period marked by the horizontal bar at the foot of the figure. Note that contracture tension was maximal about 5 min after the discontinuation of lactate.

Table 5 Effects of various drugs on the responses of atria to the sudden discontinuation of the presence of lactate in normal superfusate (NS)

Drug (μM)	*Glucose* (mM)	*Ca* (mM)	*ERP* (ms)	*CV* ($m\ s^{-1}$)	*DT* (mg)	*ATP* ($\mu mol\ g^{-1}$)
Control	10	2	18 ± 3	0.20 ± 0.07	488 ± 35	1.96 ± 0.29
	0	2	9 ± 2*	0.11 ± 0.02	562 ± 50	1.04 ± 0.12*
	20	2	29 ± 4*	0.45 ± 0.12*	210 ± 14*	3.24 ± 0.35*
	10	0	28 ± 4*	0.40 ± 0.11*	207 ± 22*	2.02 ± 0.13
Sulphinpyrazone (50)	10	2	30 ± 5*	0.39 ± 0.08*	182 ± 35*	2.57 ± 0.14*
Pyruvate (10,000)	10	2	33 ± 5*	0.50 ± 0.12*	151 ± 29*	2.61 ± 0.16*
Flufenamate (5)	10	2	10 ± 2*	0.08 ± 0.02*	582 ± 46*	0.96 ± 0.17*
Dinitrophenol (3)	10	2	8 ± 2*	0.09 ± 0.03*	576 ± 55*	0.89 ± 0.10*
Lanthanum (1000)	10	2	26 ± 3*	0.62 ± 0.13*	132 ± 18*	2.08 ± 0.14

Atria were exposed for 20 min to NS containing 20 mM lactate. Tabulated values represent means of 6–18 observations and were recorded 5 min after the sudden discontinuation of the presence of lactate in the NS. A significant difference exists ($P < 0.05$) between a value marked * and the corresponding value for control atria.
ERP = effective refractory period; CV = conduction velocity; DT = diastolic tension; ATP = adenosine triphosphate content.

pyruvate both inhibited the responses (Table 5), whereas flufenamate and dinitrophenol both potentiated them (Table 5).

Effects of lanthanum

In order to explore further the involvement of calcium in the reponses to SI, experiments were conducted in the presence of added lanthanum chloride (1 mM), a classical calcium antagonist. Systolic developed tension in NS was approximately zero in the presence of this concentration of lanthanum. Lanthanum protected against the mechanical and electrical responses normally produced by SI (Table 3), by ouabain in NS and by a low K concentration in NS. Lanthanum failed to alter the depletion of ATP, however, that was normally observed during SI (Table 3). The protective effects of lanthanum during SI were observed both in the presence and in the absence of added lactate (Table 3), and so differ from the protective effects of sulphinpyrazone. Contractures due to discontinuation of lactate were inhibited by lanthanum but the depletion of ATP was unaltered (Table 5). Again, this is different from the protective effect of sulphinpyrazone (Table 5).

Discussion

Involvement of calcium

Two lines of evidence from the present experiments suggest that during the various conditions chosen to create Ca-loading an increased diastolic Ca concentration in the myoplasm was responsible for contracture and the associated changes in ERP and CV. Firstly, when a contracture was produced by exposure to NS containing ouabain or a low K concentration the changes in ERP and CV occurred in parallel with contracture development (Table 2). Milder forms of these same interventions, sufficient to produce positive inotropism but insufficient to cause contracture, caused no significant change in ERP or CV (Table 2). Secondly, those treatments that protected atria against contracture development, such as a lack of extracellular Ca, also protected against the associated reductions in ERP and CV (Table 2).

Conditions of SI, in common with several others that are known to be associated with rapid depletion of the myocardial stores of ATP, are associated with a progressive rise in the myoplasmic concentration of Na (Murphy *et al.*, 1987a) and Ca (Murphy *et al.*, 1985; Haworth *et al.*, 1987; Steenbergen *et al.*, 1987, Barry *et al.*, 1987; Smith & Allen, 1988). The myoplasmic Ca concentration during diastole has been suggested by several of these investigators to be partly responsible for the observed contracture. Other workers have suggested that there is a similar basis for the slowing of CV (Pressler *et al.*, 1982) and for the shortening of ERP (Northover, 1987; Northover & Northover, 1988). The total cellular Ca content has been reported to rise during these and related circumstances (Murphy *et al.*, 1987b). A rapidly exchanging pool of intracellular Ca also increases under these circumstances (Murphy *et al.*, 1987a), although the sarcoplasmic reticular store of Ca may actually decline (Hasin *et al.*, 1984; Haworth *et al.*, 1987). It seems probable that ATP depletion is the primary disturbance during SI. Since the extrusion of Na and Ca from the myoplasm into the extracellular compartment, and the uptake of Ca from the myoplasm into the sarcoplasmic reticulum are ATP-dependent processes, it follows that ATP-depletion will cause a rise in the myoplasmic concentrations of Ca and Na, particularly during diastole.

In the present experiments, when SI was superimposed upon a situation that had already produced Ca-overload and hence contracture, the electrical and mechanical responses during the period of SI were enhanced (Table 1), although the depletion of ATP stores was not significantly altered (Table 1). Pretreatment of the atria with isoprenaline significantly inhibited the development of contracture during SI (Table 1). The ability of isoprenaline and other β-adrenoceptor agonists to inhibit contractures due to Ca-overload has been observed previously (Kavaler & Morad, 1966; Graham & Lamb, 1968; Lakatta & Lappe, 1981). A possible explanation is stimulation of glycolysis (MacLoed & Prasad, 1969), but this is unlikely to have contributed in the present experiments since ATP-depletion during SI was unaltered (Table 1). Alternatively, a raised concentration of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) may have reduced the sensitivity of the contractile proteins to Ca, as suggested by Ray & England (1976) and by Endoh & Blinks (1988). This would also account for the early fading of the positive inotropic response to isoprenaline shown in Figure 1. Other works have concluded, however, that the early fading of inotropic responses to isoprenaline was due to a partial failure of systolic release of Ca from the sarcoplasmic reticulum (Erne & Hermsmeyer, 1988).

All of the measured electrical and mechanical responses to SI in the present experiments were inhibited by the absence of Ca from the superfusate or by the presence of lanthanum (Table 3). The myoplasmic Ca concentration is known to be reduced by lanthanum during ATP-depletion (Hasin *et al.*, 1984; Haworth *et al.*, 1987; Murphy *et al.*, 1988) as well as under more normal conditions (Barry & Smith,

1982). In the present experiments the absence of Ca from the superfusate or the presence of lanthanum failed to alter the SI-induced depletion of ATP significantly, yet both reduced all the other measured components of the response to SI (Table 3). The electrical and mechanical responses to SI, therefore, seem to be secondary to changes in the myoplasmic Ca concentration, which in turn are the result of the ATP depletion.

Involvement of lactate

Although lactate is a fuel for the aerobic heart (Takenaka & Watanabe, 1976) it potentiates the SI-induced reduction in ERP (Northover, 1987), contracture development, slowing of CV and ATP depletion (Table 3). Lactate is known to inhibit the glycolytic synthesis of ATP in ventricular muscle (Rovetto *et al.*, 1973; 1975; Mochizuki & Neely, 1979; Neely & Grotyohann, 1984). The present experiments provide two lines of evidence that a similar effect is produced in atrial muscle. In the first place, the SI-potentiating effects of lactate were absent in previously glycogen-depeleted muscle (Table 4). During SI there would be no fuel for glycolysis so inhibitors of glycolysis would not be expected to be deleterious. In the second place, muscle exposed to a supra-physiological concentration of glucose was protected against SI (Table 3). One might expect that the more exogenous glucose was available during SI the less vulnerable would the glycolytic synthesis of ATP become to partial inhibition of any of the enzymes in this pathway. Indeed, Table 3 shows that muscle exposed to SI in the presence of 20 mM glucose had an ATP content almost as high as that of muscle in NS, and significantly higher than that of muscle exposed to SI in the absence of glucose. In contrast, the presence of 20 mM glucose did not protect against contractures caused by ouabain or a low K concentration. Presumably this was due to the normally preserved ATP content in the latter 2 situations (Table 2). Pyruvate is a fuel for ATP synthesis, but only under aerobic conditions, and would not be expected to protect under the anaerobic conditions of SI (Table 3).

Abrupt discontinuation of a supply of lactate in NS produced a brief contracture, a depletion of ATP and a reduction in both ERP and CV (Table 5). Probably, therefore, for a time after the supply of lactate ended the glycolytic pathway was still inhibited. Oxygenated muscle, however, would be expected to obtain adequate amounts of ATP from triglyceride-derived fatty acids. Lactate, however, also inhibits the oxidation of fatty acids in the mitochondria (Bielefeld *et al.*, 1983). For a short period after discontinuation of lactate, therefore, ATP synthesis from both glucose and fatty acids would be impaired, accounting for the ATP depletion observed under these circumstances (Table 5). This explanation is also consistent with the fact that exogenous glucose at high concentration protected against all components of the response to discontinuation of lactate (Table 5), and this protection was shared by pyruvate (Table 5).

Non-steroidal anti-inflammatory drugs

The present experiments furnish four lines of evidence that sulphinpyrazone protects muscle against SI by preventing the inhibition of glycolysis by lactate. In the first place, sulphinpyrazone protected against the depletion of ATP during SI (Table 3). Secondly, it failed to protect previously glycogen-depleted muscle against SI (Table 4) and failed to protect in the absence of lactate (Table 3). Thirdly, the drug failed to modify responses to interventions which, unlike SI, elevate the diastolic Ca concentration in the myoplasm without causing ATP depletion (Table 2). Finally, sulphinpyrazone protected against responses to discontinuation of lactate (Table 5). A possible alternative site of action would be the mitochondrial oxidation of fatty acids, but this is unlikely for 2 reasons. Firstly, fatty acid oxidation would be inoperative during the anaerobiosis of SI, yet sulphinpyrazone is still protective (Table 3). Secondly, it fails to explain why previously glycogen-depleted muscle is not protected by sulphinpyrazone (Table 4).

Flufenamate potentiated all the measured responses to SI (Table 3) and to discontinuation of lactate (Table 5). Evidence was obtained from the present experiments which suggests that flufenamate exerted actions that were not just the opposite, however, of those shown by sulphinpyrazone. Thus, unlike sulphinpyrazone, the effects of flufenamate were independent of the presence of lactate (Table 3), and exerted in circumstances of overload with Ca unaccompanied by ATP depletion, such as in the presence of ouabain or a low K concentration in NS. The action of flufenamate seemed to resemble that of dinitrophenol, both of which agents are known to interfere with Ca storage by the mitochondria (Burch *et al.*, 1983; McDougall *et al.*, 1988). Flufenamate and dinitrophenol, therefore, may have enhanced the responses to SI, to ouabain and to lactate discontinuation by raising the myoplasmic Ca concentration by interfering with the storage of Ca in the mitochondria. At the concentrations used in the present experiments, however, both flufenamate and dinitrophenol are known to uncouple oxidative phosphorylation (McDougall *et al.*, 1988). Both are likely, therefore, to have interfered with

ATP production. This probably explains their ability to potentiate the SI-induced depletion of the stores of ATP (Table 3), an action likely to have been due in part at least to the activation of a mitochondrial ATP-ase enzyme (Saeki *et al.*, 1972; McDougall *et al.*, 1983).

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Effects of endogenous and synthetic prostanoids, the thromboxane A_2 receptor agonist U-46619 and arachidonic acid on [^{3}H]-noradrenaline release and vascular tone in rat isolated kidney

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1 Rat kidneys were perfused with Krebs-Henseleit solution and the perfusion pressure was monitored. After incubation with [^{3}H]-noradrenaline the renal nerves were stimulated. The stimulation-induced (S-I) outflow of radioactivity was taken as an index of noradrenaline release. The effect of prostaglandins on perfusion pressure, pressor responses to renal nerve stimulation (RNS) and S-I outflow of radioactivity was assessed.

2 Prostaglandin E_2 (PGE_2, 0.06 and 0.6 μM), $PGF_{2\alpha}$ (0.6 μM), PGI_2 (0.6 and 3 μM) and iloprost (0.6 μM) increased perfusion pressure and enhanced pressor responses to RNS. These facilitatory effects of the prostaglandins were not a result of an enhanced transmitter release. In contrast, PGE_2 dose-dependently inhibited, whereas the other prostaglandins failed to modulate S-I outflow of radioactivity. PGE_2 (0.6 μM) also enhanced pressor responses to exogenous noradrenaline.

3 Arachidonic acid (1 μM) increased perfusion pressure and enhanced pressor responses to RNS. These effects were abolished in the presence of indomethacin (10 μM) suggesting that local production of prostaglandins from exogenous arachidonic acid was responsible for this facilitation. However, arachidonic acid (1 μM) did not modulate S-I outflow of radioactivity. Arachidonic acid (10 μM), despite causing a marked increase in perfusion pressure, failed to alter pressor responses to RNS and only slightly inhibited S-I outflow of radioactivity.

4 The thromboxane A_2 (TxA_2) receptor agonist U-46619 (0.1 μM) increased vascular tone and enhanced pressor responses to RNS. These effects were blocked by the newly developed selective TxA_2 receptor antagonist, daltroban (BM 13505; 3 μM), suggesting that these facilitatory effects of U-46619 were due to activation of TxA_2 receptors. However, U-46619 failed to alter the S-I outflow of radioactivity from rat isolated kidney.

5 The α_1-adrenoceptor agonist methoxamine (1 μM) also increased perfusion pressure and enhanced pressor responses to RNS without affecting the S-I outflow of radioactivity in the presence of the prostaglandin synthesis inhibitor indomethacin (10 μM).

6 The results suggest that PGE_2 modulates noradrenaline release through an inhibitory prejunctional receptor mechanism. There is no evidence for prejunctional $PGF_{2\alpha}$, PGI_2 or TxA_2 receptors in the rat isolated kidney. All prostaglandins increased vascular tone in the rat isolated kidney and this alone may provide a condition for enhanced pressor responses to RNS since methoxamine also enhanced pressor responses to RNS without affecting S-I outflow of radioactivity. It is probable that postjunctionally active $PGF_{2\alpha}$ and PGI_2 is formed locally from exogenous arachidonic acid, but not enough prejunctionally active PGE_2 is synthesized to modulate renal transmitter release.

Introduction

The kidney receives a dense innervation by the sympathetic nervous system. These renal sympathetic nerves which reach afferent and efferent arterioles, distal and proximal tubules and juxtaglomerular cells regulate renal blood flow, renin secretion and sodium reabsorption (DiBona, 1982). Although the ultimate control over these cardiovascular functions of the kidney is determined by the sympathetic nerve

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activity of the central nervous system to the kidney, there also may be local mechanisms modulating renal noradrenaline release. In many other tissues postganglionic sympathetic nerve terminals possess a variety of receptors which when activated by appropriate agonists either enhance or inhibit the amount of noradrenaline released per nerve impulse (Starke, 1977; Westfall, 1977; Rand *et al.*, 1980; Langer, 1981). Some of these prejunctional receptor systems seem also to operate in the kidney (Rump, 1987).

The most interesting modulators of renal noradrenaline release are endogenous substances which can be formed locally and released within the kidney. Those substances should be potent, physiological modulators of renal noradrenergic transmission. Neuronally released noradrenaline itself has been shown to inhibit its own release via activation of prejunctional α_1- and α_2-adrenoceptors in the rat kidney (Rump & Majewski, 1987a). Other prime candidates for intrarenal modulators of noradrenaline release are prostaglandins. They have been shown to be released from the kidney by renal nerve stimulation (RNS) (Needleman *et al.*, 1974) and through activation of α_1-adrenoceptors by exogenous α_1-adrenoceptor agonists (Cooper & Malik, 1985). Moreover, prostaglandins can be formed locally in the kidney from exogenous arachidonic acid (Miller *et al.*, 1986). Generally it has been shown that prostaglandins, mainly prostaglandin E_2 (PGE_2), inhibit noradrenaline release by activating prejunctional inhibitory prostaglandin receptors (Starke, 1977; Hedqvist, 1977). However, the pre- and postjunctional effects of the prostaglandins seem to vary between species in one and the same organ and the different prostaglandins may have different effects in one species. Regarding the kidney the data are especially conflicting (Malik, 1978). The aim of this study was to evaluate the effect of the most abundant renal prostaglandins, PGE_2, PGI_2 and $PGF_{2\alpha}$ (Schlondorff & Ardaillou, 1986) on renal vascular tone, pressor responses to RNS and noradrenaline release in the rat isolated kidney. Furthermore, the effect of the TxA_2 receptor agonist U-46619 on renal vascular tone, pressor responses to RNS and noradrenaline release was investigated since this compound has recently been shown to enhance noradrenaline release in the rabbit vas deferens (Trachte, 1988) and it has been postulated that TxA_2 may play a role in the developmental stage of hypertension in the spontaneously hypertensive rat (Stier & Itskovitz, 1988).

Methods

Preparation of the kidney

Male Wistar rats weighing 250–340 g were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}) intraperitoneally (i.p.). An abdominal midline incision was made and the lower aorta was cannulated with polyethylene tubing. The animal was then given 500 u heparin intraarterially (i.a.). The left suprarenal and spermatic vessels were ligated and cut. After ligation of the upper aorta proximal to the left renal artery the kidney was flushed with warmed Krebs-Henseleit solution through the aortic cannula. The renal artery was then cannulated with polyethylene tubing and the perfusion was switched to the renal artery cannula and maintained at a constant rate of 6.0 ml min^{-1}.

All connective tissue including the capsule was carefully removed before the renal artery and vein and the ureter were cut. A bipolar platinum electrode was placed around the renal artery and the kidney was transferred to a jacketed glass chamber maintained at a temperature of 37°C. The perfusion medium was gassed continuously with a mixture of 95% O_2 and 5% CO_2 and passed through an 0.8 μm filter before it reached the kidney. The perfusate was allowed to drip out of the cut end of the renal vein and ureter and was collected in 3 min samples with a fraction collector for estimation of radioactivity.

Experimental protocol (Part A)

After a stabilization period of 20 min the kidney was perfused with (−)-[ring-2,5,6-^{3}H]-noradrenaline (2 μCi ml^{-1}, 0.2 μM) for 15 min. During this perfusion the effluent was filtered through glass wool and a 0.8 μm filter before it was recirculated into the kidney. After this incubation the kidney was perfused with drug-free Krebs-Henseleit solution for 90 min to remove loosely bound radioactivity. After 70 min of this washing procedure a priming stimulation at 5 Hz for 30 s was given. Then 18 consecutive 3 min samples of the effluent were collected. There were two stimulation periods (S_1 and S_2) at 1 Hz for 30 s (1 ms duration, 50 V), applied 9 and 39 min after the start of collection of the perfusate, respectively. Perfusion pressure was measured continuously with a Statham P23 Db pressure transducer coupled to a Watanabe pen recorder. Drugs were added to the perfusion solution 15 min before the second stimulation period (S_2). In some experiments a drug was present for both stimulation periods (throughout). In this case the drug was added to the perfusion solution directly after the priming stimulation.

Determination of radioactivity

The radioactive content of the effluent was estimated by liquid scintillation counting and corrected for counting efficiency as determined by automatic external standardization. Aliquots (4 ml) were mixed with 10 ml of scintillation fluid (Picofluor 40, Packard Instruments) for measurement of radioactivity.

Calculation of data

The spontaneous outflow of radioactivity from the tissue was determined as the mean of the amount of radioactivity in the effluent collected during the 3 min collection period immediately before stimulation and the 3 min collection period starting 9 min after the onset of stimulation. The S-I outflow of radioactivity was calculated by subtracting the spontaneous outflow of radioactivity from the radioactivity present in the three 3 min samples collected immediately after the start of stimulation. The S-I outflow in the second stimulation period (S_2) was expressed as a percentage of that in the first stimulation period (S_1). S-I pressor responses were measured as the maximum increase of perfusion pressure above basal perfusion pressure. The results were expressed as the difference of the pressor response during S_2 from that during S_1 ($S_2 - S_1$, mmHg). Drugs were added 15 min before S_2. The effect of the drugs on basal perfusion pressure were expressed as the difference of basal perfusion pressure before addition of the drug from that measured just before S_2. All data were analyzed by two tailed Student's t test. Probability levels of less than 0.05 were considered statistically significant.

Experimental protocol (Part B)

After a stabilization period of 20 min, noradrenaline infusions (60 s, 0.3 μM) directly into the perfusion line or electrical stimulations at 2 Hz (30 s, 1 ms duration, 50 V) were given to produce pressor responses. When constant pressor responses were achieved either 9 consecutive noradrenaline infusions or 9 consecutive electrical stimulations were started, each 3 min apart (P_1–P_9). The effect of PGE_2 was tested by adding it to the perfusion solution immediately after P_6. Pressor responses to noradrenaline or to electrical stimulation were calculated as the maximum increase of perfusion pressure above basal perfusion pressure. The mean of the three pressor responses (P_4–P_6) preceding the addition of PGE_2 was compared to the mean of the three pressor responses (P_7–P_9) following the addition of PGE_2. The data were analyzed by Student's paired t test. Probability levels of less than 0.05 were considered statistically significant.

Drugs and vehicles

The Krebs-Henseleit solution had the following composition (mmol l^{-1}): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.45, $NaCO_3$ 25, KH_2PO_4 1.03, D-(+)-glucose 11.1, disodium edetate 0.067 and ascorbic acid 0.07.

The following drugs were purchased: (−)-[ring-2, 5,6-^{3}H]-noradrenaline (NEN, Dreieich, F.R.G.). PGE_2, $PGF_{2\alpha}$, arachidonic acid, indomethacin and methoxamine (Sigma, St. Louis (MO), U.S.A.), PGI_2 (Wellcome, U.K.). The following drugs were generously donated: iloprost (Schering, Berlin, F.R.G.); daltroban (BM 13505) (Boehringer, Mannheim, F.R.G.); U-46619 (11,9 epoxymethano-PGH_2) was a generous gift from Dr Häussinger (Dept. of Gastroenterology, Univ. of Freiburg, F.R.G.).

PGE_2, $PGF_{2\alpha}$, arachidonic acid and indomethacin were dissolved in absolute ethanol before being diluted with Krebs-Henseleit solution. U-46619 was dissolved in dimethyl sulphoxide (DMSO) and methoxamine was dissolved in distilled water before being diluted in Krebs-Henseleit solution. PGI_2 was dissolved in a solution containing sodium chloride (BP 0.147% w/v) and glycine (BP 0.188% w/v) with a pH of 10.5. PGI_2 was infused at a low perfusion rate (0.08 ml min^{-1}) directly into the perfusion line by a perfusion apparatus (Braun, Melsungen). Iloprost before being diluted with Krebs-Henseleit solution was dissolved in a solution with the following composition: Tris buffer 0.2424 mg, ethanol (96%) 10 μl and 1 ml sodium chloride (0.9%). The effect of the drugs was always compared to their respective vehicle control.

Results

Kidneys were isolated from rats, perfused with Krebs-Henseleit solution and incubated with [^{3}H]-noradrenaline. After a washing procedure there were two stimulation periods (S_1 and S_2). The absolute S-I outflow of radioactivity and the S-I pressor response for the first stimulation period (S_1) in the presence and absence of drugs is given in Table 1. In some experiments there was no incubation with [^{3}H]-noradrenaline and only pressor responses to either exogenous noradrenaline or RNS at 2 Hz were assessed. The mean basal perfusion pressure was 41 ± 2.0 mmHg ($n = 91$).

Effects of prostaglandins E_2 and $F_{2\alpha}$ on renal vascular tone and renal sympathetic nerve stimulation at 1 Hz

PGE_2 (0.06 μM and 0.6 μM) and $PGF_{2\alpha}$ (0.6 μM) increased basal perfusion pressure (Figure 1). PGE_2 and $PGF_{2\alpha}$ when given 15 min before the second stimulation period enhanced pressor responses to RNS (Figure 1). However, PGE_2 dose-dependently inhibited the S-I outflow of radioactivity despite causing a marked enhancement of pressor responses (Figure 1). $PGF_{2\alpha}$ did not affect S-I outflow of radioactivity (Figure 1). Neither drug had an effect on the spontaneous outflow of radioactivity (Table 2).

Table 1 Absolute S-I outflow of radioactivity and S-I pressor responses in rat isolated kidney preincubated with [^{3}H]-noradrenaline

	n	*S-I outflow of radioactivity in S_1* (d.p.m.)	*S-I pressor response in S_1* (mmHg)
No drug	67	25117 ± 1420	13.4 ± 1.1
Indomethacin (10 μM)	16	42798 ± 2577*	6.5 ± 0.9*
BM 13505 (3 μM)	9	36140 ± 4021*	17.2 ± 5.1

There were two stimulation periods (S_1 and S_2) at 1 Hz for 30 s. The S-I outflow of radioactivity and the S-I pressor response in S_1 is given as the mean ± s.e. for experiments in the absence and the presence of drugs.
* Significant difference from no drug experiments, $P < 0.05$, Student's t test.

Effect of prostaglandin E_2 on pressor responses to exogenous noradrenaline and to renal sympathetic nerve stimulation at 2 Hz

Exogenous noradrenaline (0.3 μM) given as short lasting infusions (P_1–P_6, each for 60 s) at 3 min intervals caused constant pressor responses in rat kidney (Figure 2b). PGE_2 (0.6 μM) when added to the perfusion solution immediately after P_6 increased basal perfusion pressure by 13.3 ± 4.3 mmHg and significantly enhanced pressor responses to exogenous noradrenaline (Figure 2b) from 60 ± 14 mmHg ($n = 4$) to 131 ± 13 mmHg ($n = 4$, Student's paired t test, $P < 0.05$). RNS at 2 Hz for 30 s in three min intervals also caused constant pressor responses in rat kidney (P_1–P_6, Figure 2a). PGE_2 given immediately after P_6 increased basal perfusion pressure by 14.2 ± 4.4 mmHg and significantly enhanced pressor responses (Figure 2a) from 31 ± 9 mmHg ($n = 6$) to 86 ± 20 mmHg ($n = 6$, Student's paired t test, $P < 0.05$).

Effects of prostaglandin I_2 and the PGI_2 analogue iloprost on renal vascular tone and renal sympathetic nerve stimulation at 1 Hz

PGI_2 (0.6 μM and 3 μM) markedly and dose-dependently increased basal perfusion pressure and enhanced pressor responses to sympathetic nerve stimulation in rat kidney (Figure 3). PGI_2 at either concentration, however, failed to modulate S-I outflow of radioactivity (Figure 3) and had no effect on the spontaneous outflow of radioactivity (Table 2). Iloprost (0.6 μM) slightly increased basal perfusion pressure and enhanced pressor responses to renal sympathetic nerve stimulation (Figure 3); however, it altered neither the S-I outflow of radioactivity nor the spontaneous outflow of radioactivity (Table 2).

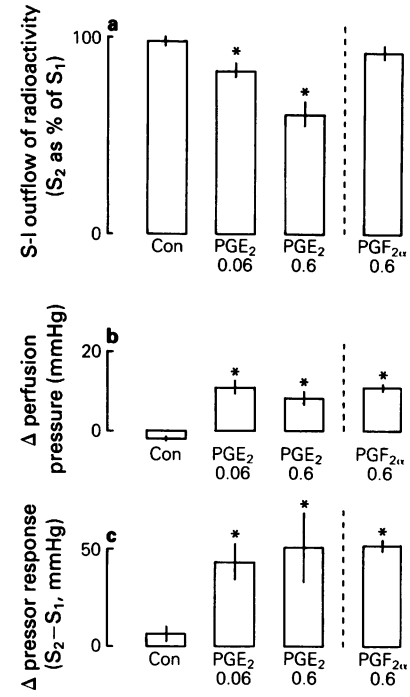

Figure 1 Effects of prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ on the S-I outflow of radioactivity (a), perfusion pressure (b) and S-I pressor responses (c), in rat kidney preincubated with [^{3}H]-noradrenaline. There were two stimulation periods (S_1 and S_2) each at 1 Hz for 30 s. Drugs were added 15 min before S_2: PGE_2 (0.06 μM, $n = 5$), PGE_2 (0.6 μM, $n = 4$), $PGF_{2\alpha}$ (0.6 μM, $n = 6$). The S-I outflow of radioactivity is expressed in S_2 as a percentage of that in S_1. Perfusion pressure is expressed as the difference between the basal perfusion pressure just measured before S_2 and that measured before the addition of the drug. S-I pressor responses are expressed as the difference between the pressor response in S_2 and that in S_1. Vertical lines represent s.e. *Significant difference from control (Con, $n = 6$), $P < 0.05$, Student's t test.

Effect of arachidonic acid on renal vascular tone and renal sympathetic nerve stimulation at 1 Hz

Arachidonic acid (1 μM and 10 μM) markedly and dose-dependently increased basal perfusion pressure (Figure 4). However, only arachidonic acid (1 μM) enhanced pressor responses to RNS (Figure 4). In the presence of indomethacin (10 μM) the arachidonic acid (1 μM and 10 μM) induced increase in perfusion pressure and the arachidonic acid (1 μM)-induced

Table 2 Effects of drugs on the spontaneous (resting) outflow of radioactivity from rat isolated kidney preincubated with [^{3}H]-noradrenaline

Drug	*R2/R1* (%)	n
Control	71.1 ± 5.5	6
PGE_2 (0.06 μM)	61.7 ± 4.0	5
PGE_2 (0.6 μM)	68.5 ± 5.3	4
$PGF_{2\alpha}$ (0.6 μM)	63.0 ± 4.0	6
AA (1 μM)	72.0 ± 7.5	4
AA (10 μM)	61.2 ± 1.6	5
Control	65.6 ± 4.3	7
Iloprost (0.6 μM)	69.8 ± 7.5	4
Control	60.5 ± 4.1	5
PGI_2 (0.6 μM)	71.5 ± 7.5	4
PGI_2 (3 μM)	62.2 ± 2.1	3
Control	65.6 ± 3.0	4
U-46619 (0.1 μM)	73.1 ± 3.1	5
BM 13505 (3 μM) throughout		
Control	71.0 ± 4.7	5
U-46619 (0.1 μM)	80.7 ± 3.7	4
Indo (10 μM) throughout		
Control	73.4 ± 4.4	4
AA (1 μM)	66.2 ± 1.5	4
AA (10 μM)	72.1 ± 6.6	4
Met (1 μM)	68.1 ± 6.1	4

There were two stimulation periods (S_1 and S_2), 30 min apart. Drugs were added 15 min before S_2. The results are expressed as R_2 (resting outflow during S_2) as a percentage of that in R_1 (resting outflow in S_1) and are given as mean ± s.e. There was no significant difference between drug experiments and corresponding controls, $P > 0.05$, Student's t test. Drugs: control, prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin I_2 (PGI_2), iloprost, arachidonic acid (AA), indomethacin (Indo), methoxamine (Met), daltroban (BM 13505) and U-46619.

enhancement of pressor responses to RNS was totally abolished (Figure 4). Only arachidonic acid (10 μM) slightly but significantly inhibited S-I outflow of radioactivity (Figure 4). This small effect was blocked by indomethacin. Neither concentration of arachidonic acid affected the spontaneous outflow of radioactivity (Table 2).

Effect of the thromboxane A_2 analogue U-46619 on renal vascular tone and renal sympathetic nerve stimulation at 1 Hz

U-46619 (0.1 μM) markedly increased basal perfusion pressure and enhanced pressor responses to RNS without affecting the S-I outflow of radioactivity (Figure 5). When the TxA_2 receptor antagonist daltroban (BM 13505; 3 μM) was present throughout the whole experiment the facilitatory effects of U-46619 on perfusion pressure and on pressor responses were abolished (Figure 5). U-46619 did not affect the spontaneous outflow of radioactivity (Table 2).

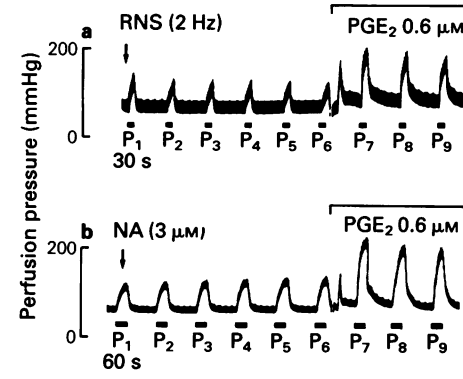

Figure 2 Effect of prostaglandin E_2 (PGE_2) on pressor responses to exogenous noradrenaline (NA) (b) and to renal sympathetic nerve stimulation (RNS) (a) in rat isolated kidney. There were nine pressor responses (P_1–P_9, 3 min intervals) elicited in each experiment either by infusion of exogenous noradrenaline (0.3 μM, 60 s) or by electrical stimulation of the renal sympathetic nerves (RNS, 2 Hz, 30 s). PGE_2 (0.6 μM) was added immediately after P_6.

Effect of the α_1-adrenoceptor agonist methoxamine on renal vascular tone and renal sympathetic nerve stimulation at 1 Hz in the presence of indomethacin

When indomethacin (10 μM) was present throughout the whole experiment methoxamine (1 μM) increased basal perfusion and markedly enhanced pressor responses to RNS (Figure 6). In the presence of indomethacin, methoxamine neither affected the S-I outflow of radioactivity (Figure 6) nor the spontaneous outflow of radioactivity (Table 2).

Discussion

In the present study, kidneys were isolated from rats, incubated with [^{3}H]-noradrenaline and perfused at a constant rate with Krebs-Henseleit solution. The S-I outflow of radioactivity was taken as an index of noradrenaline release (Langer, 1970; Starke, 1977). PGE_2 significantly increased vascular tone in the rat isolated kidney. This vasoconstrictor effect of PGE_2 is in accord with results of other workers using isolated rat tissues. In the rat liver (Häussinger *et al.*, 1987) and kidney (Malik & McGiff, 1975; Foy & Nuhu, 1984) PGE_2 has been shown to increase

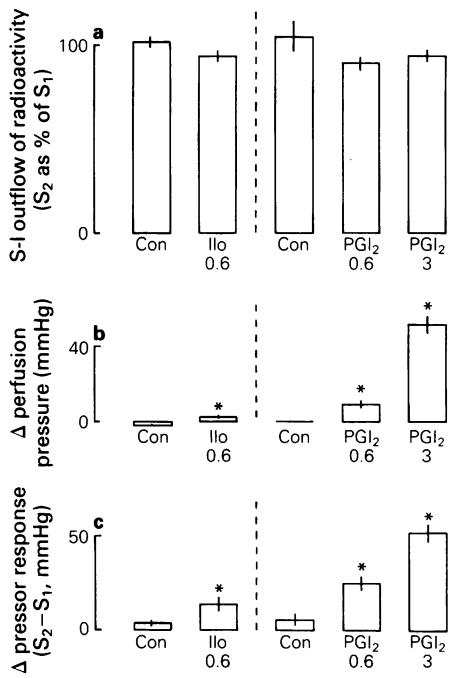

Figure 3 Effects of iloprost and prostaglandin I_2 (PGI_2) on the S-I outflow of radioactivity (a), perfusion pressure (b) and S-I pressor responses (c) in rat kidney preincubated with [^{3}H]-noradrenaline. There were two stimulation periods (S_1 and S_2) each at 1 Hz for 30 s. Drugs were added 15 min before S_2. There were two sets of experiments. (1) Control (Con, $n = 7$), iloprost (Ilo, 0.6 μM, $n = 4$). (2) Control (Con, $n = 5$), PGI_2 (0.6 μM, $n = 4$) and PGI_2 (3 μM, $n = 3$). The S-I outflow of radioactivity is expressed in S_2 as a percentage of that in S_1. Perfusion pressure is expressed as the difference between the basal perfusion pressure measured just before S_2 and that measured before the addition of the drug. S-I pressor responses are expressed as the difference between the pressor response in S_2 and that in S_1. Vertical lines represent s.e. *Significant difference from corresponding control, $P < 0.05$, Student's t test.

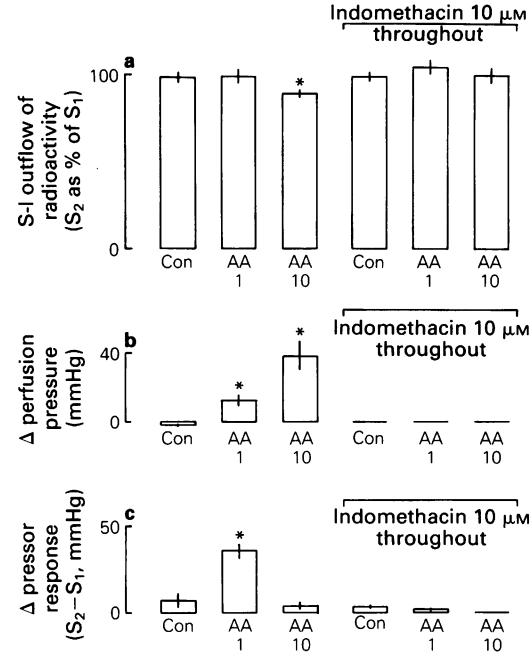

Figure 4 Effects of arachidonic acid on the S-I outflow of radioactivity (a), perfusion pressure (b) and S-I pressor responses (c) in rat kidney preincubated with [^{3}H]-noradrenaline. There were two stimulation periods (S_1 and S_2) each at 1 Hz for 30 s. Drugs were added 15 min before S_2. There were two sets of experiments. (1) In the absence of other drugs: Control (Con, $n = 6$), arachidonic acid (AA, 1 μM, $n = 4$), arachidonic acid (AA, 10 μM, $n = 5$). (2) Indomethacin (10 μM) present for both stimulation periods: Control (Con, $n = 4$), arachidonic acid (AA, 1 μM, $n = 4$), arachidonic acid (AA, 10 μM, $n = 4$). The S-I outflow of radioactivity is expressed in S_2 as a percentage of that in S_1. Perfusion pressure is expressed as the difference between the basal perfusion pressure measured just before S_2 and that measured before the addition of the drug. S-I pressor responses are expressed as the difference between the pressor response in S_2 and that in S_1. Vertical lines represent s.e. *Significant difference from corresponding control, $P < 0.05$, Student's t test.

vascular tone. *In vivo*, however, PGE_2 has been shown to cause either vasoconstriction (Gerber & Nies, 1979) or vasodilatation (Inokuchi & Malik, 1984) in the rat kidney. In the latter study PGE_2 also inhibited pressor responses to RNS. The vasoconstrictor effect of PGE_2 in the isolated kidney may be due to its stimulation of angiotensin II synthesis (Schör *et al.*, 1981). However, although there may be a local production of angiotensin II within the rat isolated kidney (Rump & Majewski, 1987b), neither the angiotensin converting enzyme inhibitor, captopril (0.1 and 5 μM) nor the angiotensin II receptor blocker, saralasin (0.1 and 1 μM) could prevent the PGE_2-mediated increase in basal perfusion pressure (data not shown). There may, however, be another explanation. PGE_2 may have both smooth muscle vasodilator and vasoconstrictor components which may be mediated by two different prostaglandin (EP_1 and EP_2) receptors (Coleman *et al.*, 1986; Vermue *et al.*, 1987). Therefore it is possible that in the absence of vascular smooth muscle tone (as in this study in the rat isolated kidney) PGE_2 mediates vasoconstriction by preferentially activating EP_1 receptors. However, when there is a substantial vascular tone in the rat kidney (*in vivo* or *in vitro*, in experiments raised by exogenous vasopressin or

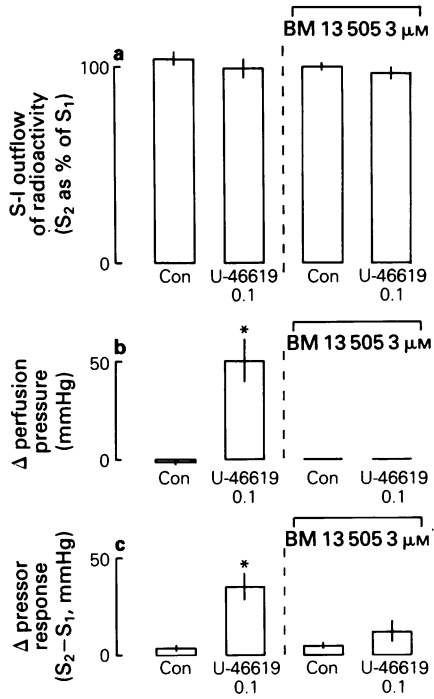

Figure 5 Effects of U-46619 on the S-I outflow of radioactivity (a), perfusion pressure (b) and S-I pressor responses (c) in rat kidney preincubated with [^{3}H]-noradrenaline. There were two stimulation periods (S_1 and S_2) each at 1 Hz for 30 s. Drugs were added 15 min before S_2. There were two sets of experiments: (1) In the absence of other drugs: Control (Con, $n = 4$); U-46619 (0.1 μM, $n = 5$). (2) In the presence of daltroban (BM 13505, 3 μM): Control (Con, $n = 5$), U-46619 (0.1 μM, $n = 4$). The S-I outflow of radioactivity is expressed in S_2 as a percentage of that in S_1. Perfusion pressure is expressed as the difference between the basal perfusion pressure measured just before S_2 and that measured before the addition of the drug. S-I pressor responses are expressed as the difference between the pressor response in S_2 and that in S_1. Vertical lines represent s.e. *Significant difference from corresponding control, $P < 0.05$, Student's t test.

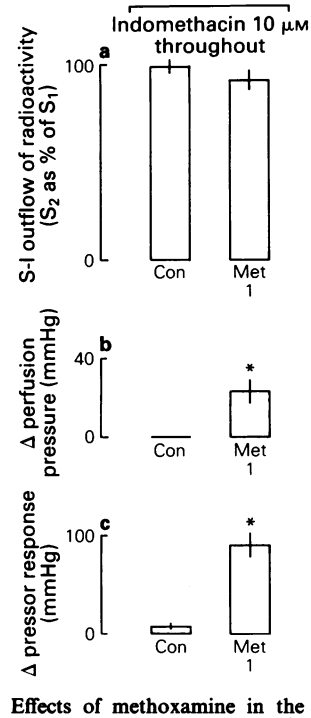

Figure 6 Effects of methoxamine in the presence of indomethacin on the S-I outflow of radioactivity (a), perfusion pressure (b) and S-I pressor responses (c) in rat kidney preincubated with [^{3}H]-noradrenaline. There were two stimulation periods (S_1 and S_2) each at 1 Hz for 30 s. Methoxamine (1 μM) was added 15 min before S_2 and indomethacin (10 μM) was present for both stimulation periods: Control (Con, $n = 4$), methoxamine (Met, 1 μM, $n = 4$). The S-I outflow of radioactivity is expressed in S_2 as a percentage of that in S_1. Perfusion pressure is expressed as the difference between the basal perfusion pressure measured just before S_2 and that measured before the addition of the drug. S-I pressor responses are expressed as the difference between the pressor response in S_2 and that in S_1. Vertical lines represent s.e. *Significant difference from corresponding control, $P < 0.05$, Student's t test.

angiotensin), PGE_2 will dilate the renal vasculature (Inokuchi & Malik, 1984; Pace-Asciak & Rosenthal, 1981) by possibly preferentially activating EP_2 receptors.

In the rat isolated kidney, PGE_2 enhances pressor responses to RNS to a greater extent than to exogenous noradrenaline (Malik & McGiff, 1975) and it was concluded by the authors that in the rat kidney, PGE_2 enhanced noradrenaline release by a facilitatory prejunctional receptor mechanism. In this study PGE_2 also enhanced pressor responses to RNS. However, this effect was not due to an enhanced noradrenaline release. Indeed, PGE_2 markedly inhibited the S-I outflow of radioactivity. This inhibitory effect on noradrenaline release is in accord with findings in many other tissues (Starke, 1977; Hedquist, 1977) including the rabbit isolated kidney (Frame & Hedquist, 1975) and is most likely due to activation of inhibitory prejunctional PGE_2 receptors.

Since in this study PGE_2 enhanced pressor responses to RNS as well as pressor responses to exogenous noradrenaline despite causing a marked inhibition of the S-I outflow of radioactivity, it is evident that PGE_2 in the rat isolated kidney has a postjunctional facilitatory and prejunctional inhibitory effect. What is the reason for this postjunctional

facilitation of pressor responses to RNS in the rat isolated kidney? The α_1-adrenoceptor agonist, methoxamine, increased vascular tone to a similar degree to PGE_2 and this effect was independent of α_1-adrenoceptor-mediated prostaglandin synthesis since the experiments were done in the presence of the prostaglandin synthesis inhibitor, indomethacin. The vasoconstrictor effect of methoxamine is due to selective activation of postjunctional α_1-adrenoceptors since in another study in rat kidney this vasoconstrictor effect was abolished by the selective α_1-adrenoceptor blocking drug, prazosin, but unaltered by the selective α_2-adrenoceptor blocking drug, idazoxan (Rump & Majewski, 1987a). When the renal nerves were stimulated in the presence of methoxamine and indomethacin, pressor responses were enhanced although methoxamine failed to alter the S-I outflow of radioactivity. Thus it is feasible that an increased vascular tone in the rat isolated kidney above basal levels, independent of which substance it is induced by (for instance by PGE_2 possibly via EP_2 receptors), allows enhanced pressor responses to RNS. Hence a slightly preconstricted renal vasculature will respond more sensitively to neuronally released noradrenaline than a renal vasculature without smooth muscle tone since a raised perfusion pressure induced by simply increasing the perfusion volume was without effect on pressor responses to RNS (data not shown). This may in fact explain the postjunctional facilitatory effect of PGE_2 in the rat isolated kidney. However, the possibility that this postjunctional effect of PGE_2 is still due to a specific PGE_2-mediated effect cannot be ruled out entirely. Moreover, this facilitatory effect of PGE_2 may be species-dependent since in the rabbit isolated kidney, PGE_2 inhibited pressor responses to RNS (Frame & Hedqvist, 1975).

In this study in the rat isolated kidney $PGF_{2\alpha}$, PGI_2 and the PGI_2 analogue, iloprost, increased vascular tone and enhanced pressor responses to RNS. This facilitatory effect on pressor responses to RNS is not due to a prejunctional modulatory action of these compounds in the rat isolated kidney, since $PGF_{2\alpha}$, PGI_2 and iloprost failed to modulate S-I outflow of radioactivity of kidneys preincubated with [^{3}H]-noradrenaline. This lack of modulatory actions of the above mentioned prostaglandins on noradrenaline release is in accord with findings in other isolated tissues (Hedqvist, 1977) including rabbit kidney (Hedqvist, 1979). Thus, there is no evidence from the present study that there are prejunctional receptors for either PGI_2 or $PGF_{2\alpha}$ on sympathetic nerve endings in the rat kidney. The enhanced pressor responses to RNS by $PGF_{2\alpha}$ and PGI_2 are possibly due to their direct vasoconstrictor effects in the rat renal vasculature (see above).

The physiological prostaglandin precursor, arachidonic acid (1 μM) markedly increased vascular tone and enhanced pressor responses to RNS in this study in the rat isolated kidney. This facilitatory effect of arachidonic acid (1 μM) on vascular tone and pressor responses seems to be due to its local conversion into prostaglandins since both effects were totally prevented by the cyclo-oxygenase inhibitor, indomethacin. However, those prostaglandins formed intrarenally from exogenous arachidonic acid failed to modulate S-I outflow of radioactivity. Therefore the effect of a 10 times higher concentration of arachidonic acid (10 μM) was investigated in order to raise the levels of locally produced prostaglandins. This high concentration of arachidonic acid markedly increased vascular tone and this effect was also totally prevented by indomethacin. However, arachidonic acid (10 μM) failed to alter pressor responses to RNS and only slightly inhibited the S-I outflow of radioactivity. Since in this study only PGE_2 has been shown to possess a modulatory prejunctional action, it is feasible that insufficient amounts of PGE_2 are formed locally from exogenous arachidonic acid to inhibit S-I outflow of radioactivity. The marked postjunctional effect of arachidonic acid would then be possibly due to a production of PGI_2 and $PGF_{2\alpha}$. Indeed, in the rabbit kidney (Miller *et al.*, 1986) the main prostaglandins formed from exogenous arachidonic acid infused into the renal artery were $PGF_{2\alpha}$ and PGI_2, the latter measured as the stable metabolite $PGF_{1\alpha}$. Thus, in the present study this may explain, why arachidonic acid failed to modulate S-I outflow of radioactivity since both $PGF_{2\alpha}$ and PGI_2 failed to modulate the S-I outflow of radioactivity. The reason why arachidonic acid (10 μM) was ineffective in enhancing pressor responses to RNS despite causing a marked increase in basal perfusion pressure is unclear.

However, RNS and activation of α-adrenoceptors by exogenous agonists has been reported to cause a local preferential formation of PGE_2 in rabbit and rat kidney (Needleman *et al.*, 1974; Cooper & Malik, 1985). Thus, in this study when the renal nerves were stimulated in the presence of indomethacin, the absolute S-I outflow of radioactivity was enhanced. This suggests that in rat kidney, neuronally-released noradrenaline induces PGE_2 formation and inhibits its own release through a transjunctional PGE_2-mediated effect and forms in addition to the autoinhibition via prejunctional α-adrenoceptors (Rump & Majewski, 1987a) another important internal modulatory mechanism of transmitter release in the kidney. This is in accord with another recent study where the α_1-adrenoceptor agonist, methoxamine, inhibited noradrenaline release and this effect was also in part due to α_1-adrenoceptor-mediated local production of prostaglandins (Rump & Majewski, 1987c).

The TxA_2 analogue U-46619 (Coleman *et al.*, 1981) markedly increased vascular tone in the rat kidney. This effect was blocked by the selective TxA_2 receptor antagonist, daltroban (BM 13505; Stegmeier *et al.*, 1986; Patscheke *et al.*, 1989) and was therefore due to activation of TxA_2 receptors. Since TxA_2 serum levels are increased in spontaneously hypertensive rats as compared to age-matched Wistar-Kyoto controls (Stier & Itskovitz, 1988), TxA_2 may be involved in the development of hypertension through a direct postjunctional vasoconstrictor effect. However, it may also enhance noradrenaline release from sympathetic nerves, since the thromboxane A_2 analogue U-46619 facilitates neurotransmitter release from the rabbit vas deferens (Trachte, 1988). A similar effect on the kidney could contribute to the development of hypertension in the spontaneously hypertensive rat. In the present study U-46619, in the same concentration which had a facilitatory effect on transmitter release in the rabbit vas deferens, enhanced pressor responses to RNS and this effect was blocked by daltroban (BM 13505). However, this was not due to activation of prejunctional facilitatory TxA_2 receptors since U-46619 failed to modulate the S-I outflow of radioactivity. The facilitatory effect of U-46619 on pressor responses to RNS was therefore solely postjunctional in nature in agreement with results obtained in the rat anococcygeus muscle (Timini *et al.*, 1978) where U-46619 enhances contractile responses to both electrical stimulation and to exogenous noradrenaline.

In summary, in the present study all prostaglandins tested increased vascular tone and enhanced pressor responses to RNS. Only PGE_2 inhibited the S-I outflow of radioactivity and may therefore be an important inhibitory modulator of renal transmitter release. The facilitatory effect of the prostaglandins on pressor responses seems to be due solely to their postjunctional vasoconstrictor action on the smooth muscle in the rat renal vasculature.

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Postjunctional α_2-adrenoceptors mediate vasoconstriction in human subcutaneous resistance vessels

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1 *In vitro* studies have been performed on human medium-sized muscular arteries (internal diameter 1–4 mm) in a classical organ bath and with human subcutaneous resistance arteries (internal diameter 103–626 μm) in a microvascular myograph.

2 Although the medium-sized muscular arteries showed no response to either of the α_2-agonists B-HT 933 or UK 14304 in concentrations up to 10 μM, the subcutaneous resistance arteries from all regions examined showed well-pronounced and concentration-dependent responses to B-HT 933, the pD_2 ($-\log EC_{50}$) being 5.11 ± 0.09.

3 In the resistance arteries the α_2-antagonist yohimbine caused a parallel shift to the right of the B-HT 933 concentration-response curve; the yohimbine pA_2 for the B-HT 933 receptor was 7.86 ± 0.12.

4 There was an inverse relationship between the maximum response to B-HT 933 and the calibre of the resistance vessels.

5 These results indicate the presence of a postjunctional α_2-adrenoceptor in human subcutaneous resistance arteries and not in medium sized muscular arteries.

Introduction

Based on *in vitro* experiments with human arteries, the postsynaptic adrenoceptor mediating vasoconstriction in man is generally believed to be of the α_1-subtype (e.g. Glusa & Markwardt, 1983; Steen *et al.*, 1984a,b; Skarby & Andersson, 1984). There have been some indications of an additional adrenoceptor mediating vasoconstriction. Moulds & Jauernig (1977) observed a prazosin-resistant component to the noradrenaline response in isolated post-mortem strips of human digital arteries, which was subsequently interpreted as a demonstration of human vascular α_2-adrenoceptors (McGrath, 1982). *In vivo* experiments on responses to systemic infusions of selective α_1- and α_2-agonists in man (Elliot & Reid, 1983; Goldberg & Robertson, 1984) have also supported the concept of heterogeneity of postjunctional α-adrenoceptors in the human vasculature.

The *in vitro* evidence, however, for a postjunctional α_2-adrenoceptor in man at the resistance level is slender. In this study we have investigated the possible distribution characteristics of vasoconstrictor α_2-adrenoceptors in man by comparing large muscular arteries isolated from a variety of sites with resistance arteries taken from subcutaneous fat. A pressor response was classified as α_2-adrenoceptor-mediated provided it could be evoked by the selective α_2-agonist, B-HT 933 (Timmermans & van Zwieten, 1980).

Methods

Human blood vessels were obtained from tissues or organs resected at surgery. Material was collected fresh at the time of operation and placed in cold Krebs buffer.

Medium-sized muscular arteries (internal diameter 1–4 mm) were obtained from a total of 23 patients (age range 20–80 years, 10 male) from colic ($n = 34$),

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splenic ($n = 12$), renal ($n = 8$), brachial ($n = 3$), femoral ($n = 2$) and gastric ($n = 2$) sites; where n = number of segments. The arteries were prepared as ring segments (in total 61 segments, length 3–5 mm) within 3 h from the time of collection, and mounted between pairs of stainless steel hooks in 6 ml organ baths (Thom *et al.*, 1987). The segments were put under resting tension as described by Towart (1982), and were then allowed to equilibrate for 1 h before starting experiments. The baths contained Krebs buffer, aerated with 95% O_2, 5% CO_2. The mechanical responses were measured by Grass PT03 transducers, amplified and recorded by Grass polygraph. The segments were first cumulatively exposed to (0.1 μM–10 μM) noradrenaline, and subsequently to cumulative concentrations (0.1 μM–10 μM) of the selective α_2-adrenoceptor agonists UK 14304 ($n = 57$) or B-HT 933 ($n = 12$).

Resistance arteries were obtained from 21 patients (age 22–70 years, 17 male); 34 vessels, internal diameter 103–626 μm, were dissected under the microscope from subcutaneous fat from ventral abdominal wall (23 vessels), thigh (5 vessels) or calf (6 vessels). The vessels were subsequently mounted as ring segments (approximately 2 mm long) in a microvascular isometric myograph (Mulvany & Halpern, 1977) by threading them onto two 40 μm stainless steel wires and securing the wires across the supports. The vessels were equilibrated in Krebs buffer within the myograph chamber at 37°C for 1 h and were then, on the basis of passive tension-length characteristics set to a normalised internal circumference L_{100} estimated to be 0.9 times the circumference they would maintain if relaxed and exposed to 100 mmHg (Mulvany & Halpern, 1977). The chamber was bubbled with 95% O_2, 5% CO_2. At the start of each study the vessels were stimulated in turn with (a) K^+-Krebs (Krebs buffer, but with 118 mM KCl substituted for NaCl), (b) 10 μM noradrenaline alone, (c) K^+-Krebs plus 10 μM noradrenaline. These served as standardized control responses, from which the effective active pressures (equal to pressure against which vessels could contract (Mulvany & Halpern, 1977)) were calculated. Vessels which failed to produce more than 100 mmHg effective active pressure for any of these responses were excluded. In 6 vessels two consecutive, cumulative B-HT 933 concentration-response curves were generated in ordinary Krebs buffer (Figure 1a), showing significant loss of efficacy and sensitivity from the first to the second concentration-response curve. This tachyphylaxis could, however, be circumvented (Figure 1b) by partially depolarizing the vessels with 30 nM K^+-Krebs (Krebs buffer with 30 mM KCl substituted for NaCl) as earlier described by Juul *et al.* (1987) for angiotensin. Partial depolarization was accordingly employed for examination of possible yohimbine antagonism of the B-HT 933 responses in 5 different vessels, but all other contractile responses to B-HT 933 were elicited in normal Krebs buffer.

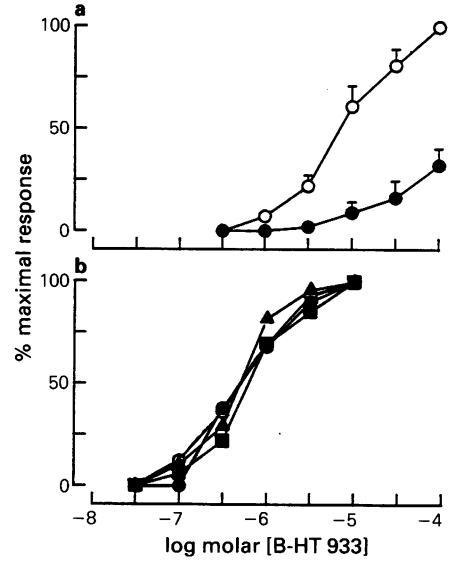

Figure 1 (a) Two sequential cumulative B-HT 933 concentration-response curves (CRCs) generated in ordinary Krebs buffer with 15–20 min intervals demonstrate the problem of tachyphylaxis to B-HT 933 in these vessels: (○) first CRC; (●) second CRC. Values are means normalised as percentage of maximal response obtained in the first concentration-response curve, (s.e.mean shown by vertical bars). Doxazosin (1 μM) was present. Data from 6 different vessels. (b) The tachyphylaxis could be circumvented by generating the B-HT 933 CRCs in partially depolarizing Krebs buffer. CRCs were generated with 15–20 min intervals: (○) first CRC; (●) second CRC; (▲) third CRC; (■) fourth CRC. Values are means normalised as percentage of maximal response obtained in the first CRC. Error bars have been omitted for clarity, but did not exceed 16.7% of the maximal response. Doxazosin (1 μM) was present. Data from 3 different vessels.

Statistics

Individual concentration-response curves were fitted to a logistic function by use of a computer programme. Sensitivities to drugs were calculated on the basis of data from individual vessels and are expressed as $pD_2 = -\log EC_{50}$, the EC_{50} being the agonist concentration needed to produce 50% of the maximal response. Antagonist pA_2's were assessed by Schild analysis (Arunlakshana & Schild, 1959) of

data derived from individual vessels. The results are expressed as means ± s.e.mean.

Drugs and solutions

The Krebs buffer was of composition (mM): NaCl 118, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, $NaH_2PO_4 \cdot 2H_2O$ 1.0, glucose 11.1, $NaHCO_3$ 25.0, $CaCl_2 \cdot 6H_2O$ 2.5, Na_2EDTA 0.03. B-HT 933-2 HCl (2-amino-6-ethyl-4,5,7,8-tetrahydro-6H-oxazolo-(5,4-d)-azepin dihydro-chloride) was a gift from Boehringer Ingelheim. UK 14304 HCl (5-bromo-6-(2-imidazolin-2-ylamino)-quinaxaline bitartrate) and doxazosin HCl were gifts from Pfizer. Noradrenaline HCl, phenylephrine HCl, and yohimbine HCl were obtained from Sigma, U.K. Fresh stock solutions were made up in isotonic saline on the day of the study.

Results

All the 61 large artery segments responded to noradrenaline, but none of them responded to either of the α_2-selective agonists B-HT 933 or UK 14304 (Figure 2).

In contrast, all the 34 subcutaneous resistance vessels from the three regions investigated showed contractile responses to B-HT 933 with 1 μM doxazosin (Figure 3). The vessels responded in a concentration-dependent manner, the pD_2 being 5.11 ± 0.09 (6 vessels), but with subsequent tachyphylaxis (Figure 1a). For the second concentration response curve the maximum response was (30 ± 8)% of that in the first concentration-response curve and the pD_2 value was 4.40 ± 0.11, with the maximum response to B-HT 933 of its own concentration-response curve used as reference. Partial depolarization of the vessels resulted in a marked decrease in pD_2 for B-HT 933 (Figure 4) to 6.37 ± 0.23 (5 vessels), and there was no tachyphylaxis following exposure to B-HT 933 (Figure 1b). The pD_2 values for the first, second, third, and fourth CRC were: 6.28 ± 0.04, 6.16 ± 0.11, 6.23 ± 0.04, and 6.29 ± 0.08, respectively (3 vessels). The maximal response to B-HT 933 in ordinary Krebs buffer was (2.07 ± 0.17) $N m^{-1}$; in partially depolarizing Krebs buffer (1.90 ± 0.18) $N m^{-1}$.

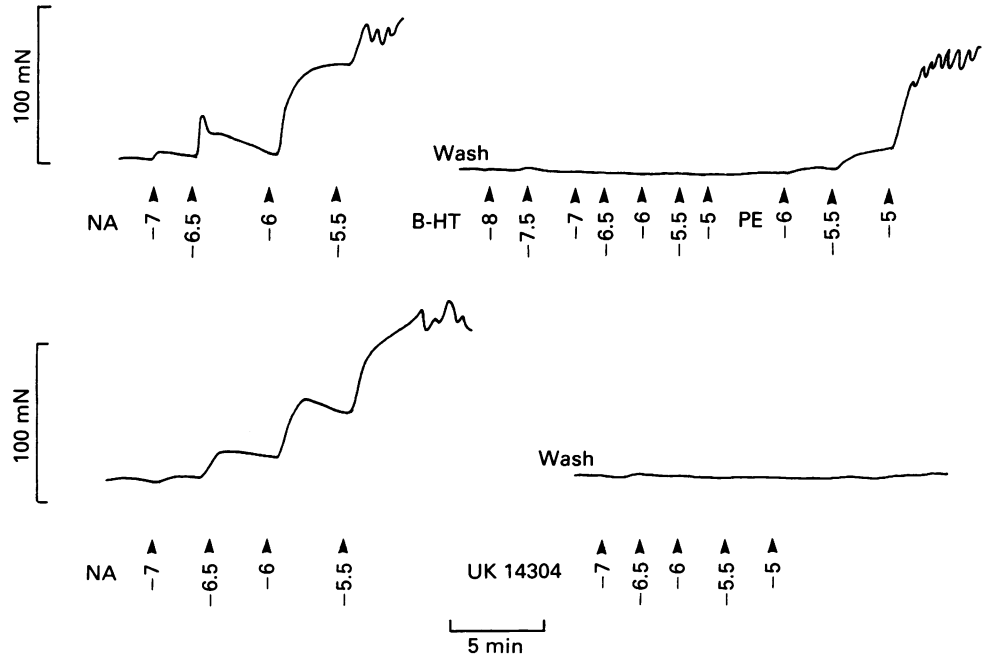

Figure 2 Trace examples of the effects of α_2-agonists B-HT 933 and UK 14304 on human isolated colic artery segments, internal diameter 2 mm. Both segments were responsive to noradrenaline (NA). The upper segment was subsequently exposed to phenylephrine (PE) to confirm α_1-adrenoceptor responsiveness. Drugs were added cumulatively at the indicated log-molar concentrations

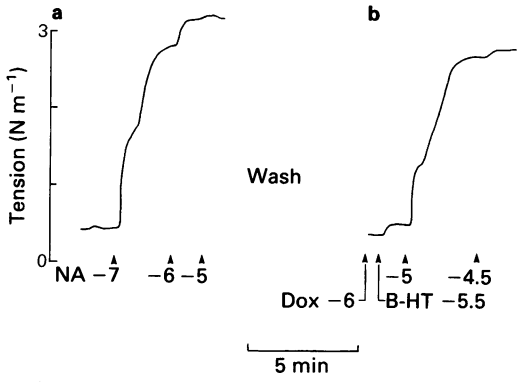

Figure 3 Noradrenaline (a) and B-HT 933 (b) concentration-response curves (CRCs) in a 231 μm subcutaneous resistance artery. Doxazosin (Dox) 1 μM was added 20 min before the generation of the B-HT 933 CRC. Drugs were added cumulatively at the indicated log-molar concentrations.

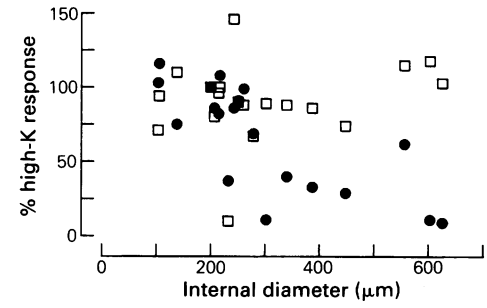

Figure 5 Maximum responses to B-HT 933 (●) and noradrenaline (□) in normal Krebs buffer in the absence of doxazosin in 19 subcutaneous arteries expressed as percentage of maximum response to 125 mM K^+-Krebs, plotted against arterial internal diameter.

Yohimbine competitively antagonized the response to B-HT 933 in the presence of 1 μM doxazosin (Figure 4). Schild analysis showed a pA_2 for yohimbine against B-HT 933 of 7.86 ± 0.12, slope 1.16 ± 0.15 (not significantly different from unity).

The amplitude of the response to 100 μM B-HT 933 in the absence of doxazosin added immediately after the standardised control responses varied with the size of the small vessels (Figure 5), such that there was an inverse relationship between the calibre of the vessels and the maximum B-HT 933 response: vessels smaller than approximately 200 μm developed a response to B-HT 933 equal to the response elicited by K^+-Krebs, in larger vessels the response declined with increasing calibre. However, there was no variation of the response to the standardized 10 μM noradrenaline with vessel size.

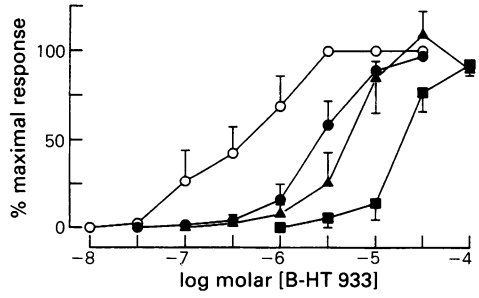

Figure 4 Antagonism of the B-HT 933 concentration-response curves (CRCs) by yohimbine. The B-HT 933 CRCs were generated in partially depolarizing Krebs buffer with 1 μM doxazosin present throughout: (○) control; (●) 3×10^{-8} M yohimbine; (▲) 10^{-7} M yohimbine; (■) 3×10^{-7} M yohimbine. Values are means normalised to the maximum response to B-HT 933 in control curve in the absence of yohimbine; s.e.mean shown by vertical bars.

Discussion

Vascular α-adrenoceptors were originally subdivided on an anatomical basis, with a postjunctional location of the α_1-subtype and a presynaptic location of the α_2-subtype (Langer, 1974). During the last decade, however, there have been several indications from *in vivo* studies of an additional postsynaptic α_2-adrenoceptor mediating vasoconstriction in human arteries (Kiowsky *et al.*, 1983; Goldberg & Robertson, 1984; Jie *et al.*, 1984, 1987). *In vitro* it has been demonstrated in digital arteries (Moulds & Jauernig, 1977; Jauernig *et al.*, 1978) and distal arteries of the foot (Flavahan *et al.*, 1987), that not all postsynaptic adrenoceptors are of the α_1-subtype, but otherwise the arterial postsynaptic α_2-adrenoceptors have been elusive.

The present study lends strong *in vitro* evidence for the presence of a postjunctional α_2-adrenoceptor in human subcutaneous resistance arteries. It is very unlikely that the responses to B-HT 933 were due to stimulation of α_1-adrenoceptors since well pronounced responses to B-HT 933 could be evoked even in the presence of 1 μM doxazosin (Figures 1, 3

and 4), which is an α_1-selective antagonist (van Brummelen *et al.*, 1985). Similarly, the competitive nature of the yohimbine antagonism of the responses to B-HT 933 in the presence of 1 μM doxazosin (Figure 4) indicates, that this response is mediated by one α-adrenoceptor subtype, and the pA_2 value (7.82 ± 0.12) suggests that this adrenoceptor is probably of the α_2-subtype. These data should not be extrapolated to the rest of the circulation, but preliminary studies have demonstrated α_2-mediated contractions in omental, pericardial fat and skeletal muscle resistance vessels (Nielsen *et al.*, 1987). In this context Steen *et al.* (1984a) working with arteries of larger size, internal diameter 1 mm, suggested a small population of postjunctional α_2-adrenoceptors at this level in omental arteries.

It has been easier to demonstrate the presence of postsynaptic α_2-adrenoceptors with *in vivo* techniques than *in vitro* techniques, and from this arises the suggestion that postjunctional α_2-adrenoceptors are present on the smaller resistance vessels and not on the larger muscular arteries. This *in vitro* study indicates that this is the case. The relationship between maximum response to B-HT 933 and vessel calibre suggests that the α_2-adrenoceptor contribution to contractile response begins in the largest of the arteries studied in the myograph and increases with diminishing size. One could argue that the complete absence of responses to B-HT 933 or UK 14304, another selective α_2-agonist (Daly *et al.*, 1988), in all the 61 segments of the large muscular arteries were due to tachyphylaxis to the drugs after exposure to noradrenaline, and this possibility cannot be rejected. On the other hand, the responses to B-HT 933 in the larger resistance arteries were consistently small and could not be enhanced by partial depolarization (H. Nielsen, unpublished observation). It is tempting to put forward the hypothesis, that only in resistance vessels and not in larger muscular arteries do postjunctional α_2-adrenoceptors contribute to contractile responses, and clearly caution is needed before extending the results of the present study to other vascular regions let alone other species, but the apparent absence of postjunctional α_2-adrenoceptors in the superior mesenteric artery of the rat (Agrawal *et al.*, 1984) compared with their presence in precapillary arterioles in the cremaster muscle of the rat (Faber, 1988) lends support to the hypothesis.

The present study has focused on (1) demonstrating responses to the α_2-selective agonist B-HT 933, (2) relating these responses to the size of the vessels, and (3) investigating the effects of an α_2-selective antagonist on these responses, not on examining the effects of selective α_1- and α_2-antagonists on the more relevant agonist, noradrenaline. Preliminary studies, though, with small subcutaneous resistance vessels and small resistance vessels from pericardial fat show that doxazosin in contrast to yohimbine, has no effect on responses to noradrenaline suggesting that small human resistance arteries at least in these tissues are predominantly subserved by α_2-adrenoceptors. Future studies are clearly needed to verify this.

Are the postjunctional α_2-adrenoceptors in human arteries of any physiological or pharmacological importance? Physiologically, the question of whether the α_2-adrenoceptors identified in this study are direct or humoral effectors remains open until further work elaborates the preferred endogenous agonist, innervation density around the relevant vessels and the role of both α_1- and α_2-subtypes in nerve stimulation responses. From a pharmacological point of view, it is interesting that selective postjunctional α_2-antagonists have recently been developed (Ruffolo *et al.*, 1987) and the apparent predilection of postjunctional α_2-adrenoceptors for small resistance vessels could make them useful as anti-hypertensive drugs provided they were without any central action.

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Mode of antinociceptive action of flupirtine in the rat

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1 Flupirtine is a novel, centrally acting, non-opioid analgesic agent. The present investigation was undertaken to ascertain which neuronal systems might be responsible for its antinociceptive effect in rodents. The antinociceptive responses to the test compounds were examined in the tail-flick test.

2 The selective destruction of noradrenergic pathways by 6-hydroxydopamine considerably reduced the flupirtine-induced inhibition of nociceptive responses but not the clonidine-induced antinociception which was significantly enhanced. Depletion of spinal 5-hydroxytryptaminergic pathways by pretreatment with 5,7-dihydroxytryptamine failed to affect the action of flupirtine and clonidine.

3 The depletion of neurotransmitters by reserpine totally abolished the antinociceptive action of flupirtine. By contrast, clonidine-induced inhibition of nociceptive responses remained unchanged.

4 Inhibition of the synthesis of noradrenaline by α-methyl-L-*p*-tyrosine attenuated the antinociception induced by flupirtine. In contrast, inhibition of the synthesis of 5-hydroxytryptamine by (±)-6-fluorotryptophan did not influence the antinociceptive activity of flupirtine.

5 Inhibition of noradrenaline uptake by imipramine led to a significant augmentation of flupirtine-induced antinociception.

6 Selective antagonists at α-adrenoceptors significantly decreased the antinociceptive action of flupirtine. Antinociception induced by clonidine was significantly diminished by idazoxan but not by prazosin.

7 The 5-hydroxytryptamine (5-HT) antagonist, ketanserin diminished the antinociceptive activity of flupirtine, probably due to its additional α_1-adrenoceptor antagonist activity. The antinociceptive effect of clonidine was not influenced by ketanserin.

8 Cholinoceptor antagonists such as mecamylamine and pirenzepine did not alter the antinociceptive action of flupirtine. Flupirtine-induced antinociception also remained unchanged after pretreatment with haloperidol.

9 Flupirtine has no pharmacologically relevant affinity for α_1-, α_2-adrenoceptors, 5-HT_1- and 5-HT_2-receptors as shown in direct binding studies.

10 The present results indicate that the antinociceptive action induced by flupirtine depends on the descending noradrenergic pain-modulating system.

Introduction

Flupirtine is a novel, non-opioid, non-addictive analgesic compound with a chemically unique structure (Engel, 1987). Jakovlev *et al.* (1985) have shown that flupirtine inhibited the nociceptive responses induced by chemical, thermal, mechanical and electrical stimuli in rodents. The antinociceptive effect of flupirtine was not abolished by naloxone (Jakovlev *et al.*, 1985; Carlsson & Jurna, 1987) and it showed no affinity for opioid receptors (Nickel *et al.*, 1985). Flupirtine, when administered intracerebroventricularly or intrathecally has been shown to possess antinociceptive activity (Nickel *et al.*, 1985; Jakovlev *et al.*, 1985; Carlsson & Jurna, 1987). Furthermore, this compound does not alter eicosanoid production (Brune, unpublished results). These previous observations indicate that flupirtine acts to produce antinociception through a central action in which opioid mechanisms play no role. The analgesic activity of

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flupirtine has also been confirmed in clinical trials (Bromm *et al.*, 1987; McMahon *et al.*, 1987; Riethmüller-Winzen, 1987). In a human pharmacodynamic study, Kobal & Hummel (1988) demonstrated that a single dose of flupirtine (200 mg) given orally significantly increased the threshold to painful stimuli. They found an intensification by flupirtine of almost all frequency bands in the EEG of healthy volunteers. Nickel & Zerrahn (1987) demonstrated that these changes in pharmaco-EEG also occurred in the rat and were similar to those induced by clonidine, an α_2-adrenoceptor agonist with analgesic activity. In addition, in animal studies, it has been demonstrated that the antinociceptive activity of flupirtine can be diminished by α-adrenoceptor antagonists (Nickel *et al.*, 1988).

There is growing evidence that noradrenergic and 5-hydroxytryptaminergic descending pathways are involved in pain modulation (Fitzgerald, 1986). The present investigation was therefore undertaken to investigate through which of the descending neuronal systems flupirtine acts to induce inhibition of nociceptive responses.

Methods

Animals

Sprague-Dawley (SIV 50) rats (190–220 g) (Fa. Savo, Kisslegg, F.R.G.) were used. Animals were maintained under standard environmental conditions (room temperature: 21–22°C; relative humidity: 55–60%; light-dark-rhythm: 12/12 h). They had free access to standard pellet food and drinking water but 18 h before the experiments they were deprived of food.

Tail flick test

Ten min after intraperitoneal or subcutaneous administration of flupirtine or clonidine, the antinociceptive action was evaluated by the tail flick test as described by D'Amour & Smith (1941) using an automated unit (Hugo Sachs Electronic, Hugstetten, F.R.G.). The intensity of the light beam was adjusted so that base-line readings were generally at about 5–6 s, and a 20 s cut-off was imposed to avoid excessive tissue damage.

Pretreatment with inhibitors of neurotransmitter uptake and synthesis, neurotoxins, or receptor antagonists

Inhibitors of neurotransmitter synthesis, α-methyl-L-*p*-tyrosine (α-MT), (200 mg kg^{-1}) and (±)-6-fluorotryptophan (6-F-Trp) (120 mg kg^{-1}) were given intraperitoneally 4 and 3 h, respectively, before nociception testing. Neurotoxins were injected intrathecally (i.th.). Twenty min before giving 6-hydroxydopamine (6-OHDA) (20 μg per rat i.th.), 20 mg kg^{-1} pargyline was administered intraperitoneally. Thirty min before the administration of 5,7-dihydroxytryptamine (5,7-DHT) (50 μg per rat i.th.), 25 mg kg^{-1} desipramine was injected intraperitoneally. Animals pretreated with neurotoxins were used 3, 6 and 11 days later. Reserpine was given intraperitoneally at a dose of 2 mg kg^{-1} and the tail flick latency was measured 16 h later. Imipramine (20 mg kg^{-1}) and different receptor antagonists were given intraperitoneally 20 min before administration of flupirtine or clonidine and 10 min later, the tail flick latency was measured.

Receptor binding assays

Receptor binding studies were carried out according to standard procedures as described previously (Borbe & Zierenberg, 1985). Briefly, crude homogenates of rat brain without cerebellum for α_1- and α_2-adrenoceptors and homogenates of rat brain cortex for 5-hydroxytryptamine$_1$ (5-HT$_1$)- and 5-HT$_2$-receptors were used. Male Sprague-Dawley rats (150–200 g; Ivanovas, Kißlegg, F.R.G.) were decapitated and the brains rapidly removed and dissected. The preparations were stored frozen at −50°C and used within 14 days.

For each binding study one preparation ($\approx$1.5 g) was homogenized (Ultra Turrax, 10 s, setting 6) in 20 ml of ice-cold washing buffer (α_1, 0.05 mol l^{-1} Tris-HCl pH 7.4 with 0.25 mol l^{-1} sucrose, 0.001 mol l^{-1} $MgCl_2$ and 0.05% ascorbic acid; α_2, 0.05 mol l^{-1} Tris-HCl pH 7.7; 5-HT$_1$, first centrifugation in 0.32 mol l^{-1} sucrose, further centrifugation in 0.05 mol l^{-1} Tris HCl, pH 7.5; 5-HT$_2$, 0.05 ml l^{-1} Tris HCl, pH 7.7). For α_1-, α_2- and 5-HT$_2$-receptor binding the homogenates were washed twice by centrifugation for 10 min at 48,000 *g*.

For 5-HT$_1$-receptor binding the sucrose plus homogenate was centrifuged for 10 min at 900 *g* and then the supernatant centrifuged for 15 min at 70,000 *g*, the pellet resuspended in the washing buffer, incubated for 10 min at 37°C and centrifuged once more as before. The resulting pellets were resuspended in the following incubation buffers (α_1, 0.05 mol l^{-1} Tris-HCl pH 7.4 with 0.01 mol l^{-1} $MgCl_2$, 0.05% ascorbic acid; 5-HT$_1$, 0.05 mol l^{-1} Tris HCl pH 7.7 with 0.004 mol l^{-1} $CaCl_2$, 0.1% ascorbic acid and 10 μmol l^{-1} pargyline; α_2 and 5-HT$_2$, the washing buffer).

For the receptor binding assays 200 μl of the suspension was incubated with 25 μl of the specific ^{3}H-ligand and 25 μl of the displacer. Non-specific binding was determined in the presence of an excess

Table 1 Protocol of receptor-binding assays

Receptor	*^{3}H-ligand conc.* ($nmol\,l^{-1}$)		*Tissue conc.* ($mg\,ml^{-1}$)		*Blank conc.* ($nmol\,l^{-1}$)	
α_1	Prazosin	1	Brain	66.7	Phentolamine	0.01
α_2	Clonidine	5	Brain	50.0	Phentolamine	0.1
5-HT_1	5-HT	6	Frontal cortex	36.7	5-HT	0.01
5-HT_2	Mesulergine	2	Frontal cortex	20.0	Ketanserin	0.001

The temperature and duration of incubation were as follows: α_1, 25°C and 25 min; α_2, 25°C and 45 min; 5-HT_1, 37°C and 30 min; 5-HT_2, 25°C and 60 min.

of an unlabelled displacer (for details see Table 1). The incubations were terminated by filtration using Whatman GF/C glass fibre filters. After washing the filters with ice-cold incubation buffer the radioactivity was measured by liquid scintillation counting (LKB Rack Beta II) at a counting efficiency of 69%. IC_{50} values were determined by log-probit-analysis using 8 increasing concentrations of the displacers. Each determination was done in triplicate and repeated four times.

Drugs

The drugs used in this study were from the following sources: clonidine hydrochloride, desipramine hydrochloride, 5,7-dihydroxytryptamine creatinine sulphate (5,7-DHT), (±)-6-fluorotryptophan(6-F-Trp), imipramine hydrochloride, 6-hydroxydopamine hydrochloride (6-OHDA), ergotamine tartrate, α-methyl-L-*p*-tyrosine (α-MT), pargyline hydrochloride, pirenzepine dihydrochloride (Sigma Chem. Corp., Munich, F.R.G.); prazosin hydrochloride, reserpine, haloperidol, ketanserin tartrate (Janssen Life Sci., Nettertal, F.R.G.); T61 (Hoechst AG, Frankfurt, F.R.G.). We are grateful to Reckitt & Colman (Hull, U.K.) for the gift of idazoxan.

Clonidine, desipramine, flupirtine, 6-F-Trp, haloperidol, 6-OHDA, idazoxan, imipramine, ketanserin, mecamylamine, α-MT and pirenzepine were dissolved in physiological saline (0.9% w/v). 5,7-DHT was prepared in saline containing $0.2\,mg\,ml^{-1}$ ascorbic acid. Reserpine was dissolved in a mixture containing dimethylacetamide, polyethylenglycol 400, benzyl alcohol and dilute lactic acid. The composition of the final solution corresponds to that of Rausedyl-Ampoules manufactured by G. Richter, Budapest, Hungary.

Labelled compounds [^{3}H]-prazosin (sp. act. 26.2 $Ci\,mmol^{-1}$) and [^{3}H]-5-HT (sp. act. 27.3 $Ci\,mmol^{-1}$) were obtained from NEN (Dreieich, F.R.G.); [^{3}H]-clonidine (sp. act. 24.1 $Ci\,mmol^{-1}$) and [^{3}H]-mesulergine (sp. act. 85.0 $Ci\,mmol^{-1}$) from Amersham (Braunschweig, F.R.G.).

Statistical analysis

Student's *t* test (two-tailed) was employed for analysis of the significant differences between treatment and placebo groups.

Results

Antinociceptive activity of flupirtine and clonidine

Both flupirtine and clonidine increased the tail flick latency in a dose-dependent manner 10 min after their administration (Figure 1). The dose that elevated the nociceptive threshold established in control animals by 50% was $4.11\,mg\,kg^{-1}$ i.p. for flupirtine and $0.16\,mg\,kg^{-1}$ s.c. for clonidine. Based on these results, equipotent doses ($10\,mg\,kg^{-1}$, i.p. of flupirtine, $0.4\,mg\,kg^{-1}$, s.c. of clonidine) were used in subsequent investigations.

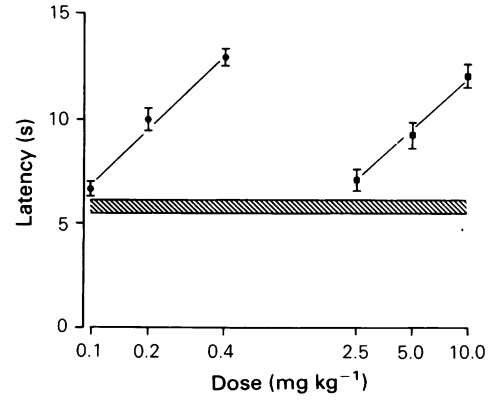

Figure 1 Dose-response curves for the antinociceptive effects caused by flupirtine (■) given intraperitoneally and clonidine (●) administered subcutaneously established in the tail flick latency test on rats. Ordinate scale: tail flick latency in s. Abscissa seale: doses of flupirtine and clonidine. The hatched area corresponds to the mean latency ± s.e.mean of control animals. Each point represents the mean of 12 animals; vertical lines show s.e.mean.

Table 2 Effect of neurotoxins, synthesis and uptake inhibitors on antinociception induced by flupirtine or clonidine in the rat tail flick test

Pretreatment	*Saline* —	*Flupirtine* (10 mg kg^{-1} i.p.)	*Clinidine* (0.4 mg kg^{-1} s.c.)
Neurotoxins			
Saline	5.65 ± 0.22	14.31 ± 0.81	12.50 ± 0.91
6-Hydroxydopamine	4.04 ± 0.13*	6.68 ± 0.21*	16.85 ± 0.68*
Saline	5.92 ± 0.28	12.87 ± 0.57	14.43 ± 0.62
5,7-Dihydroxytryptamine	6.32 ± 0.56	13.51 ± 0.73	13.53 ± 0.51
Depletor			
Saline	5.91 ± 0.28	10.62 ± 0.95	13.98 ± 0.78
Reserpine	3.78 ± 0.38*	4.63 ± 0.22*	12.27 ± 1.02
Synthesis inhibitors			
Saline	5.75 ± 0.18	14.13 ± 0.72	12.45 ± 0.82
α-Methyl-L-*p*-tyrosine	4.17 ± 0.22*	6.84 ± 0.62*	14.17 ± 0.78
Saline	6.31 ± 0.26	12.79 ± 0.88	12.90 ± 0.45
±-o-Fluorotryptophan	5.40 ± 0.31	14.24 ± 1.42	11.85 ± 0.75
Inhibition of noradrenaline uptake			
Saline	5.42 ± 0.23	12.62 ± 0.84	13.38 ± 0.72
Imipramine	6.08 ± 0.33	17.43 ± 1.54*	12.19 ± 1.04

Numbers given represent the tail flick latency of rats in s. All results are the mean ± s.e.mean of at least 10 individual values. Asterisks indicate that the values after drug administration differ significantly from the corresponding controls (saline-treated) ($P < 0.05$). For details of pretreatment see Methods. Clonidine and flupirtine were given 30 min before the measurement of the tail flick latency. Imipramine was injected 20 min before clonidine or flupirtine and 10 min after their administration, tail flick test was performed.

Effect of neurotoxins

After pretreatment with 6-OHDA, tail flick latency was diminished on the 3rd day and remained significantly reduced up to the 11th post-treatment day. Based on these results, tail flick measurements performed on the 3rd, the 6th and the 11th day were pooled (Table 2). The antinociceptive effect of flupirtine was significantly diminished in animals pretreated with 6-OHDA. In contrast, clonidine-induced inhibition of the nociceptive response was slightly but significantly potentiated in 6-OHDA-pretreated rats.

On the 11th day after pretreatment with 5,7-DHT, the nociceptive threshold was unchanged. The antinociceptive action of flupirtine and clonidine were not altered by 5,7-DHT treatment (Table 2).

Effect of reserpine

Sixteen h after giving reserpine (2 mg kg^{-1} i.p.) tail flick latency was significantly reduced in animals receiving saline. The anticociception induced by flupirtine was totally abolished in animals pretreated with reserpine (Table 2). In contrast, the antinociceptive action of clonidine was not influenced by reserpine pretreatment (Table 2).

Effect of synthesis inhibitors

α-MT (200 mg kg^{-1} i.p.) significantly reduced the nociceptive threshold. Furthermore, the antinociceptive effect of flupirtine was almost abolished. By contrast, clonidine-induced antinociception was not influenced by pretreatment with α-MT. The competitive inhibitor of 5-HT-synthesis, 6-F-Trp (120 mg kg^{-1} i.p.) did not affect the tail-flick latency of rats receiving either vehicle, flupirtine or clonidine (Table 2).

Effect of imipramine

The noradrenaline uptake inhibitor, imipramine (20 mg kg^{-1}) given intraperitoneally 30 min before the tail-flick test did not affect the nociceptive threshold in the rat. The antinociceptive activity of flupirtine was, however, significantly enhanced after inhibition of noradrenaline uptake by imipramine (Table 2).

Effect of receptor antagonists

Antagonists at α-adrenoceptors did not affect the tail-flick latency in control animals. However, both α_1- and α_2-adrenoceptor antagonists (prazosin, 2.5 mg kg^{-1} i.p.; idazoxan, 2.5 mg kg^{-1} i.p.) signifi-

Table 3 Effect of receptor antagonists on antinociception induced by flupirtine or clonidine in the rat tail flick test

Pretreatment	*Saline* —	*Flupirtine* (10 mg kg^{-1} i.p.)	*Clonidine* (0.4 mg kg^{-1} s.c.)
α-Adrenoceptor			
Saline	6.30 ± 0.50	13.63 ± 0.88	14.20 ± 0.92
Prazosin	5.92 ± 0.41	6.43 ± 0.35*	14.37 ± 0.75
Saline	5.50 ± 0.25	13.75 ± 0.78	14.92 ± 0.68
Idazoxan	4.82 ± 0.41	8.03 ± 0.21*	8.65 ± 0.43*
Dopamine receptor			
Saline	5.93 ± 0.31	12.50 ± 1.08	12.55 ± 0.98
Haloperidol	6.77 ± 0.45	10.30 ± 1.22	12.73 ± 1.02
5-HT receptor			
Saline	5.71 ± 0.28	12.91 ± 1.21	15.41 ± 1.02
Ketanserin	4.77 ± 0.21*	9.09 ± 1.05*	16.20 ± 1.12
Cholinoceptor			
Saline	5.68 ± 0.33	11.97 ± 1.09	13.22 ± 1.05
Mecamylamine	5.71 ± 0.21	10.93 ± 1.10	11.98 ± 0.78
Saline	5.54 ± 0.33	11.76 ± 0.79	14.12 ± 1.11
Pirenzepine	5.32 ± 0.21	12.14 ± 0.92	13.67 ± 0.87

Numbers given represent the tail flick latency of rats in s. All results are the mean ± s.e.mean of at least 10 individual values. Asterisks indicate that the values after drug administration differ significantly from the corresponding saline-treated controls ($P < 0.05$). The following doses of antagonists (mg kg^{-1}, i.p.) were used: haloperidol, 1.0; idazoxan, 2.5; ketanserin, 3.0; mecamylamine, 0.5; pirenzepine, 20; prazosin, 2.5. All antagonists were given intraperitoneally 20 min before administration of flupirtine or clonidine; 10 min after giving flupirtine or clonidine, the tail flick test was performed.

cantly diminished the flupirtine-induced inhibition of the nociceptive response to the thermal stimulus in rats (Table 3). Prazosin did not alter the antinociceptive activity of clonidine. The specific α_2-adrenoceptor antagonist, idazoxan, however, significantly attenuated the effect of clonidine (Table 3). The dopamine antagonist haloperidol (1.0 mg kg^{-1} i.p.) did not alter either flupirtine- or clonidine-induced antinociception. The 5-HT$_2$-receptor antagonist ketanserin (3.0 mg kg^{-1} i.p.) shortened the tail flick latency in control rats and significantly reduced the antinociceptive activity of flupirtine. It did not alter the antinociceptive activity of clonidine. Antinociception induced by flupirtine and clonidine remained unchanged after administration of the cholinoceptor antagonist, mecamylamine (0.5 mg kg^{-1} i.p.) (Table 3). Pirenzepine 20 mg kg^{-1} i.p. had no effect on the antinociceptive action of either flupirtine or clonidine (Table 3).

Receptor binding studies

The affinity of flupirtine for α_1-, α_2-adrenoceptors, 5-HT$_1$- and 5-HT$_2$-receptors of rat brain was investigated. The results summarized in Table 4 indicate that this drug has no pharmacologically relevant affinity for these receptors. For each receptor assay the IC_{50} value of one pharmacologically relevant displacer is also given in Table 4.

Discussion

Flupirtine has been postulated to produce antinociception by a central action (Jakovlev *et al.*, 1985; Nickel *et al.*, 1985; Szelenyi & Nickel, 1987). The fact

Table 4 Displacement by flupirtine and other ligands of α_1-, α_2-adrenoceptor and 5-HT$_1$-, 5-HT$_2$-receptor binding to rat brain homogenates.

	Concentration (nmol l^{-1})	*Inhibition* (%)
α_1-Adrenoceptor		
Flupirtine	10,000	21.30 ± 1.90
Prazosin	1.74 ± 0.08*	50
α_2-Adrenoceptor		
Flupirtine	10,000	20.30 ± 5.56
Clonidine	10.6 ± 0.53*	50
5-HT$_1$-receptor		
Flupirtine	100,000	No effect
Ketanserin	10,000	5.05 ± 2.43
Ergotamine	16.1 ± 2.1*	50
5-HT$_2$-receptor		
Flupirtine	75,100 ± 2020	50
Ketanserin	5.4 ± 0.50	50

The data given represent means ± s.e.mean of 4 individual experiments each performed in triplicate.
* IC_{50} value.

that the antinociceptive effect of flupirtine injection systemically or i.th. reached its maximum 5 min after administration may also indicate a central site of action (Carlsson & Jurna, 1987). Since the drug has practically no inhibitory activity on arachidonic acid metabolism (Brune, unpublished results; Darius & Schrör, 1985), it is unlikely that a non-steroidal anti-inflammatory drug-like mechanism would be involved in its antinociceptive action. Moreover, on the isolated perfused ear of rabbits, flupirtine inhibited the pain reaction (fall in blood pressure) (Juan & Lembeck, 1974) to bradykinin over a concentration range far greater than the blood levels observed to reduce nociception *in vivo* (Schweizer, unpublished results; Hlavica & Niebch, 1985). All previous experimental and human pharmacodynamic results suggest that flupirtine acts centrally and that its mode of action clearly differs from that of opioids.

The involvement of central 5-hydroxytryptaminergic and noradrenergic pathways in the mediation of pain transmission is well established. A possible interaction of descending noradrenergic and 5-hydroxytryptaminergic pathways has also been implied (Zemlan *et al.*, 1980; Archer *et al.*, 1986). The main purpose of the present study was to investigate whether or not these pathways are involved in the action of flupirtine. Flupirtine significantly prolonged the latency of the tail-flick in rats. The inhibitor of the storage of monoamines, reserpine and depletion of noradrenaline by 6-OHDA in the spinal cord abolished the flupirtine-induced antinociception. α-MT, an inhibitor of noradrenaline synthesis also attenuated the antinociceptive effect of flupirtine. Inhibition of noradrenaline uptake by imipramine resulted in an enhancement of flupirtine-induced antinociception. Blockers of α-adrenoceptors such as prazosin and idazoxan significantly decreased the antinociception induced by flupirtine. The 5-HT_2-antagonist, ketanserin diminished slightly but significantly the flupirtine-induced antinociception. Neither the dopamine receptor antagonist, haloperidol nor the cholinoceptor antagonists, mecamylamine and pirenzepine altered the antinociceptive activity of flupirtine.

In general, all interventions with the noradrenergic system resulted in diminution or abolition of the antinociceptive effect of flupirtine, suggesting that noradrenergic mechanisms are involved in its antinociceptive action. The only exception was the inhibition of noradrenaline uptake which increased the tail-flick latency to flupirtine. Although some results obtained with flupirtine are different from those with clonidine, there are several lines of evidence indicating the possible involvement of the noradrenergic system in the mode of antinociceptive action of flupirtine. In contrast to clonidine, depletion of noradrenaline by reserpine or 6-OHDA resulted in an attenuation of the antinociception induced by flupirtine. It is very likely that clonidine-induced antinociception is mediated by direct activation of postsynaptic α_2-adrenoceptors (Hayes *et al.*, 1986a,b). There is no doubt that α_1-adrenoceptors are located postsynaptically. In the central nervous system, however, α_2-adrenoceptors are apparently presynaptic as well as postsynaptic. Howe & Yaksh (1982) demonstrated that the antinociceptive effect of clonidine was potentiated by depletion of spinal noradrenergic terminals with 5-OHDA. Our present results confirm this and imply that the receptors that mediate the antinociceptive effect of clonidine are α_2-adrenoceptors located postsynaptically, as suggested by U'Prichard *et al.* (1979). Reserpine ($2\,mg\,kg^{-1}$, i.p.) induced a reduction of threshold in saline-treated control animals in the present study. Kulkarni & Robert (1982) also found hyperalgesia in rats using the tail-flick test 8 h after administration of reserpine, followed by normalisation of the nociceptive threshold by 24 h post application. However, they did not measure the reaction time to radiant heat 16 h after administration of reserpine. In contrast to the hypersensitivity of α_2-adrenoceptors after pretreatment with 6-OHDA, treatment with reserpine did not result in an α_2-adrenoceptor hypersensitivity in our investigations. Watanabe *et al.* (1982) have demonstrated that reserpine treatment causes an increase in the number of clonidine binding sites in the postsynaptic region of rat vasa deferentia. The apparent disagreement between our and Watanabe's results can be explained by the different experimental design. Watanabe *et al.* (1982) treated the animals with reserpine on two consecutive days and the measurement of binding sites was performed on the 3rd day. In our study, the animals were killed 16 h after giving reserpine. Thus, it is likely that the time after administration of reserpine was too short for the development of an α_2-adrenoceptor hypersensitivity.

Direct receptor binding studies for α_1-, α_2-adrenoceptors, 5-HT_1- and 5-HT_2-receptors were performed using rat brain preparations. No relevant affinity for either receptor was found for flupirtine. From these results, it is unlikely that a direct activation of spinal and/or supraspinal α-adrenoceptors by flupirtine is involved in its antinociceptive effects. Hence, the question arises as to whether other types of receptor can be involved in mediating the antinociceptive activity of flupirtine.

Ketanserin has widely been assumed to be a specific 5-HT_2 receptor antagonist. Recent pharmacological investigations clearly demonstrate that ketanserin also possesses antagonistic activities at α_1-adrenoceptors but not at α_2-adrenoceptors in porcine and canine vascular smooth muscle (Nishimura *et al.*, 1987; Sinanovic & Chiba, 1988).

These findings indicate that the antihypertensive effect of ketanserine, to a certain extent, depends on the blockade of α_1-adrenoceptors (Ikeda *et al.*, 1987). Binding studies show that [^{3}H]-ketanserin binds in the nanomolar concentration range to both 5-HT_2- and α_1-adrenoceptors in rat brain membranes (Hoyer *et al.*, 1987). Thus, the diminution of the antinociceptive response to flupirtine could be explained by the α_1-adrenoceptor antagonist activity of ketanserin. The clonidine-induced antinociceptive response remained unchanged upon ketanserin treatment due to the lack of affinity of ketanserin for α_2-adrenoceptors (Nishimura *et al.*, 1987). Our present binding studies clearly demonstrated that flupirtine has no relevant affinity either for 5-HT_1- or for 5-HT_2-receptors. Thus, in agreement with earlier results (Nickel *et al.*, 1985), the involvement of descending 5-hydroxytryptaminergic pain modulating pathways in the antinociceptive effect of flupirtine seems to be unlikely.

Besides the nicotinic antagonist mecamylamine, we also investigated the effect of the muscarinic (M_1) receptor antagonist, pirenzepine. The antinociceptive effect of flupirtine was not reversed by systemic administration of mecamylamine and pirenzepine, suggesting that central nicotinic (Molinero & Del Rio, 1987) and muscarinic (M_1) receptors are not involved in mediating the flupirtine-induced antinociception. The dopamine receptor antagonist haloperidol also did not alter the antinociceptive response induced by flupirtine. Therefore, it is unlikely that dopaminergic mechanisms are involved in the flupirtine-induced antinociception.

Whether flupirtine inhibits the re-uptake of noradrenaline, remains to be investigated. A noradrenaline releasing effect of flupirtine must also be considered. Since the flupirtine-induced antinociception was enhanced by imipramine, a tyramine-like release mechanism can be excluded. If flupirtine does, in fact, release noradrenaline then it occurs via another mechanism which is still unknown.

It can be concluded that the novel non-opioid analgesic, flupirtine is not an α_2-adrenoceptor agonist. However, flupirtine needs an intact noradrenergic system in the central nervous system to elicit its antinociceptive activity. Further investigations are needed to clarify the role of other mechanisms that may be involved in the flupirtine-induced antinociception.

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Physical dependence on diazepam in the dog: precipitation of different abstinence syndromes by the benzodiazepine receptor antagonists Ro 15-1788 and ZK 93426

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1 The effects of the benzodiazepine receptor antagonists Ro 15-1788 (flumazenil) and the β-carboline ZK 93426 were compared in dogs before and after chronic treatment with diazepam.

2 In diazepam-naive dogs, the most prominent behavioural alterations occurring during or after i.v. infusion of Ro 15-1788 up to a dose of 20 mg kg^{-1} were transient sedation, ataxia, and 'hot foot' behaviour, whereas behavioural alterations observed after ZK 93426 were not different from those observed after i.v. infusion of vehicle alone. This indicates that, in contrast to Ro 15-1788, ZK 93426 did not exert partial agonistic activity at benzodiazepine receptors.

3 In dogs treated 3 times daily with diazepam, 1 mg kg^{-1} orally, for 1 week, both benzodiazepine antagonists precipitated abstinence symptoms but the number and severity of withdrawal signs induced by Ro 15-1788 were greater than with ZK 93426.

4 In dogs treated 3 times daily with diazepam, 2 mg kg^{-1} orally, for 2 weeks, severe abstinence symptoms were precipitated in all animals by infusion of either antagonist but differences were found in the type of the symptoms: Ro 15-1788 induced rigid postures or rigid walking with increased muscle tone, tremor, twitches and jerks, whereas ZK 93426 did not alter motility but induced generalized myoclonic jerks and tonic-clonic seizures. A generalized tonic-clonic seizure was also observed in one dog of the trial with infusion of Ro 15-1788.

5 Plasma level determinations during chronic treatment with diazepam showed marked accumulation of the major active metabolite desmethyldiazepam, whereas diazepam levels were at least 15 times lower, which might suggest that desmethyldiazepam was responsible for the development of physical dependence on diazepam.

Introduction

Benzodiazepines, like other sedative-hypnotics, can induce tolerance and physical dependence (cf., Owen & Tyrer, 1983; Smith & Wesson, 1985). Abrupt withdrawal from prolonged treatment with benzodiazepines can lead to an abstinence syndrome with symptoms depending on duration of treatment and daily dosage of the benzodiazepine. In patients treated with high doses of diazepam or chlordiazepoxide for a month or more, severe withdrawal symptoms, such as tonic-clonic seizures (sometimes leading to death), confusion, psychosis and hyperpyrexia may occur, whereas discontinuation of treatment with lower doses may cause more diffuse withdrawal signs, such as anxiety, insomnia, hallucinations, perceptual disturbances, muscle twitching, tremor, anorexia, nausea, tachycardia and increased blood pressure (Smith & Wesson, 1985). Similar symptoms can be seen in animals either upon abrupt withdrawal from chronic benzodiazepine treatment (Turnbull *et al.*, 1981; Martin *et al.*, 1982; McNicholas *et al.*, 1983; 1985; Lamb & Griffiths, 1984; Scherkl & Frey, 1986; 1988) or upon injection of benzodiazepine receptor antagonists, such as Ro 15-1788 (flumazenil; ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo(1,5a)(1,4)benzodiazepine-3-carboxylate), during or shortly after chronic benzodiazepine treatment (Cumin *et al.*, 1982; McNicholas *et al.*, 1983; Lukas & Griffiths, 1984; Scherkl & Frey, 1986; Giorgi *et al.*, 1988; Wilson & Gallager, 1988). Withdrawal symptoms precipitated by benzodiazepine antagonists in monkeys, dogs, cats, rats and mice include retching and vomiting, rigidity, decreased food intake, increased body temperature,

anxiety, tremors, changes in motility, and generalized myoclonic or tonic-clonic seizures (Cumin *et al.*, 1982; McNicholas *et al.*, 1983; Lukas & Griffiths, 1984; Scherkl & Frey, 1986; Giorgi *et al.*, 1988; Wilson & Gallager, 1988).

Although clinicians, in particular anaesthesiologists, have a considerable interest in drugs that would effectively and safely reduce or abolish the pharmacological effects of benzodiazepines, the risk that such drugs, e.g. Ro 15-1788, might precipitate severe withdrawal symptoms in benzodiazepine dependent patients reduces their therapeutic utility. It is therefore of both experimental and clinical interest that recent experiments by Giorgi *et al.* (1988) in cats have indicated that the ability of benzodiazepine antagonists to precipitate withdrawal symptoms in diazepam-dependent animals may depend on the chemical class of the antagonist. While the benzodiazepine derivative Ro 15-1788 precipitated (mild) withdrawal symptoms in cats, the β-carboline ZK 93426, which, like Ro 15-1788, antagonized the acute pharmacological effects of diazepam, did not precipitate withdrawal signs. It was therefore suggested by the authors that benzodiazepine receptor antagonists with a β-carboline structure, such as ZK 93426 (ethyl 5-isopropoxy-4-methyl-β-carboline-3-carboxylate), might be a useful tool for reversing benzodiazepine overdosage without inducing a withdrawal syndrome in benzodiazepine-dependent patients (Giorgi *et al.*, 1988). However, Giorgi *et al.* (1988) studied only one dose of the β-carboline in diazepam-dependent cats leaving the possibility that brain concentrations of ZK 93426 were not high enough to compete with diazepam or its active metabolites for benzodiazepine recognition sites because of drug accumulation during chronic treatment.

In the present study, the effects of Ro 15-1788 and ZK 93426 were compared in dogs chronically treated with different doses of diazepam for different periods of time. The data demonstrate that both benzodiazepine antagonists precipitate severe withdrawal symptoms in diazepam-dependent animals when infused intravenously in sufficiently high doses, but that differences exist in the number, type and intensity of symptoms induced by the antagonists.

Methods

Animals

Six female Beagle dogs (Winkelmann Versuchstierzuchtanstalt, Borchen, F.R.G.), aged 2.5 years and weighing 10 to 11 kg, were used for the experiments.

Acute experiments

For testing of precipitated withdrawal, it was planned to infuse solutions of the respective benzodiazepine antagonists at a constant rate until the occurrence of (severe) abstinence symptoms, such as seizures, as recently described by Wilson & Gallager for rats (1988). Therefore, it was necessary to determine the maximum tolerable volume of vehicle and vehicle plus antagonist in diazepam-naive dogs prior to the chronic experiments with diazepam. Ro 15-1788 and ZK 93426 were dissolved in 50% glycofurol in water at a concentration of $10\,mg\,ml^{-1}$, which was the maximum concentration that could be dissolved. Vehicle (50% glycofurol in water) or vehicle with solubilized antagonist were infused with an infusion pump (Braun, Melsungen) through a thin polyethylene catheter into the cephalic vein of one foreleg at several rates of infusion and different total volumes of vehicle or total doses of antagonist, respectively. From these experiments, an infusion rate of $3\,ml\,min^{-1}$ up to a total volume of $2\,ml\,kg^{-1}$ (equivalent to a total dose of $20\,mg\,kg^{-1}$ of the antagonist) was chosen for the chronic experiments with diazepam. Higher infusion rates resulted in too marked effects of vehicle alone (mainly ataxia). The maximum dose of antagonists chosen ($20\,mg\,kg^{-1}$) corresponded to the maximum dose infused intravenously for withdrawal precipitation in diazepam-dependent rats by Wilson & Gallager (1988). For final evaluation, all 6 diazepam-naive dogs received vehicle infusion at the chosen rate and total volume and, 3–5 days later, infusion of vehicle plus antagonist (3 dogs Ro 15-1788 and 3 dogs ZK 93426). The experiment with antagonist infusion was repeated by a crossover design so that all 6 dogs received each antagonist. The animals were closely examined for changes in general behaviour for several hours after the infusion. Rectal body temperature was measured with an electronic thermometer at 5, 15, 30, 60, 90, and 120 min after infusion.

Chronic experiments

Four to 5 days after the last acute experiment (see above), the first chronic diazepam trial was started, with doses and dosing intervals chosen on the basis of previous experiments in dogs (Löscher & Frey, 1981; Frey *et al.*, 1984). The 6 dogs were given diazepam orally at a dose of $1\,mg\,kg^{-1}$ administered 3 times daily at 07 h 00 min, 15 h 00 min and 23 h 00 min for 1 week. Blood (2 ml) for drug analysis was sampled 1, 2 and 3 h after the first dose as well as immediately before, and 1 and 3 h after the morning doses on days 3 and 5. On day 7, blood was sampled before and 1 h after the last dose of diazepam in the morning. Following blood sampling after 1 h, the respective antagonist was infused at a rate of $3\,ml\,min^{-1}$ with a concentration of $10\,mg\,ml^{-1}$ as described above. In all dogs, the respective antagonist (3 dogs Ro 15-1788 and 3 dogs ZK 93426) was

Table 1 Behavioural effects induced by benzodiazepine receptor antagonists before and after chronic treatment with diazepam in dogs

	Drug-naive dogs			*After 1 week diazepam* (1 mg kg^{-1} orally 3 × daily)		*After 2 weeks diazepam* (2 mg kg^{-1} orally 3 × daily)	
Behavioural effects	Vehicle (n = 6)	Ro 15-1788 (n = 6)	ZK 93426 (n = 6)	Ro 15-1788 (n = 3)	ZK 93426 (n = 3)	Ro 15-1788 (n = 5)	ZK 93426 (n = 6)
Ataxia	5	6	5	1	1	0	3
Sedation	0	5	0	2	0	0	0
Vocalization	0	4	0	3	0	3	1
Increase in body temperature (of at least 0.5°C)	2	2	3	1	0	1	5
Retching, vomiting	0	0	0	1	1	0	0
Hyperventilation	0	0	0	1	0	3	0
Circling	0	0	0	1	0	2	0
'Hot foot' behaviour	0	3	0	0	0	1	2
Disturbed gait with retarded setting of paws (especially forepaws)	0	1	0	1	0	4	0
Rigid postures	0	0	0	0	0	4	0
Immobility	0	0	0	0	0	4	0
Agitation/ hyperexcitation	0	2	0	1	0	2	2
Gross tremor	0	0	0	2	0	5	3
Twitches and jerks	0	0	0	1	1	5	6
non-epileptic	0	0	0	0	1	4	3
epileptic (myoclonic)	0	0	0	1	0	1	3
Generalized tonic-clonic seizures	0	0	0	0	0	1	1
Total number of animals with withdrawal signs	0	0	0	2	2	5	6

Ro 15-1788 or ZK 93426 were solubilized in 50% glycofurol in water at a concentration of 10 mg ml^{-1} and were infused intravenously at a rate of 3 ml min^{-1} up to a total dose of 20 mg kg^{-1}. Infusion of vehicle at the same rate and total volume (2 ml kg^{-1}) was carried out in the same dogs for control. Infusion of the antagonists was undertaken either in drug-naive dogs or after chronic treatment of dogs with diazepam (1 h after the last oral dosing). The listed values indicate the number of dogs displaying each behavioural sign during or after infusion of vehicle or antagonist. Behavioural signs, i.e. ataxia, sedation, vocalization (groaning, growling), increase in body temperature, agitation, and 'hot foot' behaviour, induced by vehicle or antagonist in both drug-naive dogs and diazepam-pretreated dogs were not regarded as signs of a withdrawal syndrome for calculation of total number of animals with withdrawal syndrome in diazepam-pretreated dogs. Twitches and jerks comprise single rapid movements of the head, limbs or body which were further subdivided into twitches and jerks which did not look epileptic and epileptic (generalized myoclonic) jerks which looked like the typical epilepsy-like generalized myoclonic twitches of head and body which can be induced by pentylentetrazol in dogs or other species (Löscher, 1983).

infused up to the maximum dose of 20 mg kg^{-1}, because no severe abstinence symptoms (e.g. seizures) were precipitated during the time of infusion (see Results). Observation of changes in general behaviour and determination of body temperature were carried out as described for the acute experiments. In addition, on the days after the last day of diazepam treatment (and antagonist infusion), dogs were examined closely for behavioural alterations, and body weight and temperature were measured each morning up to 1 week.

Three months after the first diazepam trial, a second experiment with diazepam was undertaken in the same dogs with higher daily doses and longer duration of treatment. In this trial, the animals were treated orally 3 times daily with 2 mg kg^{-1} diazepam for 2 weeks. Blood was sampled 1, 2 and 3 h after the first dose as well as immediately before and 1 and 3 h

Table 2 Effects of benzodiazepine receptor antagonists on body temperature before and after chronic treatment with diazepam in dogs

Pretreatment with diazepam	*I.v. infusion*	*Number of dogs*	*Body temperature* (°C) *Time after i.v. infusion* Control	5 min	15 min	30 min	60 min	90 min	120 min
None	Vehicle	6	38.7 ± 0.1	39.0 ± 0.3*	39.1 ± 0.3*	39.0 ± 0.3*	38.9 ± 0.2*	38.8 ± 0.2	38.8 ± 0.2
None	Ro 15-1788	6	38.5 ± 0.3	38.6 ± 0.5	38.6 ± 0.5§	38.5 ± 0.4§	38.4 ± 0.4	38.4 ± 0.3	38.4 ± 0.2§
None	ZK 93426	6	38.5 ± 0.4	38.6 ± 0.3§	38.7 ± 0.4*§	38.9 ± 0.6*	38.8 ± 0.6*	38.7 ± 0.5	38.7 ± 0.5
1 mg kg^{-1} 3 times daily for 1 week	Ro 15-1788	3	38.6 ± 0.4	38.6 ± 0.2	38.6 ± 0.4	38.7 ± 0.3	38.6 ± 0.2	38.7 ± 0.2	38.7 ± 0.1
1 mg kg^{-1} 3 times daily for 1 week	ZK 93426	3	39.0 ± 0.4	39.0 ± 0.3	39.0 ± 0.4	39.1 ± 0.4	38.9 ± 0.2	38.6 ± 0.2	38.3 ± 0.2
2 mg kg^{-1} 3 times daily for 2 weeks	Ro 15-1788	5	38.5 ± 0.3	38.7 ± 0.3*	38.7 ± 0.2	38.7 ± 0.3	38.7 ± 0.1	38.6 ± 0.2	38.5 ± 0.2
2 mg kg^{-1} 3 times daily for 2 weeks	ZK 93426	6	38.4 ± 0.4	38.8 ± 0.4*	38.9 ± 0.3*#	39.0 ± 0.3*	38.8 ± 0.4*	38.8 ± 0.4*	38.7 ± 0.3*

Ro 15-1788 or ZK 93426 were dissolved in 50% glycofurol in water at a concentration of 10 mg ml^{-1} and were infused intravenously at a rate of 3 ml min^{-1} up to a total dose of 20 mg kg^{-1}. Infusion of vehicle at the same rate and total volume (2 ml kg^{-1}) was carried out in the same dogs for control. Data are means ± s.d. of the number of dogs indicated. Infusion of the antagonists was undertaken either in diazepam-naive dogs or after chronic treatment of the same dogs with diazepam (1 h after the last oral dosing). Control data for the antagonists were determined immediately before the start of infusion of the respective antagonist. Significance of differences from the individual control data determined in each group before infusion of vehicle or antagonist is indicated by asterisks ($P < 0.05$). Significance of differences between data determined in diazepam-naive dogs after vehicle alone and vehicle plus antagonist is indicated by § ($P < 0.05$), while significance of differences between data determined after antagonist infusion before and after diazepam treatment is indicated by # ($P < 0.05$).

after the morning dose on days 3, 8 and 11. On day 15, blood was sampled before and 1 hour after the last dose of diazepam in the morning and the respective antagonist was then infused as in the foregoing experiments. Observation of behavioural changes and determination of body temperature and body weight were carried out as described above.

This second experiment with diazepam ($2\,mg\,kg^{-1}$ 3 times daily for 2 weeks) was repeated after 7 months by a crossover design so that all dogs received each antagonist after high dose diazepam. However, only 5 of the 6 dogs could be used for this crossover experiment, because one dog had an infectious disease at the time of the experiment.

Plasma level determinations

Diazepam and its metabolites desmethyldiazepam and oxazepam were determined in plasma by gas chromatography with electron capture determination as described previously (Löscher & Frey, 1981).

Drugs

For chronic treatment, diazepam was used in the form of commercial tablets containing 2, 5 or 10 mg of diazepam (Valium; Hoffmann-La Roche, Grenzach-Whylen, F.R.G.). Ro 15-1788 was a gift from Hoffmann-La Roche (Basle, Switzerland) and ZK 93426 (ethyl 5-isopropoxy-4-methyl-β-carboline-3-carboxylate) was a gift from Schering AG (Berlin, F.R.G.). Pure samples of diazepam, desmethyldiazepam, oxazepam and medazepam (used as internal standard) for gas chromatography were obtained from Hoffmann-La Roche.

Statistics

Body temperature is given as mean $\pm$ s.d.; significance of differences between data was calculated by the Wilcoxon signed rank test for paired replicates.

Results

Experiments in diazepam-naive dogs

In the acute experiments with i.v. infusion of benzodiazepine antagonists or vehicle alone in diazepam-naive dogs, it was found that at the infusion rate ($3\,ml\,min^{-1}$) and maximum infusion volume ($2\,ml\,kg^{-1}$) chosen, the glycofurol vehicle alone caused transient ataxia (slight weakness in hind legs for about 30–120 s after termination of infusion) in 5 of 6 dogs examined (Table 1) and a moderate but significant increase in body temperature (Table 2). After infusion of Ro 15-1788 ($20\,mg\,kg^{-1}$) ataxia was much more marked and of longer duration than ataxia observed with vehicle alone and the dogs showed sedation, vocalization, 'hot foot' behaviour (i.e. tentative placement and rapid lifting of paws during locomotion or lifting of paws during standing), and (1 dog) disturbed gait with retarded setting of paws, indicating that Ro 15-1788 exerted effects of its own (Table 1). Two dogs showed agitated behaviour during the infusion of Ro 15-1788. In contrast, the only symptom occurring after infusion of ZK 93426 ($20\,mg\,kg^{-1}$) was transient ataxia, which was not different from that observed in the same dogs with vehicle alone, indicating that ZK 93426, at least in dogs, is a more neutral antagonist than Ro 15-1788. With respect to body temperature (Table 2), the rise determined after infusion of vehicle was not observed in the experiments with Ro 15-1788. After ZK 93426, a significant increase in body temperature was found, which, however, was less marked than that observed with vehicle alone (Table 2).

Experiments in diazepam-pretreated dogs

Three times daily treatment of the animals with diazepam, $1\,mg\,kg^{-1}$, for 1 week did not cause any observable side-effects nor did it change body temperature or body weight. Plasma concentrations of diazepam and its metabolites during the period of treatment are shown in Figure 1. Diazepam was extensively metabolized in the animals so that, even after the first dose, levels of desmethyldiazepam and oxazepam were several times higher than those of the parent drug. After repeated dosing, maximum drug and metabolite levels were higher than those determined after the first dosing with diazepam, indicating accumulation. However, average diazepam levels did not exceed $30\,ng\,ml^{-1}$, whereas maximum levels of oxazepam and desmethyldiazepam of about 100 and $400\,ng\,ml^{-1}$, respectively, were determined during prolonged diazepam treatment. Immediately before infusion of benzodiazepine antagonists, i.e. 1 h after the last oral dosing with diazepam at day 7 of the trial, average levels of diazepam, oxazepam and desmethyldiazepam were 8, 62, and $280\,ng\,ml^{-1}$, respectively. Infusion of Ro 15-1788 or ZK 93426 induced behavioural symptoms in some of the animals which were not present after injection of either vehicle or antagonist in diazepam-naive dogs, indicating that only 1 week of treatment with relatively low doses of diazepam had led to the development of physical dependence (Table 1). Thus, with

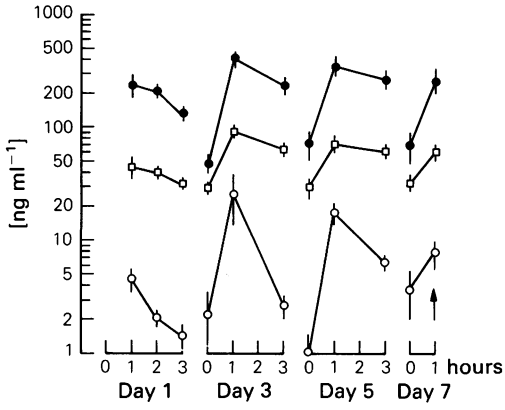

Figure 1 Plasma concentrations of diazepam (○) and its active metabolites desmethyldiazepam (●) and oxazepam (□) during treatment of dogs with diazepam (1 mg kg^{-1}, orally) three times daily for 1 week. Data shown as ng per ml plasma on a logarithmic scale are means (s.e. shown by vertical lines) of 6 dogs. Concentrations were determined 1, 2 and 3 h after the first oral dose of diazepam at day 1 as well as immediately before ('0') and 1 and 3 h after the morning doses at day 3 and 5 of the treatment period. In the morning of day 7, the last dose of diazepam was administered and immediately after blood sampling at 1 h, the benzodiazepine antagonists were infused as indicated by the arrow.

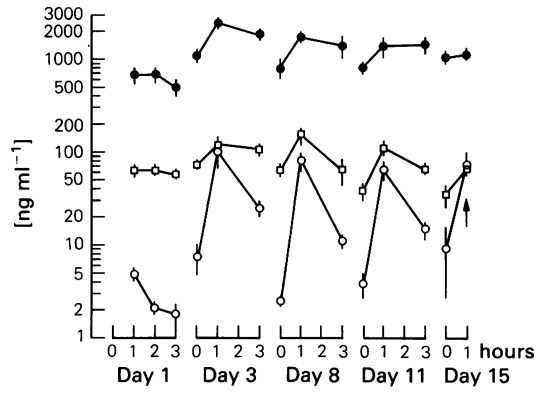

Figure 2 Plasma concentrations of diazepam (○) and its active metabolites desmethyldiazepam (●) and oxazepam (□) during treatment of dogs with diazepam (2 mg kg^{-1}, orally) three times daily for 2 weeks. Data shown as ng per ml plasma on a logarithmic scale are means (s.e. shown by vertical lines) of 6 dogs. Concentrations were determined 1, 2 and 3 h after the first oral dose of diazepam on day 1 as well as immediately before ('0') and 1 and 3 h after the morning doses at day 3, 8 and 11 of the treatment period. In the morning of day 15, the last dose of diazepam was administered and immediately after blood sampling at 1 h, the benzodiazepine antagonists were infused as indicated by the arrow.

Ro 15-1788 in one dog marked hyperexcitation was induced with generalized tremor, myoclonic jerks, hyperventilation, disturbed gait with retarded setting of paws, transient circling and retching. A second dog showed generalized tremor, whereas the third dog tested with Ro 15-1788 did not exhibit abstinence symptoms. With ZK 93426, behavioural changes observed were less pronounced compared to Ro 15-1788. One dog vomited, whereas a second dog showed twitches and jerks, mainly of the head. There were no marked changes in body temperature after infusion of the antagonists (Table 2). On the days after the last administration of diazepam, no further behavioural alterations and no changes in body temperature or body weight were noted.

During three times daily treatment of dogs with diazepam, 2 mg kg^{-1}, for 2 weeks, no side-effects were observed, although maximum plasma concentrations of desmethyldiazepam reached during treatment were about 4–6 times higher than those determined in the first trial with three times daily administration of 1 mg kg^{-1} diazepam (Figure 2) indicating marked accumulation at this daily dosage. Immediately prior to infusion of antagonists, i.e. 1 h after the last dose of diazepam at day 15 of the trial, average levels of diazepam, oxazepam and desmethyldiazepam were 75, 67 and 1200 ng ml^{-1}, respectively. Infusion of antagonists precipitated marked withdrawal symptoms in all dogs of the trial, but differences were observed between the two antagonists (Table 1 and 2). With Ro 15-1788, all 5 dogs showed agitation, hyperexcitation and/or generalized tremor during the i.v. infusion, which, however, was continued up to the maximum dose of 20 mg kg^{-1}. Two of the animals exhibited jerks during the last minute of infusion. After the infusion, 4 of the 5 dogs were immobile with rigid postures (3 dogs in a prone position) and increased muscle tone in limbs (as determined by palpation) and all 5 dogs exhibited generalized tremor, and twitches and jerks of head, limbs or body. In addition, 3 dogs showed pronounced hyperventilation. Immobility lasted for 5 to 30 min after which 3 of the animals still had a disturbed gait with retarded setting of paws and/or rigid walking. In 2 dogs, transient circling was seen. Twitches and jerks were observed for up to 3 h after the infusion. One dog exhibited a generalized tonic-clonic seizure 4.5 h after the infusion. Body temperature was not changed to any significant extent after infusion of Ro 15-1788 (Table 2).

With ZK 93426, 3 dogs exhibited tremor and 4 dogs myoclonic jerks during the last minute of infu-

sion. After the infusion, in contrast to Ro 15-1788 the dogs were able to run around normally (although hot foot walking with tentative placement and rapid lifting of paws was observed in one animal), but showed rhythmic twitches, predominantly of the head (3 dogs), or marked generalized myoclonic jerks (3 dogs) of head and body during forward locomotion. The myoclonic jerks resembled the typical generalized epileptic myoclonic jerks of head or body which can be induced by pentylenetetrazol in diazepam-naive dogs (Löscher, 1983). In the experiments with Ro 15-1788, such epileptic myoclonic jerks had been observed only in one of the animals, and jerks observed in this dog were less marked than those induced by ZK 93426. Three hours after infusion of ZK 93426, one dog had a generalized tonic-clonic seizure, while another dog still showed myoclonic jerks. The dog with the tonic-clonic seizure was not the animal which had had a tonic-clonic seizure after infusion of Ro 15-1788; in other words 2 of a total of 6 dogs, that had been examined by the crossover design, exhibited such severe seizures after infusion of benzodiazepine receptor antagonists.

Body temperature was significantly increased after ZK 93426 infusion (Table 2). At 15 min after infusion, the increase in rectal temperature was significantly higher than that determined with ZK 93426 in diazepam-naive dogs (Table 2).

On the days after the last day of diazepam treatment, all 6 dogs behaved normally and showed no alterations in body weight or body temperature, indicating that the decline in diazepam and metabolite levels did not cause withdrawal symptoms in addition to those already precipitated by the antagonist at times of maximum drug levels.

In both experiments with chronic administration of diazepam, there were no significant differences in drug and metabolite levels between the dogs used for Ro 15-1788 or ZK 93426 withdrawal precipitation, so that differences in abstinence symptoms observed after infusion of the antagonists were not secondary to differences in benzodiazepine levels.

Discussion

It has been demonstrated previously that abstinence symptoms can be precipitated in dogs by benzodiazepine antagonists after prolonged administration of benzodiazepines (McNicholas *et al.*, 1983; Scherkl & Frey, 1986). Using the triazoloquinone benzodiazepine antagonist CGS 8216, McNicholas *et al.* (1983) precipitated abstinence symptoms, such as myoclonic jerks and clonic seizures, in dogs which were given diazepam ($15\,mg\,kg^{-1}$) orally, 4 times daily for at least 2 weeks. However, the selectivity of CGS 8216 as a benzodiazepine antagonist appears to be less than that of the imidazobenzodiazepinone Ro 15-1788, since it also blocks adenosine receptors in reasonable doses (Haefely, 1985). This action, together with a slight negative ('inverse agonistic') efficacy at benzodiazepine receptors, endows CGS 8216 with a potent stimulant and proconvulsive activity (Jensen *et al.*, 1984; Haefely, 1985). When the selective antagonist Ro 15-1788 was used for withdrawal precipitation, anxiety, tremor and increases in body temperature were observed after i.v. injection of the antagonist in dogs chronically treated with clonazepam ($0.5\,mg\,kg^{-1}$ orally, twice daily), whereas abstinence symptoms induced by abrupt discontinuation of clonazepam treatment in dogs were much more severe (Scherkl & Frey, 1986), which might suggest that the dose of the antagonist used for withdrawal precipitation was not high enough. In fact, Wilson & Gallager (1988) have shown that myoclonic twitches and tonic-clonic seizures can be precipitated by i.v. infusion of high doses of Ro 15-1788 ($3–25\,mg\,kg^{-1}$) in rats following continuous exposure to relatively low levels of diazepam and its active metabolites after only 1 week of oral diazepam treatment. Similar results were obtained by Lukas & Griffiths (1984) with Ro 15-1788 ($5\,mg\,kg^{-1}$, i.m.) in diazepam-dependent baboons. Interestingly, in dogs it was shown that 3 times daily oral treatment with 0.25 or $0.5\,mg\,kg^{-1}$ diazepam resulted in marked decrease of the anticonvulsant effect of the treatment after only 1 week of treatment (Frey *et al.*, 1984). It was therefore of interest to see whether physical dependence developed in parallel to tolerance in dogs after this short treatment period.

Indeed, it was found in the present study that i.v. infusion of Ro 15-1788 precipitated abstinence symptoms in dogs after only 1 week of treatment with relatively low doses of diazepam ($1\,mg\,kg^{-1}$, 3 times daily). However, the severity of the withdrawal syndrome and the number of withdrawal signs markedly increased with longer duration of treatment and higher daily doses, which is consistent with previous observations in rats and baboons (Lukas & Griffiths, 1984; Wilson & Gallager, 1988). Interestingly, after precipitation of withdrawal symptoms by the benzodiazepine antagonist, no additional withdrawal signs were observed on the days after discontinuation of chronic diazepam treatment (after abrupt discontinuation of chronic benzodiazepine treatment in dogs, the withdrawal syndrome is most pronounced after 2–3 days; Scherkl & Frey, 1986), possibly indicating a 're-setting' of benzodiazepine receptors by the antagonist. Plasma level determinations in the dogs suggested that physical dependence on diazepam

may have been related to the accumulation and actions of its major metabolite, N-desmethyldiazepam, because levels of this compound reached during chronic treatment with diazepam were at least 15–20 times higher than those of the parent drug. The other metabolite, oxazepam, was present in much lower concentrations. Desmethyldiazepam and diazepam have about the same affinity for benzodiazepine receptors, but the pharmacological potency of desmethyldiazepam is somewhat lower than that of diazepam (Frey & Löscher, 1982). Indeed, more recent data of Gobbi *et al.* (1987) have substantiated that desmethyldiazepam is a partial agonist at the central type of benzodiazepine receptors when compared with the effects of diazepam. Interestingly, chronic treatment with desmethyldiazepam or its pro-drug clorazepate induces physical dependence in dogs and discontinuation of treatment has been shown to result in severe abstinence symptoms, including tonic-clonic seizures and death (McNicholas *et al.*, 1985; Scherkl & Frey, 1989). Accumulation of desmethyldiazepam, as observed in the present experiments, also occurs during chronic treatment with diazepam in man and plasma levels of desmethyldiazepam reached are similar to those determined by us in dogs (cf. Guentert, 1984). Thus, the propensity of some benzodiazepines for forming active metabolites which accumulate may play a role in the development of physical dependence.

When the β-carboline ZK 93426 was used for withdrawal precipitation, abstinence symptoms observed were qualitatively and quantitatively different from those precipitated by Ro 15-1788. ZK 93426 is also a selective benzodiazepine receptor antagonist with an *in vitro* and *in vivo* potency similar to Ro 15-1788 (Jensen *et al.*, 1984). In contrast to Ro 15-1788, which, depending on dose and species examined, exhibits slight partial agonist or inverse agonist activity at high doses (Bonetti *et al.*, 1982; Haefely, 1985), ZK 93426 is an almost neutral benzodiazepine receptor ligand (Jensen *et al.*, 1984; Löscher *et al.*, 1985; De Deyn & Macdonald, 1987). This is also demonstrated by the present experiments in diazepam-naive dogs, in which Ro 15-1788 induced sedation, ataxia and other gait disturbances in high doses, whereas ZK 93426 was devoid of typical benzodiazepine receptor-related effects. In diazepam-dependent dogs, ZK 93426 precipitated myoclonic jerks and, in one animal, generalized tonic-clonic seizures. Although jerks were also induced by Ro 15-1788, they were qualitatively different from those induced by the β-carboline, which, in 3 of 6 animals, precipitated myoclinic fits resembling those caused by chemical convulsants, such as pentylenetetrazol, in dogs. Thus, after the high dose diazepam treatment studied with a crossover design, 4 dogs showed epileptic (myoclonic or generalized tonic-clonic) seizures after infusion of ZK 93426 compared to 2 dogs with (milder) myoclonic or generalized tonic-clonic seizures precipitated by Ro 15-1788. However, the most prominent difference between behavioural symptoms precipitated by Ro 15-1788 and ZK 93426 in diazepam-dependent dogs was the increase in muscle tone associated with long lasting immobility and rigid postures which was observed after Ro 15-1788 but not after ZK 93426. Rigid postures and/or increased muscle tone after withdrawal precipitation by Ro 15-1788 have also been examined recently in diazepam-dependent cats (Giorgi *et al.*, 1988) and baboons (Lukas & Griffiths, 1984).

The reasons for the differences in abstinence symptoms precipitated by the two receptor antagonists are not clear. It has been suggested that β-carbolines display binding properties different from those of benzodiazepines and that the coupling of the β-carboline recognition sites to the GABA-chloride ionophore complex is not identical to that modulated by benzodiazepine derivatives (cf. Biggio & Costa, 1983). However, these suggestions were based on brain receptor studies in which full agonists of the benzodiazepine group were compared with partial or full inverse agonists of the β-carboline group, which could explain most differences in binding or coupling characteristics found. Although more recent experiments provided some evidence that in the spinal cord there may be differences in the actions of β-carbolines and benzodiazepines at the GABA/benzodiazepine receptor complex (Santi *et al.*, 1988), no such differences were found when Ro 15-1788 and ZK 93426 were compared in *in vitro* and *in vivo* binding studies with brain tissue, thus indicating that both antagonists display comparable binding properties in the brain (Jensen *et al.*, 1984). Nevertheless, Giorgi *et al.* (1988) proposed that ZK 93426 interacts with a domain of the benzodiazepine recognition site in the brain which is different from the domain(s) at which benzodiazepines interact. This proposal was based on experiments in diazepam-dependent cats in which mild abstinence symptoms could be precipitated by Ro 15-1788 but not by ZK 93426, although both antagonists were injected at a dose ($10\,mg\,kg^{-1}$, i.p.) which antagonized the *acute* pharmacological effects induced by a single dose ($7\,mg\,kg^{-1}$, i.p.) of diazepam (Giorgi *et al.*, 1988). The most likely explanation for the weak or absent withdrawal effects after injection of Ro 15-1788 and ZK 93426 is that the dose of antagonists used ($10\,mg\,kg^{-1}$, i.p.) was too low to displace diazepam and its active metabolites from receptor sites in cats undergoing *chronic* administration of diazepam ($7\,mg\,kg^{-1}$ twice daily for 3 weeks), because of the marked accumulation of desmethyldiazepam that takes place during prolonged treat-

ment. As shown in the present study, levels of active benzodiazepines (i.e., diazepam plus active metabolites) after chronic treatment are at least 2 times higher than those determined after a single dose of diazepam. In cats, accumulation of diazepam and desmethyldiazepam is even more pronounced than in dogs, because elimination of these benzodiazepines is much slower in this species (the half-life of diazepam is about 15–20 h in cats compared to 2–5 h in dogs; Frey, 1986). Furthermore, in cats, in contrast to dogs and other species, no tolerance develops during chronic treatment with diazepam (administered at daily doses of 0.5–2 mg kg^{-1}, orally for up to several years; Frey, 1986) which could indicate that differences also exist in development of physical dependence between cats and other species. In any case, it is important to note that we have shown that ZK 93426 is capable of precipitating severe abstinence symptoms in diazepam-dependent animals, when sufficiently high doses are administered, although due to unknown factors the precipitated symptoms differ from those observed with Ro 15-1788.

In this respect, it should be considered that abstinence symptoms precipitated by ZK 93426 in diazepam-dependent dogs were similar to those observed after abrupt discontinuation of chronic treatment with diazepam or desmethyldiazepam in the same species (McNicholas *et al.*, 1983; 1985; Scherkl & Frey, 1988), whereas several symptoms, especially the rigid postures and the immobility, induced by Ro 15-1788 were different from those induced by spontaneous withdrawal in dogs. Possibly the partial agonist and/or inverse agonist activity of Ro 15-1788 could play a role in this respect. Thus, in very high doses, Ro 15-1788 has been shown in different species to induce inverse agonist-like effects, i.e. increased skeletal muscle tone and immobility in mice (at 100 mg kg^{-1}, i.v. or 300–1000 mg kg^{-1}, i.p.), increased muscle tone, tremor and tonic seizures in rats (at 100 mg kg^{-1}, i.v. or i.p.), generalized tremor and catatonia in squirrel monkeys (at 300–1000 mg kg^{-1}, orally), and increased muscle tone and vomiting in dogs (at 300 mg kg^{-1}, orally) (Bonetti *et al.*, 1982). These symptoms were not observed in these species at the much lower doses which were sufficient to antagonize the acute effects of benzodiazepine receptor agonists. Thus, it was concluded that at antagonistic doses, Ro 15-1788 exerts no intrinsic activity (Bonetti *et al.*, 1982). However, Little *et al.* (1986) and Petersen & Jensen (1987) have recently reported that chronic exposure to benzodiazepine agonists, such as flurazepam or lorazepam, while decreasing the sensitivity of the animals to the effects of full or partial agonists, increases markedly the sensitivity of the animals to drugs with partial or full inverse agonist action. Thus, the rigid postures with increased muscle tone observed in the present experiments after infusion of Ro 15-1788 in diazepam-treated but not in diazepam-naive dogs could be explained by such an increase in sensitivity to the partial inverse agonist effects of the drug. Furthermore, since Little *et al.* (1986) and Petersen & Jensen (1987) found that both Ro 15-1788 and ZK 93426 became proconvulsant after chronic treatment with full agonists, the twitches and seizures observed with the two benzodiazepine receptor antagonists in diazepam pretreated dogs could possibly also relate to such a 'withdrawal shift' in the effect of benzodiazepine receptor ligands. In other words, it could be that the symptoms observed after infusion of the antagonists in diazepam-treated dogs were not precipitated withdrawal signs but pharmacodynamic effects of the antagonists because of altered sensitivity of the animals in response to the chronic diazepam treatment. Conversely, most if not all of the experimental findings reported by Little *et al.* (1986) and Petersen & Jensen (1987) could be explained simply in terms of development of tolerance and dependence during the chronic agonist treatment. Indeed, both groups tested the acute effects of the different receptor ligands 24 h after termination of chronic treatment with full agonists, so that e.g. the proconvulsant effects of Ro 15-1788 and ZK 93426 determined at this time could be just due to withdrawal hyperexcitability.

In any case, the present data refute the suggestion of Giorgi *et al.* (1988) that ZK 93426 might have advantages in comparison to Ro 15-1788 in terms of risk of withdrawal precipitation in benzodiazepine-dependent subjects, but clearly demonstrate that severe, although different, syndromes can be induced by high doses of both benzodiazepine antagonists.

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Effect of endothelium on basal and on stimulated accumulation and efflux of cyclic GMP in rat isolated aorta

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1 The aim of this study was to examine the possible role of the release of guanosine 3':5'-cyclic monophosphate (cyclic GMP) into the extracellular space in the regulation of rat aortic cyclic GMP content.

2 Rat aortic segments incubated in physiological solution released cyclic GMP into the medium in a time-dependent manner. This release was greatly enhanced when intact instead of tissues without endothelium were used. After 120 min of observation, a maximal 33 fold difference in extracellular cyclic GMP content was detected.

3 Treatment of rat aortic preparations with either a Ca^{2+}-free solution or methylene blue, both conditions known to inhibit endothelium-derived relaxing factor (EDRF)-mediated responses, markedly reduced the extracellular accumulation of cyclic GMP from tissues with but not without endothelium.

4 Endothelium-dependent vasodilators such as acetylcholine (10 μM) and carbachol (10 μM) greatly increased tissue cyclic GMP content, in a time-dependent manner in rat aortic preparations with endothelium, but only slightly in tissues without. Maximal increases in intact tissues were obtained after about 1 min of agonist contact and amounted to about 35 and 15 fold respectively, thereafter tissue cyclic GMP content rapidly declined. Histamine (10 μM) elicited only minor effects on tissue cyclic GMP content of both intact preparations and those without endothelium.

5 Acetylcholine (10 μM), carbachol (10 μM) and histamine (10 μM) stimulated a time-dependent release of the cyclic nucleotide into the incubation medium from tissues with endothelium. After 120 min of observation, extracellular accumulation of cyclic GMP from intact tissues was increased by about 2.6, 6.6 and 1.7 fold respectively. Carbachol and histamine induced only minor effects on release from tissues without endothelium.

6 Sodium nitroprusside (0.3 and 10 μM), a direct activator of soluble guanylate cyclase, induced a concentration-dependent accumulation of cyclic GMP in tissues with and without endothelium that was associated with a concentration-dependent accumulation of cyclic GMP in the extracellular space. Peak tissue cyclic GMP content reached similar levels in preparations with and without endothelium, while extracellular cyclic GMP levels were about two times greater when experiments were performed with intact compared to endothelium-denuded tissues.

7 Atriopeptin II, an activator of particulate guanylate cylase, increased tissue cyclic GMP content by about 8 and 18 fold respectively in tissues with and without endothelium. As was the case with sodium nitroprusside, atriopeptin II-stimulated release was markedly enhanced from intact tissues compared with those without endothelium. After 120 min of observation, there was a 16 fold difference in the amount of extracellular cyclic GMP.

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8 Exposure to a Ca^{2+}-free solution, a condition known to abolish production of EDRF by endothelial cells, did not affect either sodium nitroprusside or atriopeptin II-induced release of cyclic GMP from tissues with or without endothelium.

9 Methylene blue, an inhibitor of guanylate cyclase activation, reduced sodium nitroprusside-stimulated release of cyclic GMP to a similar extent from tissues with and without endothelium. Methylene blue also tended to reduce atriopeptin II-stimulated release of cyclic GMP from intact tissue but not from tissue without endothelium.

10 The study showed that rat aortic preparations release cyclic GMP into the incubation medium. This release of the nucleotide was markedly enhanced both from unstimulated and from agonist (histamine, acetylcholine and carbachol)-stimulated tissues with a functional endothelium compared to tissues without endothelium and seemed to be mediated by EDRF. Efflux elicited by sodium nitroprusside or atriopeptin II was also potentiated by the presence of a functional endothelium. This latter effect of endothelium is unrelated to EDRF and may possibly be due to an alteration of smooth muscle cyclic GMP metabolism by endothelial cells or to the presence of endothelial cells *per se*. Furthermore, the data indicate that release of cyclic GMP is secondary to, and perhaps dependent on, the extent of tissue cyclic GMP production.

Introduction

It is now well established, both *in vitro* and *in vivo*, that vascular endothelium mediates relaxant responses to various physiological and pharmacological agonists such as acetylcholine, histamine, bradykinin and the ionophore A23187 (for reviews see Furchgott, 1984; Peach *et al.*, 1985). These vasodilators induce the liberation of a short-lived relaxing factor or factors from endothelial cells (EDRF) which diffuses to the adjacent smooth muscle tissue and induces its relaxation. Endothelial cells also liberate a small amount of EDRF continuously in unstimulated conditions (Griffith *et al.*, 1984). The production of EDRF, its release, or both, is dependent on the presence of extracellular Ca^{2+} (Singer & Peach, 1982; Long & Stone, 1985; Spedding *et al.*, 1986). EDRF-mediated relaxation of vascular tissue is associated with an increase in tissue guanosine 3′, 5′-cyclic monophosphate (cyclic GMP) content (Holzmann, 1982; Rapoport & Murad, 1983; Diamond & Chu, 1983) resulting from the direct activation of soluble guanylate cyclase by EDRF (Förstermann *et al.*, 1986; Ignarro *et al.*, 1986). This increase in cyclic GMP levels is inhibited by methylene blue (Holzmann, 1982; Rapoport *et al.*, 1985) perhaps by an action at the guanylate cyclase level (Arnold *et al.*, 1977; Katsuki *et al.*, 1977).

Atrial natriuretic peptides, nitrovasodilators and endothelium-derived relaxing factor (EDRF) all induce vasorelaxation and increase tissue guanosine 3′,5′-cyclic monophosphate (cyclic GMP) content by increasing guanylate cyclase activity (Katsuki & Murad, 1977; Holzmann, 1982; Diamond & Chu, 1983; Rapoport & Murad, 1983; Winquist *et al.*, 1984; Fiscus *et al.*, 1985). Responses elicited by both atrial natriuretic peptides and nitrovasodilators result mostly from a direct effect on the smooth muscle cells without the involvement of endothelial cells (Rapoport & Murad, 1983; Winquist *et al.*, 1984). Production of cyclic GMP by atrial natriuretic peptides is mediated by their binding to a small fraction of high affinity cell surface receptors which then activate closely associated particulate guanylate cyclase (Waldman *et al.*, 1984; Leitman *et al.*, 1986). In the case of nitrovasodilators, production of cyclic GMP is thought to be mediated by nitric oxide radicals which directly activate soluble guanylate cyclase (Arnold *et al.*, 1977; Katsuki *et al.*, 1977) and the same may be true for EDRF (Palmer *et al.*, 1987; Ignarro *et al.*, 1987).

In various biological systems, increased cellular levels of cyclic nucleotides have been associated with their release or extrusion into the extracellular medium. Davoren & Sutherland (1963) first reported that stimulation of pigeon erythrocytes by adrenaline increased their adenosine 3′:5′-cyclic monophosphate content and induced the appearance of the nucleotide in the bathing medium. Since this initial observation, release of cyclic nucleotides has been reported from various tissues such as adipose, liver, cerebellum, pineal and posterior pituitary glands (Zumstein *et al.*, 1974; O'Dea *et al.*, 1978; Tjörnhammar *et al.*, 1983; 1986), from various cell lines such as C-6 rat glioma cells and WI-38 human fibroblasts (Kelly & Butcher, 1974; Penit *et al.*, 1974; Doore *et al.*, 1975; Rindler *et al.*, 1978), as well as from formed elements of blood (Rindler *et al.*, 1978; Brunton & Mayer, 1979; Wiemer *et al.*, 1982, Heasley & Brunton, 1985) and recently from endothelial and smooth muscle cells in culture (Goldman *et al.*, 1983).

Thus, cellular cyclic nucleotide levels might be regulated not only by their catabolism via 3′,5′-cyclic nucleotide phosphodiesterases but also by their efflux from the cell. It is also possible that these

extracellular nucleotides or their metabolites act as intercellular messengers and could affect tissues either directly or by modulating the effects of hormones and drugs thereon. It therefore seemed to be of interest to find out if increases in cyclic GMP content of vascular smooth muscle, either as a result of activation of soluble or particulate guanylate cyclase, are also associated with cyclic GMP efflux and if endothelium might modulate such an effect.

The results show that rat aortic preparations release cyclic GMP into the extracellular space. This response was greatly enhanced in tissues with an intact endothelium, whether unstimulated or agonist-stimulated (with histamine, acetylcholine, carbachol, sodium nitroprusside or atriopeptin II), as compared to tissues without endothelium. A preliminary report of some of these observations has been given (Miller *et al.*, 1987).

Methods

Female Wistar rats (11 to 15 weeks old) were killed by a blow to the neck, the thoracic aorta removed, placed in a physiological salt solution (composition in mM: NaCl 112, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1, $MgSO_4$ 0.24, $CaCl_2$ 1.25, glucose 11.5) and cleaned of all loosely adherent tissue. At all stages care was taken not to damage the endothelium. The aorta was then divided into 4 or 5 segments, of about 5 mg each, for extracellular and tissue cyclic GMP experiments respectively. When necessary, endothelium was removed by lightly rubbing the intimal surface with a wooden match stick.

Determination of extracellular cyclic GMP levels

Experiments were carried out on paired groups of 4 aortic segments, one group with and the other without endothelium. Each group comprised four aortic segments belonging to different rats and to different anatomical levels of the aorta. The tissues were immersed in the physiological salt solution (1 ml), maintained at 37°C and aerated with a gas mixture of 95% O_2 and 5% CO_2. After a 30 min rest period, during which time the medium was changed twice, a test compound or its solvent was added. Methylene blue (0.5 μM) was present during the rest period and throughout the experiment when necessary. In Ca^{2+}-free experiments, performed in physiological salt solution from which Ca^{2+} was omitted, the incubation medium was changed twice with a Ca^{2+}-free solution just before the 30 min rest period in order to reduce the residual Ca^{2+} content of the medium. Various times after addition of a test compound or solvent an aliquot was taken from the medium bathing the aortic segments and its cyclic GMP content was assayed by a radioimmunological method (Cailla *et al.*, 1976). At the end of the experiment the artery segments were placed between two sheets of filter paper and pressed four times with a roller weighing 350 g before being weighed. Results of cyclic GMP measurements were expressed as fmol mg^{-1} wet weight tissue.

Determination of tissue cyclic GMP content

Experiments included one control and four agonist-treated groups, each composed of four aortic segments, either with or without endothelium, taken from different rats as described above. The segments were incubated in physiological salt solution at 37°C and oxygenated with a gas mixture of 95% O_2 and 5% CO_2. After a 120 min rest period, during which time the medium was changed twice (at 60 and 105 min), a test compound or its solvent was added. At the appropriate time, the tissues were rapidly frozen using an aluminium clamp pre-cooled in liquid nitrogen. The segments were then thawed in 400 μl of perchloric acid (1 N), homogenized with a Potter glass/glass homogenizer followed by sonication (Ultrason-Annemasse, Type 75TS, France) for 15 s. After 5 min centrifugation at 10000 *g*, the cyclic GMP content of the supernatant was assayed. The 10000 *g* pellet was resuspended in 750 μl of ethanol/diethyl ether (3:1) and allowed to stand for 10 min at room temperature before centrifugation. The aim of this step was to extract lipids that could interfere with the determination of DNA content. The supernatant was then removed and the pellet resuspended in 1 ml of 0.5 N perchloric acid and left at 45°C for 60 h in microcentrifuge tubes in order to extract DNA completely. The extracts were then stored at 4°C for later measurement of DNA content. After centrifugation, 100 μl of the supernatant was allowed to react with 100 μl of a 300 mg ml^{-1} solution of 3,5-diaminobenzoic acid (Merck-Schuchardt, F.R.G.) in 4 N HCl at 60°C for 30 min. The volume was then adjusted to 2 ml with 0.6 N perchloric acid. Maximal fluorescence intensity was obtained with an excitation wavelength of 417 nm and an emission wavelength of 495 nm. Standard curves were constructed with solutions of calf thymus DNA in water (prepared from a 50 μg ml^{-1} stock solution), adjusted to a volume of 100 μl with 0.5 N perchloric acid and then treated in the same manner as the samples. Tissue cyclic GMP content was expressed as fmol μg^{-1} DNA.

Effect of cyclic GMP phosphodiesterase on extracellular accumulation of cyclic GMP

Four intact segments belonging to one aorta were incubated in physiological solution and stimulated

with 1 μM atriopeptin II. After a 90 min stimulation period, at which time the appearance of extracellular cyclic GMP had reached a plateau (Figure 1d), a 400 μl aliquot of the bathing solution was taken and its nucleotide content assessed as a substrate for cyclic GMP phosphodiesterase. The reaction, in siliconized tubes at 37°C, was started by the addition of either 100 μl of bovine aortic cyclic GMP phosphodiesterase solution (0.59 mg protein ml^{-1}), prepared by the method of Lugnier *et al.* (1986), or buffer (20 mM Tris-HCl, 2 mM Mg acetate, pH 7.5). An aliquot of 75 μl was taken from this reaction medium at various times and the cyclic GMP content assayed. After 5 min of treatment, cyclic GMP content was reduced to only 2% of the initial content before the addition of the enzyme, indicating that the extracellular accumulation was of cyclic GMP.

Materials

Atriopeptin II (rat sequence, Sigma Chemical Co, St Louis, U.S.A.) was prepared as a 0.1 mM stock solution in physiological solution or distilled water, fractionated into aliquots and kept at −80°C until used. Sodium nitroprusside (Merck, Darmstadt, F.R.G.), carbachol and histamine dihydrochloride (Sigma Chemical Company, U.S.A.) were prepared daily in physiological salt solution. Acetylcholine chloride (Sigma Chemical Company, U.S.A.) was prepared as a stock solution of 10 mM in 0.1 M NaH_2PO_4. Methylene blue (Ugine Kuhlmann, France) was dissolved as a stock solution of 3 mM in distilled water. All dilutions were made in physiological salt solution (without Ca^{2+} when necessary). Cyclic GMP specific antibodies were obtained from the Centre d'Immunologie, Marseille-Luminy, France. Cyclic GMP and DNA (calf thymus, type I) were obtained from Sigma. Labelled antigens were prepared by iodination of the succinyltyrosyl-methylester of cyclic GMP (Sigma). All drug concentrations are expressed in terms of the base.

Statistical analysis

The data are expressed as means ± s.e.mean. Tests of significance were made by Student's paired or unpaired *t* test where appropriate (2-tailed). *P* values less than 0.05 were considered significant. *n* refers to the number of experiments.

Results

Tissue cyclic GMP content

The aortic content of cyclic GMP in unstimulated segments with endothelium, 45.8 ± 3.6 fmol μg^{-1} DNA ($n = 27$), was significantly greater ($P < 0.001$) than that of segments without endothelium, 13.8 ± 1.6 fmol μg^{-1} DNA ($n = 20$).

Basal release of cyclic GMP

Incubation of unstimulated rat aortic segments with or without endothelium resulted in a time-dependent accumulation of cyclic GMP in the medium bathing the tissues (Figure 1a). In the case of preparations without endothelium, release of the nucleotide was modest and after 120 min of observation amounted to only 17.0 ± 4.7 fmol mg^{-1} wet weight ($n = 19$). In contrast, time-dependent accumulation of cyclic GMP into the medium bathing tissues with endothelium reached a plateau after about 60 min of observation (Figure 1a) and after 120 min of observation the extracellular level was 560 ± 82 fmol mg^{-1} wet weight ($n = 20$), a 33 fold greater increase than that obtained with tissues without endothelium.

Modulation of the activity of EDRF by removing extracellular Ca^{2+}.

In Ca^{2+}-free solution, where EDRF synthesis, release or both are abolished (Singer & Peach, 1982; Long & Stone, 1985), the time-dependent accumulation of cyclic GMP in the medium bathing tissues with, but not without, endothelium was significantly ($P < 0.05$) reduced (Figure 2a). The amount of extracellular cyclic GMP, measured after a 120 min incubation of intact tissues in a Ca^{2+}-free solution, was reduced by about 91% as compared to tissues in a Ca^{2+}-containing solution (from 448 ± 125 to 40.2 ± 12.1 fmol mg^{-1} wet weight, $n = 5$). This reduced extracellular cyclic GMP content was not significantly greater than that derived from tissues without endothelium incubated in a Ca^{2+}-free medium (8.2 ± 3.0 fmol mg^{-1} wet weight; $n = 5$).

Modulation of the activity of EDRF with methylene blue.

Treatment of rat aortic vessels with 0.5 μM methylene blue, a condition that inhibits the effects of endothelium on agonist-induced responses (Miller *et al.*, 1984), significantly ($P < 0.05$) reduced the time-dependent release of cyclic GMP from vessels with, but not from those without, endothelium (Figure 2b). After a 120 min observation period, the extracellular cyclic GMP level was reduced by about 78% from 484 ± 188 to 107 ± 17 fmol mg^{-1} wet weight ($n = 6$).

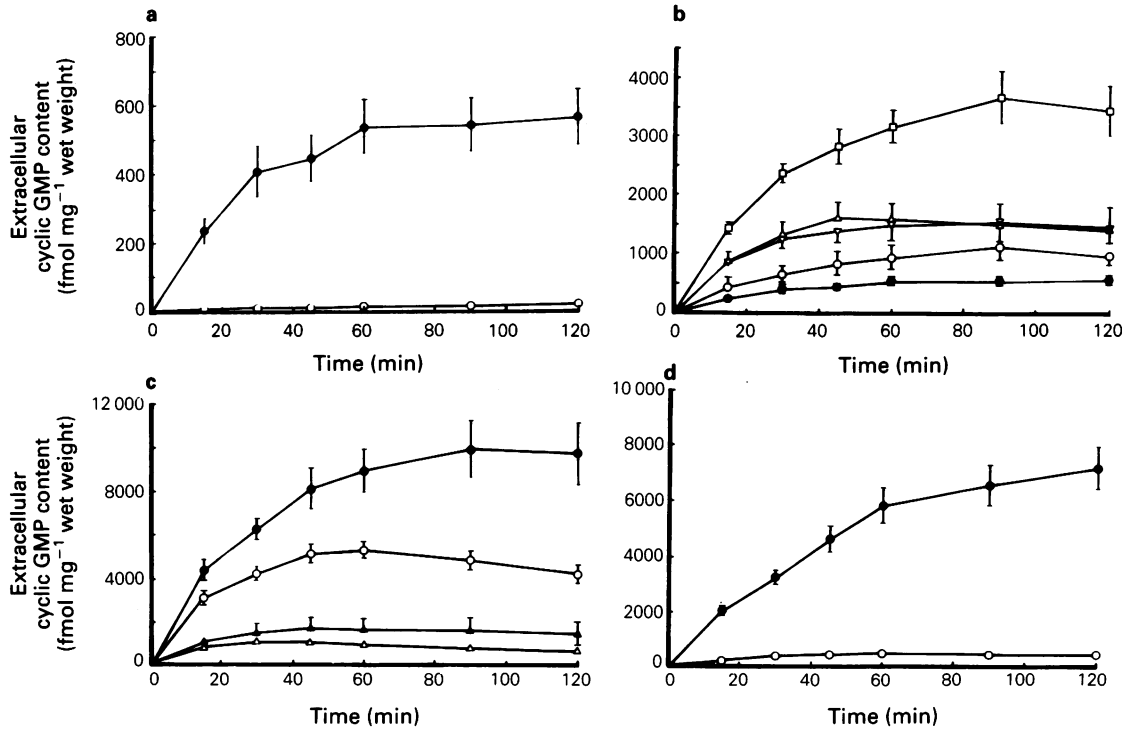

Figure 1 Accumulation of cyclic GMP in the incubation medium bathing rat aortic tissues as a function of time. (a) Basal accumulation with (●) and without (○) endothelium as a function of time. (b) Accumulation with endothelium in the absence (●) and in the presence of either histamine (10 μM, ○), acetylcholine (1 μM, △ and 10 μM, ▽) or carbachol (10 μM, □). (c) Effect of sodium nitroprusside 0.3 μM (▲, △) and 10 μM (●, ○) with (solid symbols) and without (open symbols) endothelium. (d) Atriopeptin II (1 μM) on segments with (●) and without (○) endothelium. Results are the means ± s.e.mean (vertical bars) of 5 to 17 observations.

Effects of the endothelium-dependent relaxant agents histamine, acetylcholine and carbachol on tissue cyclic GMP levels.

Incubation of intact, precontracted aortic tissues with 10 μM histamine, 10 μM acetylcholine or 10 μM carbachol is sufficient to elicit an almost maximal relaxation. Histamine (10 μM) induced a modest (about 1.7 fold, from 40.9 ± 4.5, $n = 4$ to 69.4 ± 15.7 fmol μg^{-1} DNA, $n = 3$) increase in mean tissue cyclic GMP content (Figure 3a) after 60 s of agonist contact. However, this response was not significant. Treatment of intact vessels with 10 μM acetylcholine markedly increased tissue cyclic GMP content in a time-dependent manner (Figure 3b). This increase amounted to about 15 fold after 15 s of agonist contact (from 33.7 ± 11.2 to 505 ± 201 fmol μg^{-1} DNA, $n = 4$) and reached a near maximal value of about 35 fold after 1 min (1343 ± 380 fmol μg^{-1} DNA, $n = 4$). Thereafter, tissue cyclic GMP content declined rapidly, but after 5 min of stimulation it was still significantly ($P < 0.025$) increased over the basal level and amounted to about 14% of the maximal content. Stimulation of intact aortic preparations with 10 μM carbachol also significantly increased tissue cyclic GMP content in a time-dependent manner (Figure 3c). Cyclic GMP concentration was near maximal after about 1 min of agonist contact and consisted of an approximate 15 fold increase (from 53.1 ± 12.0, $n = 4$ to 784 ± 160 fmol μg^{-1} DNA, $n = 3$). Thereafter tissue cyclic nucleotide content declined to about 44% of the maximal response after 5 min of agonist contact.

Treatment of endothelium denuded tissues with 10 μM histamine or 10 μM carbachol did not significantly affect their cyclic GMP content after 1 min of agonist contact. However, stimulation of such tissues by 10 μM acetylcholine increased cyclic GMP content significantly ($P < 0.025$) by about 1.5 fold (from 12.1 ± 0.9 to 18.2 ± 1.6 fmol μg^{-1} DNA, $n = 4$) after 1 min of agonist contact, perhaps due to the presence of a residual population of functional endothelial cells.

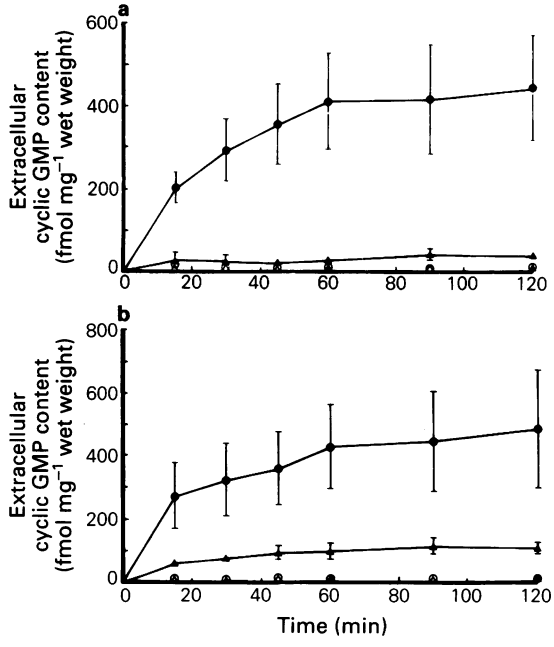

Figure 2 Basal accumulation of cyclic GMP in the incubation medium bathing rat aortic tissues with (solid symbols) and without (open symbols) endothelium as a function of time. (a) In a Ca^{2+}-containing (circles) or Ca^{2+}-free medium (triangles). (b) In the absence (circles) or presence (triangles) of 0.5 μM methylene blue. Results are the means ± s.e.mean (vertical bars) of 5 or 6 observations.

Effects of the endothelium-independent relaxant agents atriopeptin II and sodium nitroprusside

Atriopeptin II 0.1 μM, is a threshold relaxant concentration in rat aortic preparations maximally contracted by noradrenaline (Fiscus *et al.*, 1985). A 10 fold higher concentration of atriopeptin II induced a significant time-dependent accumulation of cyclic GMP in tissues with and without endothelium (Figure 4a, b). Tissue cyclic GMP content was maximal after about 2 min of agonist contact and, at this time, was induced respectively by about 8 fold (from 52 ± 8 to 413 ± 44 fmol μg^{-1} DNA, $n = 8$) and 18 fold (from 10 ± 1 to 186 ± 30 fmol μg^{-1} DNA, $n = 8$) in aortic segments with and without endothelium respectively. Atriopeptin II-induced increases in tissue cyclic GMP content were maintained for at least 5 min and, after 10 min, they amounted respectively to about 63 and 28% of their respective maximal values in tissues with and without endothelium.

Sodium nitroprusside 0.1 μM is a concentration just sufficient to relax maximally rat aortic preparations contracted by noradrenaline (Keith *et al.*, 1982). Treatment of tissues with 10 μM sodium nitroprusside elicited maximal increases in tissue cyclic GMP content after about 1 min of agonist contact which were respectively about 141 fold (from 44 ± 3 to 6126 ± 572 fmol μg^{-1} DNA, $n = 4$) and 260 fold (from 24 ± 2 to 6259 ± 859 fmol μg^{-1} DNA, $n = 4$) in intact and denuded tissues (Figure 5c, d). In contrast to atriopeptin II, this response was transient in both types of preparations. After 5 min of agonist

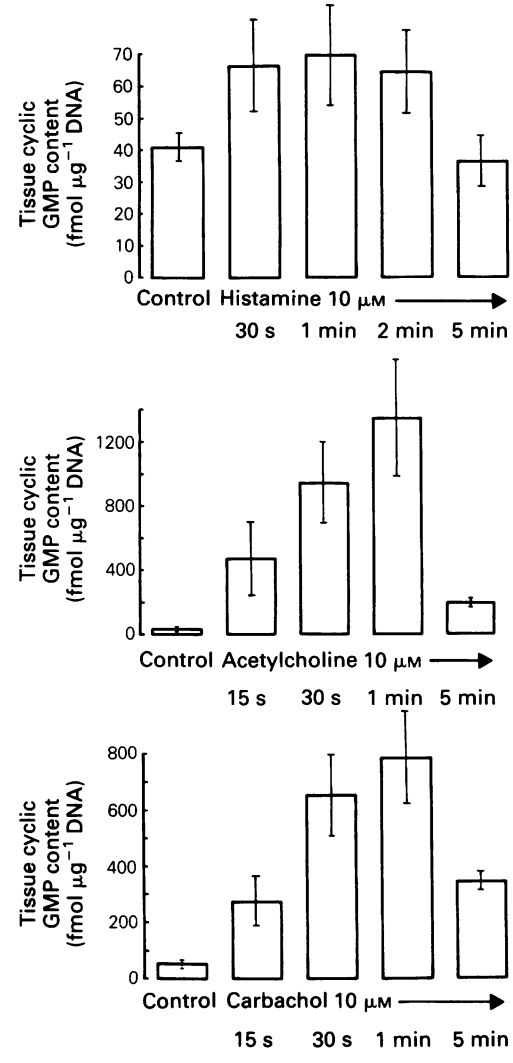

Figure 3 Effect of (a) histamine (10 μM), (b) acetylcholine (10 μM) and of (c) carbachol (10 μM) on cyclic GMP content of rat aortic tissues with endothelium at various observation times. Results are the means ± s.e.mean (vertical bars) of 3–4 observations.

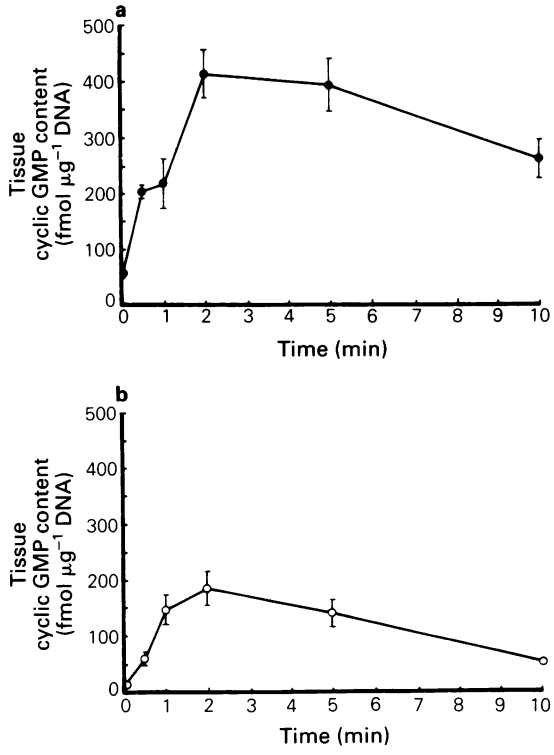

Figure 4 Effect of atriopeptin II 1 μM on cyclic GMP content of rat aortic tissues with (a) and without (b) endothelium as a function of time. Results are the means ± s.e.mean (vertical bars) of 3 to 8 observations.

contact, tissue cyclic GMP contents were still significantly ($P < 0.005$) increased, however they were only about 49% and 44% of the peak levels in tissues with and without endothelium respectively. The maximal increase in tissue cyclic GMP observed in tissues with endothelium in the presence of 10 μM sodium nitroprusside was much greater than that induced by 1 μM atriopeptin. However, a lower concentration of sodium nitroprusside (0.3 μM) elicited maximal increases in tissue cyclic GMP content after about 2 min of agonist contact and induced about 15 fold (from 45 ± 9 to 675 ± 134 fmol μg^{-1} DNA, $n = 3$) and 63 fold (from 12 ± 5 to 766 ± 169 fmol μg^{-1} DNA, $n = 4$) increases in the cyclic GMP content respectively in preparations with and without endothelium (Figure 5a, b). This increase in tissue cyclic GMP content in the presence of endothelium was similar to that observed with 1 μM atriopeptin II.

Stimulated release of cyclic GMP

Effects of the endothelium-dependent relaxant agents histamine, acetylcholine and carbachol Treatment of rat aortic tissues with 10 μM histamine increased the time-dependent accumulation of cyclic GMP into the bathing medium both from preparations without endothelium by about 8.8 fold (data not shown) and with endothelium by about 1.7 fold (Figure 1b). After 120 min of agonist contact, extracellular cyclic GMP content of the medium containing tissues without and with endothelium amounted to 150 ± 27 and 959 ± 134 fmol mg^{-1} wet weight ($n = 5$) respectively, a significant ($P < 0.005$) 6.4 fold difference. Stimulating rat aortic segments with endothelium with either 1 or 10 μM acetylcholine enhanced the time-dependent accumulation of cyclic GMP into the incubation medium (Figure 1b). Both concentrations induced similar maximal responses of 1415 ± 226 ($n = 7$) and 1449 ± 345 fmol cyclic GMP mg^{-1} wet weight ($n = 9$) respectively after 120 min of agonist contact. Treatment of aortic segments with 10 μM carbachol also markedly increased the time-dependent release of cyclic GMP from aortic segments with endothelium (by about 6.6 fold) but only moderately, by about 2 fold, from vessels without endothelium (data not shown). After 120 min of agonist contact, extracellular cyclic GMP amounted to 34.2 ± 8.5 and 3666 ± 606 fmol mg^{-1} wet weight ($n = 5$) respectively in medium bathing tissues without and with endothelium, a significant ($P < 0.005$) 107 fold difference.

Effects of the endothelium-independent relaxant agents atriopeptin II and sodium nitroprusside Treatment of rat aortic tissues with 0.3 or 10 μM sodium nitroprusside induced a time- and concentration-dependent accumulation of cyclic GMP in the incubation medium of tissues with and without endothelium (Figure 1c). In the case of 0.3 μM sodium nitroprusside, the cyclic GMP content of the medium was near maximal after about 30 min of agonist contact and remained constant for the next 90 min. In each experiment, the time-dependent accumulation of cyclic GMP in the medium was greater when intact segments rather than those without endothelium were used. After 120 min of stimulation, extracellular cyclic GMP content bathing tissues without (658 ± 136 fmol mg^{-1} wet weight, $n = 6$) and with endothelium (1475 ± 540 fmol mg^{-1} wet weight, $n = 6$) was not significantly different (Figure 1c). Increasing the concentration of sodium nitroprusside to 10 μM, markedly enhanced the time-dependent accumulation of cyclic GMP from both types of preparations (Figure 1c). This response was significantly ($P < 0.001$) greater from intact than from tissues without endothelium. Cyclic GMP content of the bathing medium was near maximal after about 45 min of stimulation and, measured after 120 min of agonist contact, it amounted to 4208 ± 430 ($n = 14$) and 9694 ± 1420

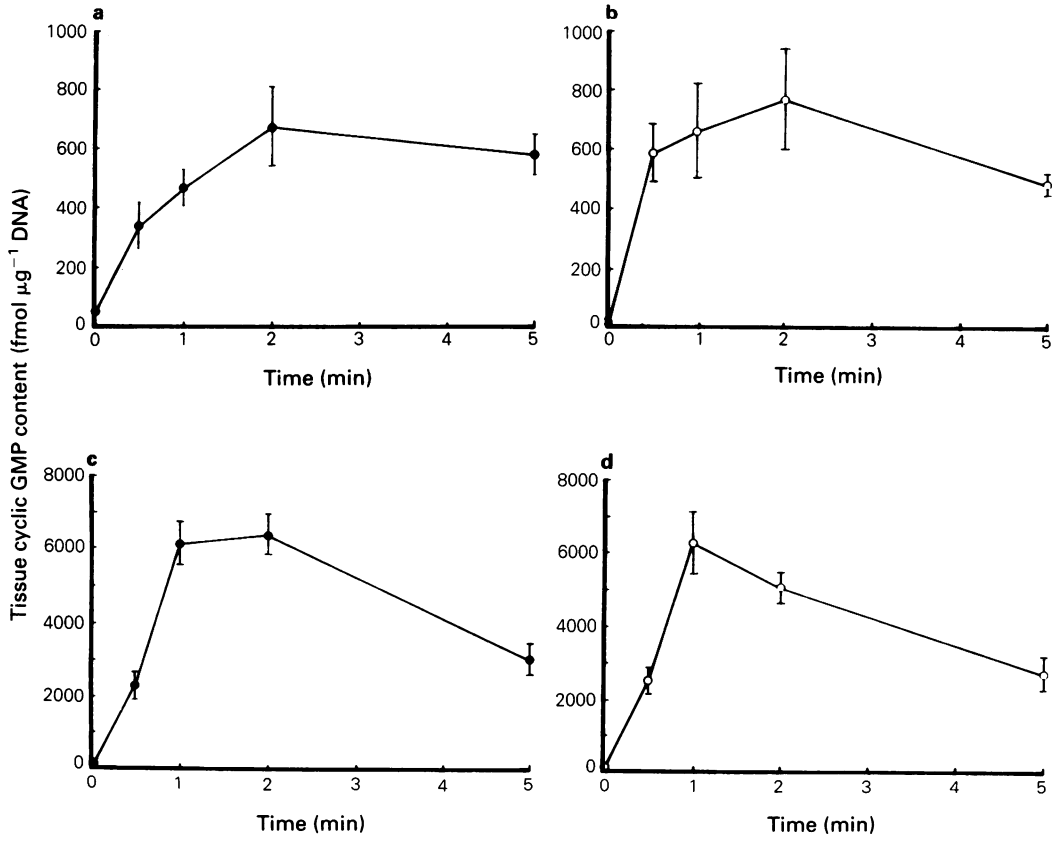

Figure 5 Effect of sodium nitroprusside 0.3 (a, b) and 10 μM (c, d) on cyclic GMP content of rat aortic tissues with (a, c) and without (b, d) endothelium as a function of time. Results are the means ± s.e.mean (vertical bars) of 3 to 4 observations.

($n = 12$) fmol mg^{-1} wet weight respectively from tissues without and with endothelium, a 2.3 fold difference.

Treatment of rat aortic tissues with 1 μM atriopeptin II also induced a time-dependent accumulation of cyclic GMP in the incubation medium of tissues with and without endothelium. As with sodium nitroprusside, this response was markedly enhanced when intact, instead of tissues without endothelium, were used (Figure 1d). Initial accumulation of cyclic GMP in the incubation medium was slower than that seen with 10 μM sodium nitroprusside and reached a plateau only after about 60 min of agonist contact. After 120 min of stimulation, the extracellular cyclic GMP level was 447 ± 69 and 7197 ± 762 fmol mg^{-1} wet weight ($n = 17$) in endothelium-denuded and intact tissues respectively; a 16.1 fold difference.

Modulation of the activity of EDRF by absence of extracellular Ca^{2+}

Incubation in a Ca^{2+}-free solution had no significant effect on the time-dependent release of cyclic GMP from vascular segments with or without endothelium stimulated by either 10 μM sodium nitroprusside (Figure 6a) or 1 μM atriopeptin II (Figure 7a).

Modulation of the activity of EDRF by methylene blue

Treatment of aortic preparations with 0.5 μM methylene blue inhibited 10 μM sodium nitroprusside-induced time-dependent accumulation of cyclic GMP in the medium bathing segments with and without endothelium (Figure 6b). The degree of inhi-

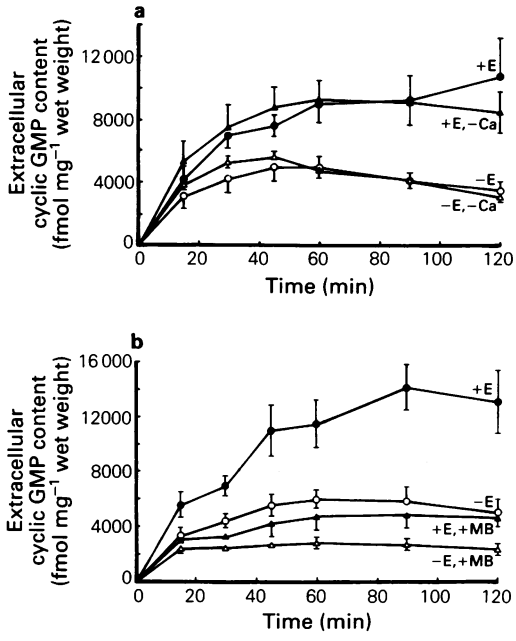

Figure 6 Sodium nitroprusside (10 μM)-stimulated accumulation of cyclic GMP in the medium bathing rat aortic preparations with (solid symbols) and without (open symbols) endothelium (E) in (a) Ca^{2+} containing (circles) or Ca^{2+}-free medium (triangles) and in (b) the absence (circles) or presence (triangles) of 0.5 μM methylene blue (MB) as a function of time. Results are shown as the means ± s.e.mean (vertical lines) of 5 observations.

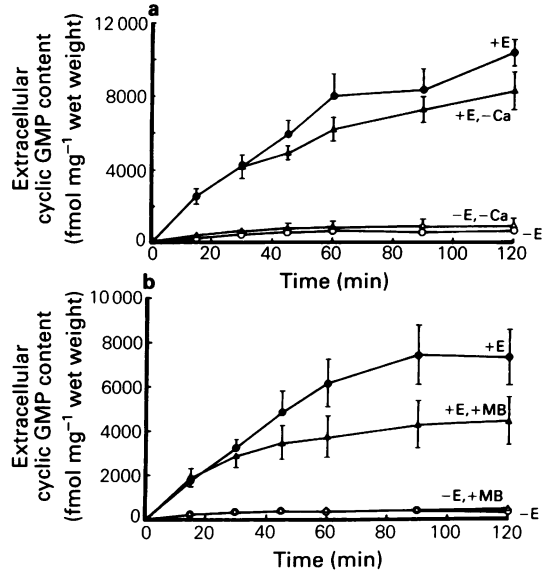

Figure 7 Atriopeptin II (1 μM)-stimulated accumulation of cyclic GMP in the medium bathing rat aortic preparations with (solid symbols) and without (open symbols) endothelium (E) in (a) Ca^{2+} containing (circles) or Ca^{2+}-free medium (triangles) or in (b) the absence (circles) or presence (triangles) of 0.5 μM methylene blue (MB) as a function of time. Results are shown as the means ± s.e.mean (vertical lines) of 5 observations.

bition was similar for both intact and tissues without endothelium and amounted to about 64% (reduction from 13090 ± 2329 to 4698 ± 611 fmol mg^{-1} wet weight, $n = 5$; $P < 0.025$) and to about 53% (from 5017 ± 1001 to 2346 ± 427 fmol mg^{-1} wet weight, $n = 5$; $P < 0.05$) respectively, after an observation period of 120 min.

Treatment of aortic segments without endothelium with 0.5 μM methylene blue had no significant effect on the time-dependent release of cyclic GMP induced by atriopeptin II (Figure 7b). However, treatment of intact tissues with methylene blue significantly reduced ($P < 0.025$) extracellular accumulation of cyclic GMP after 45, 60, 90 and 120 min of observation by about 29, 40, 43 and 39% respectively (Figure 7b).

Discussion

The cyclic AMP content of erythrocytes, glial cells, liver and cerebellar slices is regulated by hydrolysis by specific 3',5'-cyclic nucleotide phosphodiesterases and by efflux of the nucleotide into the extracellular medium (Davoren & Sutherland, 1963; Penit *et al.*, 1974; Doore *et al.*, 1975; Rindler *et al.*, 1978; Tjörnhammar *et al.*, 1983; 1986). The present study demonstrates that a time-dependent release of cyclic GMP occurred from unstimulated rat aortic tissue without endothelium and that this release was markedly enhanced when endothelium was present (Figure 1a). Unstimulated tissues with endothelium had about a 3 fold higher cyclic GMP content than those without (Holzmann, 1982; Diamond & Chu, 1983; Rapoport & Murad, 1983; Miller *et al.*, 1984; Spedding *et al.*, 1986), and this increased production of cyclic GMP in intact tissues seems to be due to basal release of EDRF from endothelial cells since it was inhibited in the absence of extracellular Ca^{2+} (Figure 2a), a condition known to inhibit EDRF synthesis, release or both (Singer & Peach, 1982; Long & Stone, 1985; Spedding *et al.*, 1986). Extracellular accumulation of the nucleotide in medium bathing tissues without endothelium was not markedly affected in Ca^{2+}-free conditions, even when nucleotide production was markedly stimulated by sodium nitroprusside or atriopeptin II (Figures 6a, 7a), indi-

cating that this efflux is not affected by the absence of extracellular Ca^{2+}. The involvement of EDRF in endothelium-enhanced efflux of cyclic GMP is further supported by the reduction in efflux from tissues with endothelium in the presence of methylene blue (Figure 2b). This compound is known to inhibit endothelium-dependent production of cyclic GMP and relaxation of vascular tissues (Holzmann, 1982; Rapoport *et al.*, 1985), perhaps by an action on soluble guanylate cyclase (Arnold *et al.*, 1977; Katsuki *et al.*, 1977).

To investigate further the involvement of EDRF in endothelium-enhanced release of cyclic GMP, its production was stimulated with maximally effective relaxant concentrations of histamine, acetylcholine or carbachol (Holzmann, 1982, Rapoport & Murad, 1983, Diamond & Chu, 1983; Van de Voorde & Leusen, 1983; Schini *et al.*, 1987). Tissue responses were near maximal after about 1 min of agonist contact and then declined (Figure 3). At the time of peak tissue cyclic GMP content the presence of extracellular nucleotide could not be estimated with certainty, but thereafter it increased steadily and reached a plateau after about 30 to 60 min of observation (Figure 1b), indicating that release of cyclic GMP is secondary to an enhancement of tissue cyclic GMP content.

A possible relationship between tissue and extracellular accumulation of cyclic GMP was examined by comparing the magnitude of responses induced by the various agonists. Comparing the extent of 10 μM agonist-stimulated maximal increases in tissue cyclic GMP content, the following order was obtained: acetylcholine $\approx$ carbachol $\gg$ histamine $\approx$ basal EDRF (Figure 3). For maximal accumulation of the nucleotide in the extracellular medium the sequence was: carbachol > acetylcholine > histamine $\approx$ basal EDRF (Figure 1). The similarity between the two orders of potency suggests that release of cyclic GMP into the extracellular space is related to tissue cyclic GMP content. The large difference in tissue and extracellular accumulation of cyclic GMP between maximal effective relaxant concentrations of acetylcholine, carbachol and histamine (Figures 3, 1b) however needs to be investigated. Thus EDRF, released under basal or stimulated conditions, increased tissue cyclic GMP content and this was associated with a time- and concentration-dependent release of the nucleotide into the extracellular medium.

Treatment of endothelium-denuded tissues with the endothelium-dependent vasorelaxant compounds acetylcholine or histamine increased their cyclic GMP content little if at all and such small effects may result from an action of the agonists on a residual population of functional endothelial cells in 'endothelium-free' preparations.

It might be expected that cyclic GMP efflux occurs mostly from smooth muscle cells as they represent the major cell population of rat aorta, but endothelium-dependent vasorelaxant compounds such as bradykinin, ATP and the ionophore A23187 increase cyclic GMP content in pig and human endothelial cells in culture (Brotherton, 1986; Martin *et al.*, 1988) and they might also participate in the enhanced release from intact tissues.

The two endothelium-independent agonists, sodium nitroprusside and atriopeptin II, induced increases in tissue cyclic GMP content which were maximal after about 1 to 2 min of agonist contact and declined slowly thereafter (Keith *et al.*, 1982; Winquist *et al.*, 1984). At the time of peak tissue levels, extracellular nucleotide content was only just detectable (unpublished results), but thereafter increased steadily and reached a plateau after about 45 to 60 min. Furthermore, sodium nitroprusside induced a concentration-dependent increase in both tissue and extracellular accumulation of cyclic GMP, further supporting the idea that cyclic GMP efflux is dependent on the cellular content of the nucleotide. However, 0.3 μM sodium nitroprusside, 1 μM atriopeptin II and 10 μM carbachol stimulated similar maximal tissue levels of cyclic GMP in the presence of endothelium, but there was a marked difference in the quantity appearing in the medium. Evidently tissue levels alone are no indication of the absolute amount of nucleotide released with time, specially as concentrations in the medium increase when tissue levels are low.

As moderate increases in tissue cyclic GMP content were also associated with its release, efflux may perhaps result not only from an overload of the cellular regulatory mechanisms and a passive efflux, but may be an active cellular process which participates, together with cyclic nucleotide phosphodiesterases, in the regulation of the cellular nucleotide content. In fact, it has been suggested that such release is an energy-dependent mechanism as it is reduced by inhibitors of organic acid transport such as probenecid (Davoren & Sutherland, 1963; Penit *et al.*, 1974. Doore *et al.*, 1975; Tjörnhammar *et al.*, 1983), it occurs against an apparent concentration gradient (Davoren & Sutherland, 1963; Brunton & Mayer, 1979) and it is inhibited by metabolic inhibitors (Doore *et al.*, 1975; Rindler *et al.*, 1978; Brunton & Mayer, 1979; Wiemer *et al.*, 1982). As already noted this release is independent of extracellular Ca^{2+}.

The time-dependent appearance of cyclic GMP in the extracellular medium during exposure to maximally effective concentrations of 10 μM sodium nitroprusside and 1 μM atriopeptin II was enhanced when experiments were performed with intact instead of denuded tissues, by about 2 and 16 fold respectively.

Basal release of EDRF does not seem to participate in this endothelium-dependent release as this effect persisted in the absence of extracellular Ca^{2+} (Figures 6, 7). The possibility that endothelial cells produce another stimulus which could modify smooth muscle cyclic GMP metabolism might warrant further investigation. Another explanation may be that endothelial cells, *per se*, participate in this potentiation perhaps by responding to nitrovasodilators and atriopeptin II by production of cyclic GMP followed by its release into the extracellular space. However, this would mean that endothelial cells must have, when stimulated, a relatively high turnover of cyclic GMP in comparison to smooth muscle cells, as they represent only a small proportion of cells in the tissue. Nitrovasodilators induce cyclic GMP production in some cultured endothelial cells but responses seem to depend not only on the agonist but also on the cultures used. Glyceryl trinitrate and sodium azide increased cyclic GMP content by about 3 to 5 fold in cultures of pig aortic endothelial cells (Martin *et al.*, 1988) and sodium nitroprusside induced 3 to 10 fold increases in human umbilical vein endothelial cells (Brotherton, 1986) but only a small (about a 2 fold increase, Ganz *et al.*, 1986) or no response at all in bovine aortic endothelial cells (Schenk *et al.*, 1985; Hamet *et al.*, 1986; Schini *et al.*, 1989). Increases of about 20 to 500 fold in cyclic GMP levels have been obtained in bovine and porcine aortic endothelial cells in culture in response to atrial natriuretic peptides (Leitman & Murad, 1986; Leitman *et al.*, 1986). Overall, the various observations suggest that endothelial cells participate in the enhanced release of cyclic GMP from tissues with a functional endothelium only under certain conditions.

Methylene blue inhibited sodium nitroprusside-induced release of cyclic GMP from rat aortic preparations with and without endothelium to about the same extent. This effect probably reflects an action of methylene blue on tissue cyclic GMP content as it has been reported to inhibit sodium nitroprusside-induced production of cyclic GMP in this tissue, perhaps by inhibiting guanylate cyclase activation (Rapoport *et al.*, 1985). Methylene blue also reduced atriopeptin II-induced accumulation of cyclic GMP in the extracellular space from intact but not endothelium-denuded tissues. This inhibitory effect may be partially explained by a reduced influence of tonic release of EDRF on tissue and extracellular accumulation of cyclic GMP in intact tissues. An action of methylene blue on cyclic GMP efflux or on the interaction of atrial natriuretic peptide with its receptors is unlikely to be important since atriopeptin II-stimulated cyclic GMP efflux from tissues without endothelium was not affected by methylene blue treatment.

Although the physiological role of extracellular cyclic nucleotides is only poorly understood, they have been reported to stimulate sodium and potassium influxes in erythrocytes (Rudolph & Greengard, 1980), to modulate proliferation of B and T lymphocytes (Diamantstein & Ulmer, 1975), adherence of granulocytes to endothelial cells (MacGregor *et al.*, 1978; Boxer *et al.*, 1980) and they may also serve as a signal for initiation of phagocytosis by neutrophils (Pryzwansky *et al.*, 1981). Further, specific high affinity uptake of cyclic AMP into brain has been described (Balcar *et al.*, 1988).

In conclusion, in rat aortic preparations, increases in tissue cyclic GMP content elicited by either the presence of endothelium, sodium nitroprusside or atriopeptin II were associated with cyclic GMP efflux. The results further indicate that release of cyclic GMP is secondary and related to the extent of tissue cyclic GMP production, but the nature of this relationship is unknown. Endothelium-dependent efflux could be enhanced by exposure to endothelium-dependent vasodilators and seems to be mediated by EDRF. As small increases in tissue cyclic GMP content are also associated with efflux, it can be suggested that efflux is not necessarily due to an overload of the intracellular regulatory mechanisms. The presence of a functional endothelium potentiated sodium nitroprusside-induced release slightly and atriopeptin II-induced release markedly, suggesting that endothelial cells may modulate smooth muscle cyclic GMP metabolism or that endothelial cells, themselves, participate in this endothelium-dependent release particularly when atriopeptin II was the agonist. This latter possibility is supported by the fact that endothelial cells respond to nitrate compounds and also to atrial natriuretic peptides by cyclic GMP synthesis. The physiological significance of this release is unknown, but apart from a role in regulating the cellular concentration of cyclic GMP, this extracellular nucleotide or a metabolite may also act as an intercellular messenger and affect tissues (including vascular) and formed elements of the blood, either directly or by modifying their reactivity to physiological agonists.

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Potassium channel blockers differentially affect carbachol and (—)-N^6-phenylisopropyladenosine on guinea-pig atria

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1 The effect of three different potassium channel blockers (tetraethylammonium, TEA; 4-aminopyridine, 4-AP; and apamin) and of variations in the concentration of K^+ and Ca^{2+} in the medium, have been studied on the responses of guinea-pig isolated atria to (−)-N^6-phenylisopropyladenosine (R-PIA), a stable adenosine A_1-receptor agonist, and to carbachol, a muscarinic agonist. R-PIA and carbachol showed the same negative inotropic effects over a similar range of concentrations (3–300 μM), both in spontaneously beating and in electrically driven atria.

2 TEA (0.1 to 20 mM) and 4-AP (0.3 to 3 mM), both antagonized the negative inotropic and chronotropic effects of carbachol in a concentration-dependent manner. In contrast, these compounds failed to inhibit the effects induced by R-PIA. Apamin, a specific blocker of a low conductance Ca^{2+}-activated K^+ channel, was ineffective in accordance with the absence of these channels in atrial tissue.

3 TEA (0.1 to 20 mM) inhibited the negative inotropic effect of carbachol, but not that of R-PIA, in atria paced and depolarized by a high K^+ medium (22 mM). In this preparation Na^+ current is abolished and the contraction induced by noradrenaline and electrical stimulation is solely dependent on Ca^{2+} influx currents.

4 Stepwise addition of Ca^{2+} to a calcium-depleted perfusing medium of electrically driven atria, induced a positive inotropic effect which was inhibited by R-PIA. In contrast, carbachol had no effect.

5 In agreement with our previous study, the data suggest that R-PIA acts on isolated atria by inhibiting Ca^{2+} influx through L-channels.

Introduction

Similar effects of adenosine and acetylcholine were observed in the early studies of atrial preparations (Johnson & McKinnon, 1956; Hartzell, 1979). In atrial cells and in the isolated SA node, adenosine (Ado) and acetylcholine (ACh) shorten the action potential, produce hyperpolarization and depression of automaticity (Belardinelli & Isenberg, 1983; West & Belardinelli, 1985). The similar effect of the two drugs is dependent on activation of K^+ channels that in turn indirectly reduce the inward calcium flux during the action potential (reviews: Nawrath *et al.*, 1985; Isenberg *et al.*, 1987; Sperelakis, 1987; West *et al.*, 1987). Although Ado and ACh act on different receptors, these may be connected with the same population of K^+ channels, via guanosine 5'-triphosphate (GTP)-binding proteins (Kurachi *et al.*, 1986; Böhm *et al.*, 1986; Cerbai *et al.*, 1988). In previous studies (Caparrotta *et al.*, 1987; Borea *et al.*, 1989) we observed that in isolated atria, stable analogues of adenosine antagonized the positive inotropic effect of Bay K 8644, a dihydropyridine Ca^{2+} L-channel activator. Carbachol, a stable cholinoceptor agonist, was ineffective. The most simple explanation was that Ado analogues and carbachol, though having similar effects in single cells, may act differently in integrated structures such as isolated atria. In view of this, the aim of the present work was to compare the effect of different potassium channel blockers and of ionic variations in the medium, on the negative inotropic actions of

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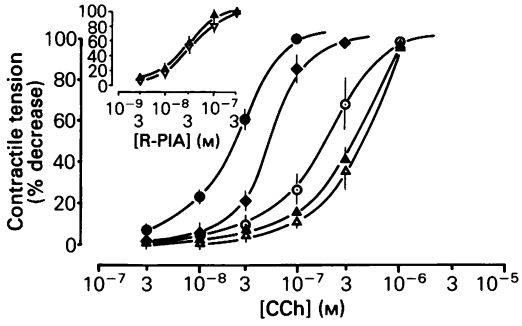

Figure 1 Inhibitory effect of tetraethylammonium (TEA) on the negative inotropic effect of carbachol (CCh) in spontaneously beating atria. Cumulative concentration-response curves for CCh in the absence (●) and in the presence of TEA 0.1 mM (◆), 1.0 mM (○), 10 mM (▲), 20 mM (△). Each point is the mean of 6–8 experiments. Vertical lines indicate s.e.mean. Inset: cumulative concentration-response curves for (−)-N^6-phenylisopropyladenosine (R-PIA) alone (▲) and in the presence of TEA 20 mM (▽).

(−)-N^6-phenylisopropyladenosine (R-PIA), an adenosine A_1-agonist, and of carbachol, in guinea-pig isolated atria.

The results show a different behaviour of carbachol and R-PIA when ionic channels and fluxes are modified by K^+ channel blocking drugs or by a change in the ion concentration. In these conditions, R-PIA, but not carbachol, is apparently able to inhibit the Ca^{2+} influx through L-channels.

Methods

The hearts were removed from guinea-pigs of either sex (300–500 g) and placed in a physiological solution (29°C) of the following composition (mM): NaCl 120, KCl 2.7, $CaCl_2$ 1.36, $MgCl_2$ 0.09, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, glucose 5.5, and gassed with 95% O_2 + 5% CO_2. The atria were dissected, suspended in a 30 ml organ bath and connected to a transducer (Basile, type DYO). An initial tension of 1 g was applied to the tissue and changes in isometric tension were recorded by a writing oscillograph (Basile, Unirecord System, cod. 7050).

Left atria were mounted on punctate electrodes with a load of 0.5 g and stimulated by square wave electrical pulses (1 Hz, 3 ms, 0.5–1.2 V) provided by a Grass stimulator (Mod. 25 S4KR). The voltage was about 20% greater than threshold. The control developed tension ranged from 0.8 to 1.3 mN. An equilibration period of 60 min was allowed before experiments were started. To investigate the calcium-dependent responses, the fast Na^+ channels were inactivated by elevating the external potassium concentration to 22 mM (Pappano, 1970; Thyrum, 1974; Sada *et al.*, 1986); equimolar NaCl was subtracted to maintain constant osmolarity. Under these conditions, contractility disappeared. After 60 min equilibration in high K^+ (22 mM) bathing solution, calcium-dependent action potentials, with consequent developed tension were induced by the addition of noradrenaline 3 μM. Stimulation frequency was lowered ten fold while voltage was increased.

Concentration-response curves were constructed by cumulative addition of drugs. Drug responses were allowed to equilibrate (4–8 min) before the subsequent addition of a higher concentration.

Results are expressed as % decrease from the control tension and rate.

Drugs and compounds used

(−)-N^6-phenylisopropyladenosine (R-PIA) (Boehringer, Mannheim) was dissolved and diluted in 50% ethanol-50% bathing solution. The total volume of ethanol never exceeded 0.05% (12.5 μl per 30 ml) in the organ bath. Carbachol, tetraethylammonium, noradrenaline (NA) and 4-aminopyridine (4-AP) were from Sigma; apamin from Serva Feinbiochemic; tetraethylammonium chloride (TEA) from Aldrich. Fresh stock solutions were prepared in distilled water and subsequently diluted with bathing solution to achieve the desired concentration.

Analysis of results

Values are presented as means ± s.e.mean. The −log concentration that produced half-maximal effects (−log EC_{50}) and its s.e.mean were determined by interpolation according to Tallarida & Murray (1987).

Results

Negative effect of (−)-N^6-phenylisopropyladenosine and carbachol on contractile tension and frequency in spontaneously beating atria

(−)-N^6-phenylisopropyladenosine, (R-PIA), a stable A_1-adenosine receptor agonist produced negative inotropic (EC_{50} = 19 nM) and chronotropic effects (EC_{50} = 42 nM) on the guinea-pig spontaneously beating atria. These effects were concentration-dependent between 3 and 300 nM. Carbachol, a muscarinic agonist also showed negative inotropic (EC_{50} = 22 nM) and chronotropic (EC_{50} = 36 nM) effects in the same range of concentrations as R-PIA.

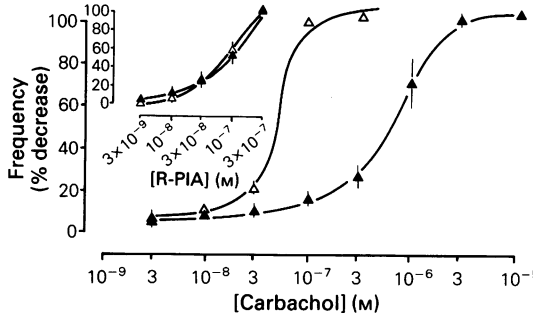

Figure 2 Effect of tetraethylammonium 20 mM (▲) on the negative chronotropic effect induced by carbachol (△, larger figure) and (−)-N^6-phenylisopropyladenosine (R-PIA, △, inset) in spontaneously beating atria. Each value is the mean of 8–12 experiments. Vertical lines indicate s.e.mean.

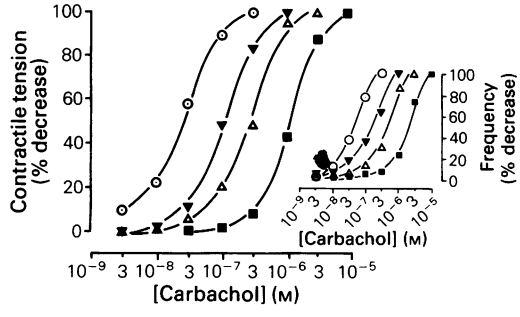

Figure 3 Concentration-response curves for carbachol alone (○) and in the presence of 4-aminopyridine 0.3 mM (▼), 0.7 mM (△) and 3 mM (■). Larger figure: % decrease of contractile tension. Inset: % decrease of frequency.

Tetraethylammonium and 4-aminopyridine on the negative inotropic and chronotropic effects of carbachol and R-PIA in spontaneously beating atria

Tetraethylammonium chloride 20 mM added to the medium 20 min before carbachol or R-PIA, slightly reduced *per se* the atrial rate (4 ± 1% of control) and increased the contractile tension (17 ± 2%), but maximally antagonized both the negative inotropic (Figure 1) and chronotropic (Figure 2) effects of carbachol. The concentration-response curve in the presence of TEA (0.1 mM to 10 mM) showed, in fact, that the inhibitory effect of carbachol was concentration-dependent (Figure 1). The slope of the Schild plot for contractility was different from unity (0.46 ± 0.13). The apparent pA_2 value was 4.47 ± 0.56 for contractility. TEA, 20 mM, failed to inhibit both the decrease of contractile tension (Figure 1) and of frequency (Figure 2) induced by PIA.

Concentrations of TEA higher than 20 mM could not be tested because of the development of arrhythmias, as also shown in other studies (Freeman, 1979; Asano *et al.*, 1985).

4-Aminopyridine (0.3 to 3 mM) was added to the organ bath 20 min before carbachol and R-PIA. 4-AP was found to cause *per se* some decrease in frequency and increase in contractility; this effect faded rapidly and the atria returned to the basal values in 2–6 min. 4-AP antagonized the negative inotropic and chronotropic effects of carbachol in a concentration-dependent manner and induced a parallel shift to the right of the dose-response curve of the drug (Figure 3). The slope of the Schild plot for contractility and frequency was not different from unity (1.10 ± 0.07 and 1.20 ± 0.15, respectively) possibly indicating a competitive antagonism. The

Table 1 Effects of 4-aminopyridine (4-AP), on the negative inotropism and chronotropism of (−)-N^6-phenylisopropyladenosine (R-PIA) in guinea-pig spontaneously beating atria

	*pD_2 ± s.e.mean (EC_{50})	
Compounds	*Contractile tension*	*Frequency*
R-PIA	7.70 ± 0.070 (19 nM)	7.37 ± 0.104 (42 nM)
R-PIA + 4-AP 0.3 mM	7.86 ± 0.134 [NS] (13 nM)	7.51 ± 0.096 [NS] (30 nM)
R-PIA + 4-AP 0.7 mM	7.87 ± 0.129 [NS] (19 nM)	7.54 ± 0.045 [NS] (28 nM)
R-PIA + 4-AP 3 mM	7.63 ± 0.075 [NS] (23 nM)	7.37 ± 0.095 [NS] (42 nM)

* pD_2 is the −log of the concentration able to induce half-maximum effect (EC_{50}). Each value was obtained by least squares method from 6–10 different curves.
NS = not significant vs R-PIA.

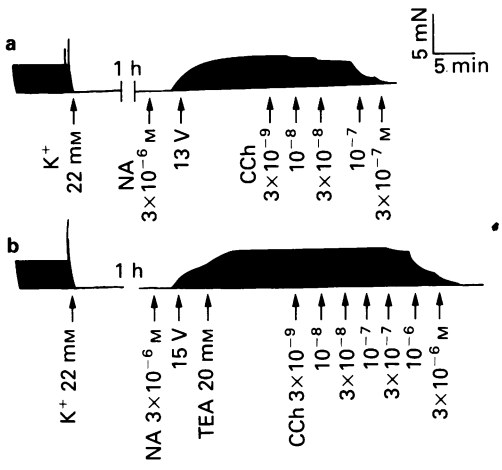

Figure 4 Effect of carbachol (CCh) in the absence (a) and presence (b) of tetraethylammonium (TEA) 20 mM on the contraction induced by noradrenaline (NA) 3 μM in the potassium-depolarized, electrically driven, left atria. Carbachol was added cumulatively and its final concentrations in the medium are indicated.

apparent pA_2 values were 4.02 ± 0.06 for contractility and 3.8 ± 0.10 for frequency, corresponding to K_is of 94 μM and 157 μM, respectively. 4-Aminopyridine 0.3 to 3 mM failed to inhibit both the inotropic and chrotropic effects of R-PIA (Table 1).

Apamin, 10–100 nM, preincubated 20 min before drug addition did not interfere with the negative ino-

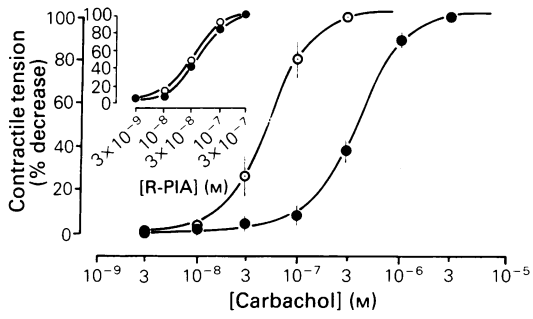

Figure 5 Effect of tetraethylammonium (TEA) on the negative inotropic effect of carbachol and of (−)-N^6-phenylisopropyladenosine (R-PIA, inset) in depolarized left atria (see Methods). Cumulative concentration-response curves for carbachol or R-PIA (inset) in the absence (○) and presence (●) of TEA 20 mM. Inotropic effects were expressed as percentage of the maximum increase induced by noradrenaline 3 μM. Each point is the mean of 6–7 experiments. Vertical lines indicate s.e.mean.

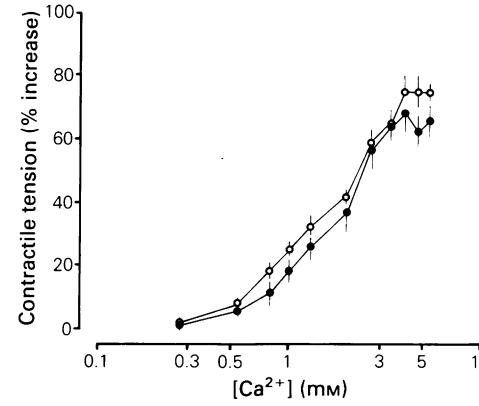

Figure 6 Effect of carbachol on the positive inotropic effect of Ca^{2+} in electrically driven left atria. Cumulative concentration-response curves for calcium in the absence (○) and presence (●) of carbachol 50 nM. Each point is the mean of 6 experiments. Vertical lines indicate s.e.mean.

tropic and chronotropic effects of carbachol and R-PIA (data not shown).

Tetraethylammonium chloride on the negative inotropic effect of carbachol and R-PIA in depolarized electrically driven atria

The effects of R-PIA and carbachol were investigated on guinea-pig left atria which were paced and depolarized (Figure 4). K^+ 22 mM abolishes the contractility of atria by inhibiting the fast Na^+ current (Thyrum, 1974). Further addition of catecholamines (or of other positive inotropic agents such as Ca^{2+}, or histamine) together with suitable conditions of electrical stimulation, induces slow action potentials chiefly due to Ca^{2+} currents (Pappano, 1970; Thyrum, 1974; Sada *et al.*, 1986). In our experiments, slow action potentials were evoked by adding noradrenaline 3 μM. In such conditions, R-PIA and carbachol showed the same effects as in non-depolarized atria; both drugs inhibited contractile tension (Figure 5) in a range of concentrations similar to those effective in spontaneously beating atria. TEA 20 mM induced *per se* an increase of contractile tension, probably due to an increase of Ca^{2+} influx (Asano *et al.*, 1985). TEA 20 mM inhibited the negative inotropic effect of carbachol (Figure 5) but not that of R-PIA (Figure 5), as previously shown in spontaneously beating atria.

Influence of calcium on the effects of R-PIA and of carbachol in electrically driven atria

In electrically driven atria the effect of R-PIA and carbachol on the contraction induced by progressive

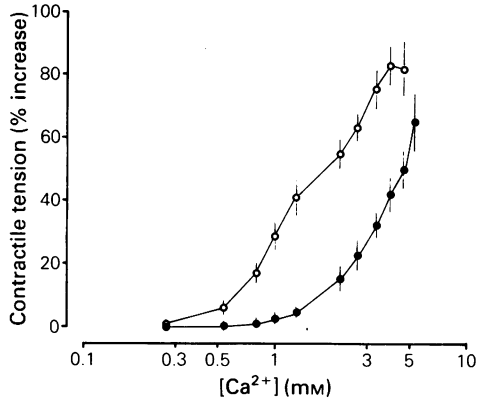

Figure 7 Inhibitory effect of (−)-N^6-phenylisopropyladenosine (R-PIA) on the positive inotropic effect of Ca^{2+} in electrically driven left atria. Cumulative concentration-response curves for calcium in the absence (○) and in presence (●) of R-PIA 50 nM. Each point is the mean of 6 experiments. Vertical lines indicate s.e.mean.

addition of Ca^{2+}, to a physiological medium previously depleted of this ion, was studied. In the absence of calcium the atrial contractility was abolished. Stepwise cumulative addition of Ca^{2+}, up to 4 mM, induced a concentration-dependent positive inotropic effect (Figure 6). Preincubation with carbachol 50 nM (EC_{75}) did not significantly affect the Ca^{2+}-dependent increase of contractile tension (Figure 6). In contrast, preincubation with R-PIA 50 nM (EC_{75}) clearly inhibited the Ca^{2+}-induced positive effect on contractile tension (Figure 7), mainly at lower Ca^{2+} concentrations (0.5–2.0 mM).

Discussion

Several types of potassium channel occur within the same cell (reviews: Hille, 1984; Cook, 1988). In cardiac tissue, at least seven potassium channels are operative (Pelzer & Trautwein, 1987). These have the function of repolarizing or hyperpolarizing the membrane potential at different moments and in the various cells. TEA blocks the majority of K^+ channels (see: Hille, 1984; Cook, 1988) such as the fast transient K^+ channels, delayed outward rectifier channels, and inward rectifiers. 4-AP is also non-selective, even if more active on transient delayed rectifier channels. In contrast, apamin, a bee venom polypeptide, is a selective blocker of the low conductance Ca^{2+}-activated K^+ channels which are insensitive to TEA and to 4-AP (Romey & Lazdunski, 1984; Cook & Haylett, 1985; Blatz & Magleby, 1986; Lazdunski *et al.*, 1987). Apamin was used in our experiments, as a negative control, because the potassium channels sensitive to this agent are present only in Purkinje fibres (Pelzer & Trautwein, 1987). The lack of effect by apamin on responses to R-PIA and carbachol thus validates the results obtained with TEA and 4-AP.

TEA antagonized in a concentration-dependent manner (0.1 to 10 mM) the negative effects of carbachol. The concentrations effective in our experiments are similar to those that prolong action potential in the guinea-pig ventricular fibres (Ochi & Nishiye, 1974), in guinea-pig ventricles (Corabeuf & Vassort, 1968) and in the heart of rat embryo (Bernard & Gargouïl, 1969). The mechanism of the negative effects of ACh in the mammalian heart (Ten Eick *et al.*, 1976) and in single atrial and pacemaker cells (Sakmann *et al.*, 1983; Iijima *et al.*, 1985) is dependent on the increase of the outward K^+ current shortening action potentials and indirectly reducing Ca^{2+} influx. The antagonism by TEA of carbachol in our experimental conditions is quite in accordance with the above data. However, TEA failed to inhibit the decrease of contraction and frequency induced by R-PIA. This result is puzzling, as the electrophysiological effects of Ado (and R-PIA) and of ACh in whole cell and patch-clamp studies are not distinguishable from each other and both substances activate the same K^+ channel population (Belardinelli & Isenberg, 1983; Kurachi *et al.*, 1986). Since our results with TEA are not in agreement with these conclusions, we studied the effect of 4-AP, by using the same protocol as for TEA. 4-AP inhibited the effects of carbachol, at concentrations which lengthened the action potential, increased the spike amplitude, and antagonized both the electrical and contractile effects of ACh in guinea-pig atria (Freeman, 1979). Again 4-AP was not able to antagonize the atrial effects of R-PIA.

A pertinent question is how can the difference between R-PIA and carbachol in atria be explained, if the two drugs are believed to affect the same population of K^+ channels? A first suggestion is that a block of K^+ channels unmasks the response of voltage-dependent Ca^{2+} channels (Hille, 1984). R-PIA is still effective in the presence of TEA or of 4-AP, implying a negative effect on Ca^{2+}-channels. This is further supported by the effect of TEA in experimental conditions where slow Ca^{2+} currents through L-channels are activated: 22 mM K^+ inhibits the fast Na^+ current thus abolishing the contractility of atria; the addition of noradrenaline together with suitable electrical stimulation induces slow action potentials chiefly due to slow Ca^{2+} currents (Pappano, 1970; Thyrum, 1974; Sada *et al.*, 1986). In such experimental conditions, carbachol is not effective, while R-PIA is still inhibitory.

R-PIA may antagonize the positive inotropism of Ca^{2+} addition by directly inhibiting the Ca^{2+} channels. These data are in accordance with previous results showing that R-PIA and other analogues of Ado are able to antagonize the positive inotropic effect of the dihydropyridine Ca^{2+} L-channel activator Bay K 8644 on isolated atria (Caparrotta *et al.*, 1987). Such effects are dependent on A_1 receptors and not on a direct interaction between Ado analogues and dihydropyridines at the level of specific binding sites on Ca^{2+} channels (Borea *et al.*, 1989). In addition, in rat cultured dorsal root ganglion neurones, 2-chloroadenosine reduces Ca^{2+} inward current activation by a direct effect on A_1-adenosine receptors (Dolphin *et al.*, 1986).

We suggest that Ado and ACh, although they have the same activity on ion currents in isolated cells or patch clamp studies, may display a different behaviour in atrial multicellular preparations under certain conditions, in that adenosine receptors may additionally inhibit Ca^{2+} channels.

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Investigation of the central sites at which morphine acts to cause hypertension in conscious rabbits

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1 In conscious rabbits intracerebroventricular (i.c.v.) morphine (10 and 50 $\mu g\,kg^{-1}$) caused a dose-related increase in plasma noradrenaline and adrenaline, respiratory depression and sedation. The increase in sympatho-adrenal outflow resulted in hypertension accompanied by bradycardia and the increase in adrenaline secretion caused hyperglycaemia. Morphine (1 $\mu g\,kg^{-1}$ i.c.v.) and i.c.v. saline had no effect.

2 The same doses of morphine given intracisternally (i.c.) caused bradycardia and a similar degree of respiratory depression to i.c.v. morphine, but no significant increase in blood pressure and only a small, gradual rise in plasma adrenaline.

3 Intravenous naloxone (1 $mg\,kg^{-1}$) did not block the hypertension, hyperglycaemia or increase in plasma catecholamines that followed i.c.v. morphine, but prevented the respiratory depression and sedation.

4 Ganglionic blockade with pentolinium prevented the rise in plasma catecholamines, blood pressure and plasma glucose induced by i.c.v. morphine.

5 These findings demonstrate that the increased sympathoadrenal outflow following i.c.v. morphine results from an action on periventricular structures. The resultant increase in plasma catecholamines, which is largely naloxone resistant, accounts for the hypertension and hyperglycaemia. The bradycardia is probably partly baroflex mediated and partly due to an increase in vagal tone as a result of stimulation of brainstem opioid receptors. The respiratory depression is probably due to an action of morphine on brainstem opioid receptors.

Introduction

Morphine given intravenously caused a centrally mediated increase in sympatho-adrenal outflow in conscious, but not anaesthetised rabbits (May *et al.*, 1988; 1989). The increased sympathetic nerve activity and increased secretion of adrenaline caused a sustained increase in blood pressure (BP) accompanied by a fall in heart rate (HR). The increased adrenaline secretion accounted for the hyperglycaemia. These effects together with the respiratory depression and sedation were prevented by intravenous (i.v.) or intracerebroventricular (i.c.v.) naloxone indicating dependence on stimulation of central opioid receptors (May *et al.*, 1988; 1989).

We have investigated the central sites at which morphine acts by comparing the responses to i.c.v. and intracisternal (i.c.) administration of morphine in conscious rabbits. The effects of a range of doses given by these two routes on cardiovascular and respiratory function and on sympatho-adrenal outflow have been compared.

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Methods

Surgical procedures

Two groups of male Sandy Half-lop rabbits (National Institute for Medical Research, Mill Hill) allowed free access to food (RHM R14, Labsure Animal Diets) and water and weighing between 2.4 and 3.8 kg were studied.

For injection into the right lateral ventricle, cannulae (Harvard Apparatus) were implanted aseptically under general anaesthesia (Saffan, Glaxovet). Coordinates were 1.0 mm caudal to bregma, 3.0 mm

lateral to midline and 8.5 mm below the dura. Cannulae were screwed into a hole drilled in the skull and fixed with dental cement which also enveloped 3 stainless steel screws screwed into the skull. Drugs were injected i.c.v. by a Hamilton syringe with a 25 g needle that protruded 1 mm from the cannula tip.

For injection into the cisterna magna a modification of the catheter described by Head *et al.* (1983) was used. Catheters were made from PP10 polythene tubing (i.d. 0.28 mm, o.d. 0.61 mm, Portex) one end of which was warmed and stretched to give an internal diameter of approximately 0.1 mm. A 4 cm length of silicone tubing (0.5 i.d., 1.0 mm o.d.) was expanded by soaking in diethyl ether which enabled it to be sleeved over the PP10 tube leaving 0.5 cm of the thinned tip protruding. The thinned end was bent back on to the silicone tube, held with tape and heated to 60°C after which it remained set in position. Animals were anaesthetised with Saffan, the atlanto-occipital membrane exposed and the tip of the cannula was inserted through a hole made with a 25 g needle. The catheter was held in place by a suture (6-0) which was passed through the silicone tube and membrane. Two further sutures were inserted through the periosteum overlying the occipital protruberance and tied around the silicone tube. The proximal end of the silicone tube was sutured to the muscle under the skin, and 4 cm of the PP10 tubing was left to protrude from the skin at the back of the neck. This was plugged and could be repeatedly used. The dead volume of the catheter was 10 μl. These catheters have remained patent and given reproducible responses for up to 6 months. Drugs were administered from a Hamilton syringe with a 30 g needle.

The injection site was confirmed at the end of a series of experiments by injection of 100 μl of bromophenol blue (1%), followed by 50 μl of saline i.c.v. or 20 μl i.c. The animal was killed by an overdose of pentobarbitone, the brain removed and the distribution of dye examined.

Animals were not used for at least 7 days post-operation. Before each experiment cannulae were inserted into the central artery and marginal vein of the ear under 1% lignocaine local anaesthesia. The arterial cannula was connected to a pressure transducer (Bell and Howell) and BP and HR were recorded on a Devices polygraph. During the experiment the rabbits sat on a grid (30 cm long) with solid plastic sides (14 cm high) adjusted to suit the width of the rabbit. Intervals of at least 7 days were allowed between studies.

Blood collection and analysis

Plasma from blood (2 ml) collected from the arterial cannula into fluoride oxalate tubes was used for the measurement of plasma glucose by a glucose oxidase method using a Chem Lab analyser. Catecholamines were measured in plasma from blood (2.5 ml) collected into cooled tubes containing 50 μl of EGTA (0.095%)/glutathione (0.06%) and kept on ice until centrifugation. Catecholamines were separated by h.p.l.c. and detected by electrochemical detection (May *et al.*, 1988). Arterial blood (100 μl) was collected into a capillary tube for the measurement of blood gases (Corning 158 pH/blood gas analyser).

Drugs

Drugs were dissolved in sterile, non-pyrogenic saline and doses refer to the salts. For i.c.v. and i.c. administration morphine sulphate (Macarthys) was given in 100 μl injected over 1 min, followed by 50 μl of saline (i.c.v.) or 20 μl of saline (i.c.). The treatments for the dose-response study were given in a randomised order. Naloxone hydrochloride (Sigma) was given either i.v. in 1 ml (0.5 or 1.0 mg kg^{-1}) via the marginal ear vein or i.c.v. in 100 μl (50 or 100 μg kg^{-1}) 15 min before morphine. Pentolinium (May and Baker) was given i.v. as a bolus dose (5 mg kg^{-1} in 1 ml kg^{-1}) 10 min before i.c.v. morphine.

Data analysis

Data are presented as means $\pm$ s.e.mean. Comparisons between treatment groups were made on differences from baseline values by use of the SAS statistical programme. The baseline values for mean arterial pressure (MAP) and HR consisted of the mean of the six readings taken during the 30 min control period. A logarithmic transformation was performed on the values for glucose, noradrenaline and adrenaline before analysis. The data were subjected to analysis of variance and where the null hypothesis was rejected Scheffe's comparisons were performed on the means; $P < 0.05$ was considered significant.

Results

Cardiovascular effects of i.c.v. and i.c. morphine

In conscious rabbits ($n = 5$) i.c.v. morphine produced a dose-related increase in MAP accompanied by a fall in HR. After i.c.v. morphine (50 μg kg^{-1}) the maximum increase in MAP of 43 ± 4 mmHg ($P < 0.05$) occurred after 15 min and MAP remained significantly elevated above baseline values for the 120 min experimental period. After i.c.v. morphine HR fell by 46 ± 17 beats min^{-1} ($P < 0.05$) after 10 min and by 70 ± 11 beats min^{-1} ($P < 0.05$) after

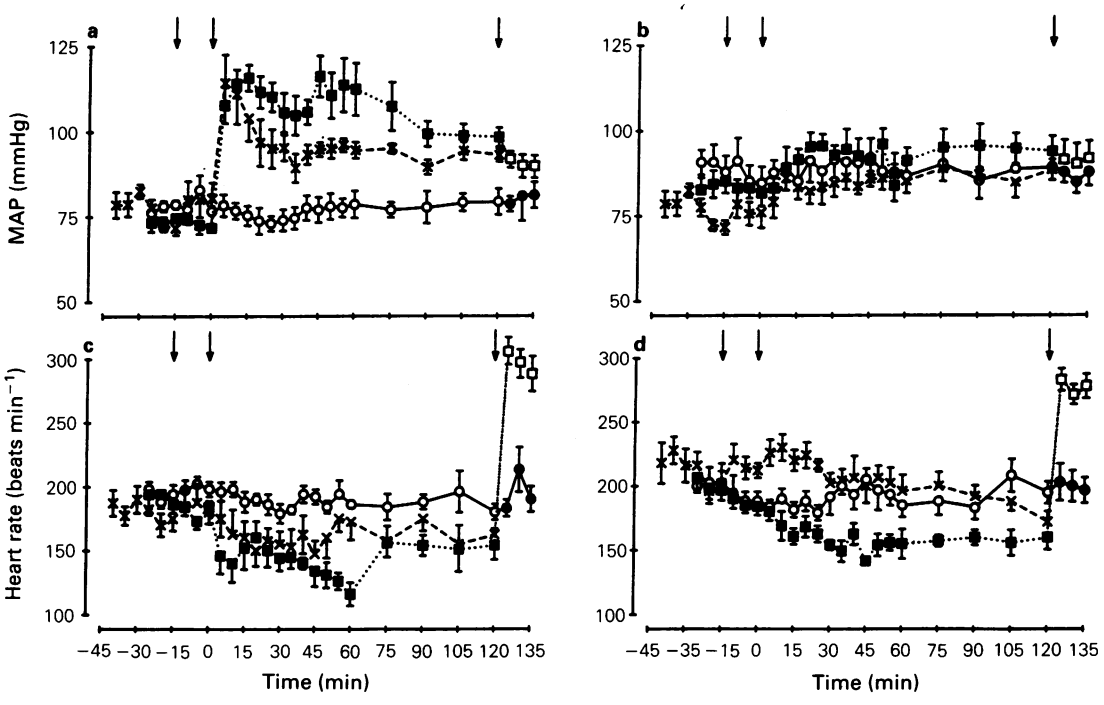

Figure 1 Cardiovascular responses to morphine or saline given either intracerebroventricularly (i.c.v.) (a, c) or intracisternally (i.c.) (b, d) in conscious rabbits. The effect on (a, b) mean arterial pressure (MAP) and (c, d) heart rate of saline (100 μl) (○——○) or morphine (50 $\mu g\,kg^{-1}$) (■·····■) given at 0 min either i.c.v. ($n = 5$) or i.c. ($n = 6$) followed by i.v. naloxone (0.5 $mg\,kg^{-1}$) given 120 min after saline (●——●) or morphine (□·····□), and the effect of i.v. naloxone (1 $mg\,kg^{-1}$) given at −15 min on the response to i.c.v. ($n = 4$) and i.c. ($n = 5$) morphine (50 $\mu g\,kg^{-1}$) (×– – –×). Each point represents the mean and vertical lines show s.e.mean.

60 min (Figure 1). After i.c.v. morphine (10 $\mu g\,kg^{-1}$) the changes in MAP and HR followed a similar time course. MAP increased by 21 ± 5 mmHg ($P < 0.05$) and HR fell by 31 ± 7 beats min^{-1} after 10 min; these changes in MAP were significantly greater than after i.c.v. saline but significantly less than after i.c.v. morphine (50 $\mu g\,kg^{-1}$). After 60 min MAP was elevated by 25 ± 8 mmHg ($P < 0.05$) and HR reduced by 43 ± 6 beats min^{-1}, and after 120 min MAP was elevated by 11 ± 5 mmHg and HR reduced by 19 ± 13 beats min^{-1}. There were no cardiovascular changes following i.c.v. morphine (1 $\mu g\,kg^{-1}$) or i.c.v. saline (100 μl).

Naloxone (0.5 $mg\,kg^{-1}$, i.v.) given 120 min after the 50 $\mu g\,kg^{-1}$ dose of i.c.v. morphine reduced MAP, although not to baseline levels, and caused an immediate increase in HR to 120 ± 12 beats min^{-1} above baseline ($P < 0.05$) (Figure 1). There was a similar rise in HR of 115 ± 10 beats min^{-1} when naloxone (0.5 $mg\,kg^{-1}$) was given 120 min after the 10 $\mu g\,kg^{-1}$ dose of morphine ($P < 0.05$), but an increase of only 10 ± 2 beats min^{-1} when it was given 120 min after the 1 $\mu g\,kg^{-1}$ dose ($P < 0.05$). After pretreatment with naloxone (1 $mg\,kg^{-1}$, i.v.), i.c.v. morphine (50 $\mu g\,kg^{-1}$) caused a similar initial increase in MAP which was followed by a more rapid fall than after morphine alone; the fall in HR was not affected by naloxone except at 55 and 60 min after morphine when HR in the naloxone-treated group was significantly greater (Figure 1). We have previously shown that, in conscious rabbits, i.v. naloxone (1 $mg\,kg^{-1}$) does not alter blood pressure, HR or plasma catecholamine levels (May *et al.*, 1988). In additional studies pretreatment with i.c.v. naloxone (100 $\mu g\,kg^{-1}$), given 15 min before i.c.v. morphine (50 $\mu g\,kg^{-1}$), or 50 $\mu g\,kg^{-1}$ naloxone given 15 min before 10 $\mu g\,kg^{-1}$ morphine, did not prevent the morphine-induced hypertension and bradycardia.

The same doses of morphine given i.c. resulted in significantly smaller changes in MAP than after i.c.v. administration. In six conscious rabbits i.c. morphine (50 $\mu g\,kg^{-1}$) caused a gradual increase in MAP and fall in HR; over 30 min MAP increased by 9 ± 4 mmHg and HR fell by 39 ± 6 beats min^{-1} but these changes were not significant (Figure 1). After i.c. morphine (10 $\mu g\,kg^{-1}$) HR fell by 20 ± 9

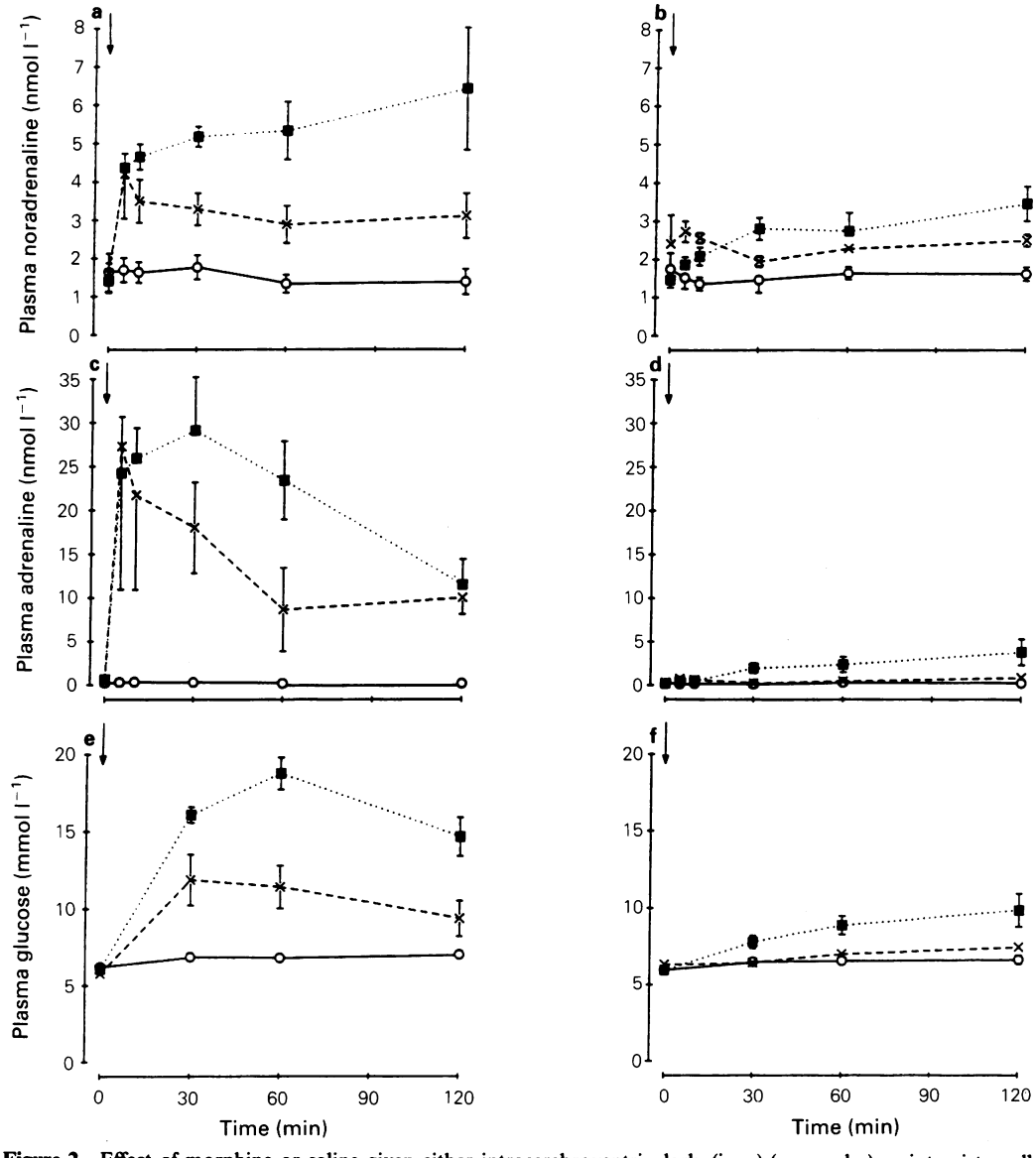

Figure 2 Effect of morphine or saline given either intracerebroventricularly (i.c.v.) (a, c and e) or intracisternally (i.c.) (b, d and f) on plasma levels of (a, b) noradrenaline, (c, d) adrenaline and (e, f) glucose in conscious rabbits. The effect of saline (100 μl) (○——○) or morphine (50 μg kg^{-1}) (■·····■) given at 0 min either i.c.v. ($n = 5$) or i.c. ($n = 6$) followed by i.v. naloxone (0.5 mg kg^{-1}) given 120 min after saline (●——●) or morphine (□·····□), and the effect of i.v. naloxone (1 mg kg^{-1}) given at −15 min on the response to i.c.v. ($n = 4$) and i.c. ($n = 5$) morphine (50 μg kg^{-1}) (×– – –×). The control samples were taken before the administration of drugs or vehicle. Each point represents the mean and vertical lines indicate s.e.mean.

beats min^{-1} over 30 min and this was accompanied by a rise in MAP of 5 ± 30 mmHg. There were no changes after i.c. morphine (1 μg kg^{-1}) or i.c. saline (100 μl).

Naloxone (0.5 mg kg^{-1}, i.v.) given 120 min after i.c. morphine (50 μg kg^{-1}) had no effect on MAP but increased HR by 90 ± 8 beats min^{-1} ($P < 0.05$) (Figure 1). HR increased by 62 ± 10 beats min^{-1}

($P < 0.05$) when naloxone (0.5 mg kg^{-1} i.v.) was given 120 min after i.c.v. morphine (10 μg kg^{-1}), but there was no effect when it was given after i.c. morphine (1 μg kg^{-1}) or i.c. saline. Pretreatment with naloxone (1 mg kg^{-1}, i.v.) reduced the BP response to i.c. morphine (50 μg kg^{-1}) and prevented the fall in HR (Figure 1).

Effects of i.c.v. and i.c. morphine on plasma catecholamines and plasma glucose

After i.c.v. morphine (50 μg kg^{-1}), plasma noradrenaline increased by 3.0 ± 0.5 nmol l^{-1} ($P < 0.05$) within 5 min and rose continually throughout the remaining 120 min to reach 5.1 ± 0.8 nmol l^{-1} above control ($P < 0.05$) (Figure 2). Plasma adrenaline reached a peak of 28.5 ± 6.3 nmol l^{-1} above control ($P < 0.05$) after 30 min and then fell, but remained significantly above control values after 120 min. After i.c.v. morphine (10 μg kg^{-1}) the initial rise in noradrenaline (2.0 ± 0.4 nmol l^{-1}) after 5 min was similar to that after the higher dose but the increases were less after 30 min (2.6 ± 0.1 nmol l^{-1}), 60 min (4.0 ± 0.5 nmol l^{-1}) and 120 min (3.0 ± 0.5 nmol l^{-1}). The increases in plasma adrenaline after 5 min (19.9 ± 6.4 nmol l^{-1}), 30 min (24.1 ± 5.7 nmol l^{-1}), 60 min (25.4 ± 5.7 nmol l^{-1}) and 120 min (12.2 ± 2.6 nmol l^{-1}) were similar to those after 50 μg kg^{-1} of morphine. There was a small transient rise in both catecholamines 5 min after 1 μg kg^{-1} morphine i.c.v. and no effect after i.c.v. saline. Pretreatment with naloxone (1 mg kg^{-1}, i.v.) did not alter the initial increase in plasma noradrenaline and adrenaline after i.c.v. morphine (50 μg kg^{-1}), but thereafter the levels of both catecholamines fell more rapidly than in the absence of naloxone (Figure 2).

After i.c. morphine (50 μg kg^{-1}) there was a small gradual increase in plasma adrenaline which was significant from 10 min after morphine, but there were no significant changes in plasma noradrenaline. The changes in adrenaline were reduced by pretreatment with naloxone (1 mg kg^{-1}, i.v.) (Figure 2). There was no change in catecholamines after the lower doses of i.c. morphine (1 and 10 μg kg^{-1}).

There was a dose-related hyperglycaemia after i.c.v. morphine (Figure 2). The maximum rise in plasma glucose of 12.7 ± 1.1 mmol l^{-1} ($P < 0.05$) occurred 60 min after the 50 μg kg^{-1} dose. This effect was significantly reduced but not abolished by pretreatment with naloxone (1 mg kg^{-1}, i.v.). After i.c.v. morphine (10 μg kg^{-1}) the maximum increase in plasma glucose was 7.9 ± 1.1 mmol l^{-1} ($P < 0.05$) after 60 min. There were no significant changes after i.c.v. morphine (1 μg kg^{-1}) or i.c.v. saline. In contrast, after i.c. morphine (50 μg kg^{-1}) there was a small gradual increase in plasma glucose reaching 3.9 ± 1.0 mmol l^{-1} ($P < 0.05$) above control after 120 min and this was prevented by pretreatment with i.v. naloxone (1 mg kg^{-1}) (Figure 2). There were no significant changes in plasma glucose after the 10 μg kg^{-1} and 1 μg kg^{-1} doses of i.c. morphine or after i.c. saline.

Effect of i.c.v. and i.c. morphine on respiration

Morphine given i.c.v. or i.c. caused a dose-related fall in respiration rate which led to an increase in $Paco_2$ and a fall in Pao_2 (Figure 3). Following morphine given via either route the maximum changes in respiration occurred after 90 min. At this time after the 50 μg kg^{-1} dose given i.c.v. (or i.c.) respiration rate had fallen by 120 ± 22 (142 ± 13) breaths min^{-1}. $Paco_2$ had increased by 22.9 ± 0.6 (23.2 ± 1.5) mmHg and Pao_2 had fallen by 25.8 ± 2.6 (35.7 ± 3.5) mmHg (Figure 3). At 90 min after 10 μg kg^{-1} morphine given i.c.v. (or i.c.) respiration rate had fallen by 116 ± 20 (143 ± 25) breaths min^{-1}, $Paco_2$ had risen by 22.4 ± 2.2 (18.7 ± 2.8) mmHg and Pao_2 had fallen by 30.7 ± 3 (24.7 ± 6) mmHg. The 1 μg kg^{-1} dose given i.c.v. (or i.c.) reduced respiration rate by 106 ± 14 (68 ± 20) breaths min^{-1}, increased $Paco_2$ by 9.2 ± 1.8 (5.7 ± 2) mmHg and reduced Pao_2 by 7.8 ± 1.4 (7.9 ± 2.5) mmHg. The changes in $Paco_2$ and Pao_2 following i.c.v. and i.c. morphine (50 and 10 μg kg^{-1}) were significantly different from those after saline. Naloxone (0.5 mg kg^{-1}, i.v.) given 120 min after morphine returned blood gases to control values within 5 min and significantly increased respiration rate above control when given after i.c.v. but not i.c. morphine (Figure 3).

Pretreatment with naloxone (1 mg kg^{-1}, i.v.) initially prevented the respiratory depression caused by i.c.v. and i.c. morphine (50 μg kg^{-1}) but after 60 min respiratory depression began to develop (Figure 3). After naloxone pretreatment, i.c.v. but not i.c. morphine caused an increase in respiration rate of 107 ± 25 beats min^{-1} ($P < 0.05$) after 5 min which was accompanied by a fall in $Paco_2$ (7.6 ± 4.1 mmHg) and a rise in Pao_2 (14.6 ± 2.6 mmHg).

Effects of i.c.v. and i.c. morphine on behaviour

Morphine (1 μg kg^{-1}) given i.c.v. produced a mild degree of sedation which lasted 1–2 h. The sedation following the 2 higher doses was deeper and longer lasting and these doses also produced catalepsy. These effects were prevented by pretreatment with naloxone. In initial studies a higher dose of morphine of 100 μg kg^{-1} was used, but when given i.c.v. or i.c. this dose caused excitation and rapid circling movements. Therefore, the highest dose was reduced to 50 μg kg^{-1}. After i.c. administration there was

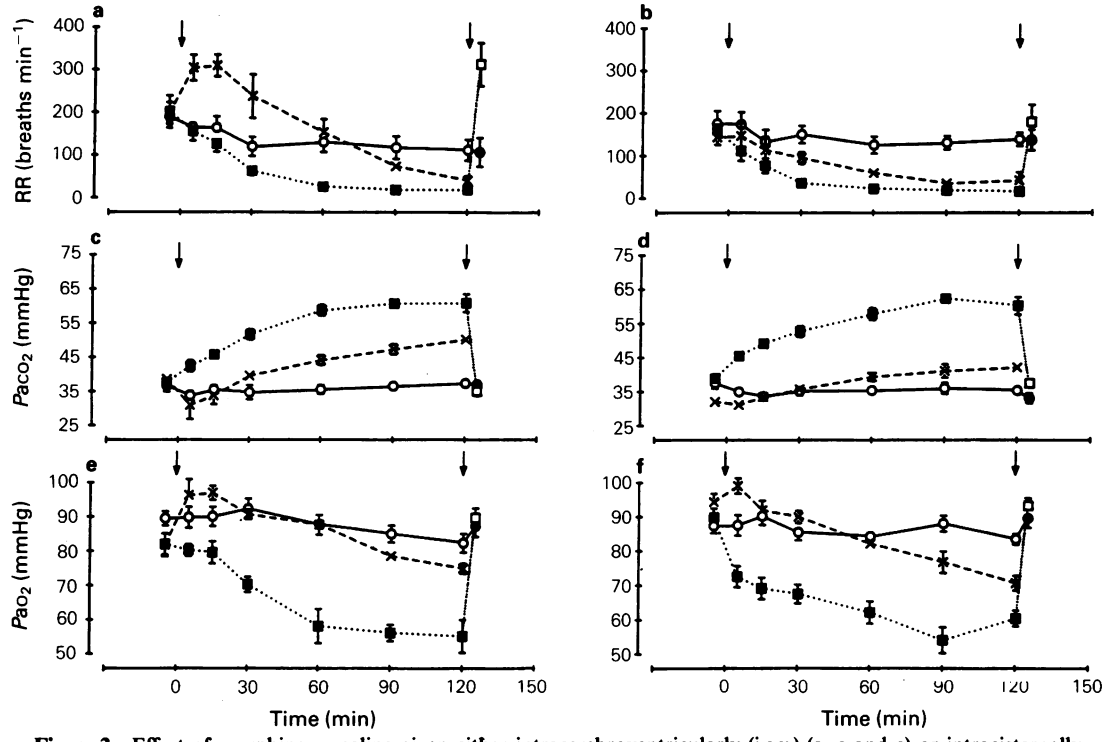

Figure 3 Effect of morphine or saline given either intracerebroventricularly (i.c.v.) (a, c and e) or intracisternally (i.c.) (b, d and f) on (a, b) respiration rate (RR), (c, d) $Paco_2$ and (e, f) Pao_2 in conscious rabbits. The effect of saline (100 μl) (○——○) or morphine (50 $\mu g\,kg^{-1}$) (■·····■) given at 0 min either i.c.v. ($n = 5$) or i.c. ($n = 6$) followed by i.v. naloxone (0.5 $mg\,kg^{-1}$) given 120 min after saline (●——●) or morphine (□·····□), and the effect of i.v. naloxone (1 $mg\,kg^{-1}$) given at −15 min on the response to i.c.v. ($n = 4$) and i.c. ($n = 5$) morphine (50 $\mu g\,kg^{-1}$) (×- - -×). The control samples were taken before the administration of drugs or vehicle. Each point represents the mean and vertical lines indicate s.e.mean.

only a mild degree of sedation which developed after the 50 $\mu g\,kg^{-1}$ dose.

Effect of ganglionic blockade on responses to i.c.v. morphine

In 5 conscious rabbits ganglionic blockade with i.v. pentolinium (5 $mg\,kg^{-1}$) reduced MAP from 93 ± 5 to 70 ± 3 mmHg and increased HR from 190 ± 8 to 226 ± 10 beats min^{-1} after 10 min. At 10 min after pentolinium, administration of i.c.v. morphine (50 $\mu g\,kg^{-1}$) had no cardiovascular effects. At 15 min after morphine, the time of the peak response, MAP (91 ± 8 mmHg) was similar to the control value and HR (244 ± 14 beats min^{-1}) remained elevated. After 60 min MAP (105 ± 9 mmHg) was not significantly different from control and the tachycardia (246 ± 14 beats min^{-1}) was unchanged. Pentolinium given alone to conscious rabbits causes a transient fall in MAP, a prolonged increase in HR and reduces plasma catecholamines (May *et al.*, 1989).

Ganglionic blockade abolished the increase in plasma noradrenaline and adrenaline that followed i.c.v. morphine (Table 1). The respiratory depressant effects of i.c.v. morphine were unaffected by pentolinium pretreatment, the changes in blood gases (Table 1) being similar to those after morphine alone (Figure 3).

Discussion

In these studies the effects of morphine given i.c.v. or i.c. to conscious rabbits have been compared. After i.c.v. morphine the changes were similar to those after systemic administration and included an increase in plasma catecholamines, hypertension, hyperglycaemia, respiratory depression and sedation. These effects were dose-related and occurred at considerably lower doses than required systemically (10 $\mu g\,kg^{-1}$ i.c.v. being equivalent to 4 $mg\,kg^{-1}$ i.v.).

Table 1 Effect of intracerebroventricular (i.c.v.) morphine ($50\,\mu g\,kg^{-1}$) on plasma catecholamines and respiration after pretreatment with i.v. pentolinium ($5\,mg\,kg^{-1}$) in 5 rabbits

		Time after morphine (min)			
	Control	5	10	30	60
Noradrenaline ($nmol\,l^{-1}$)	2.08 ± 0.60	2.00 ± 0.38	1.57 ± 0.23	2.09 ± 0.28	3.19 ± 0.57
Adrenaline ($nmol\,l^{-1}$)	0.36 ± 0.22	0.33 ± 0.22	0.51 ± 0.39	0.19 ± 0.11	0.33 ± 0.23
Respiration rate (breaths min^{-1})	166 ± 34	228 ± 19	208 ± 24	119 ± 34	39 ± 6
$Paco_2$ (mmHg)	34.3 ± 0.8	38.8 ± 1.1	42.6 ± 1.4	47.3 ± 0.9	53.4 ± 1.0
Pao_2 (mmHg)	82.4 ± 1.6	79.5 ± 3.0	71.4 ± 4.3	69.4 ± 2.3	66.1 ± 2.7

The second post treatment measurement of respiration rate and blood gases was made after 15 not 10 min.

In contrast, after i.c. morphine there was little effect on plasma catecholamines or BP, but the degree of respiratory depression and fall in HR were similar. These effects of morphine do not result from leakage into the peripheral circulation, as we have previously shown that a higher dose of morphine ($300\,\mu g\,kg^{-1}$) given i.v. had no effect (May *et al.*, 1988).

These findings are consistent with those of Conway *et al.* (1983) who demonstrated that in conscious rats i.c.v. morphine produced hypertension, catecholamine release and respiratory depression. The present finding that the morphine-induced increase in sympatho-adrenal activity and hypertension occurs after i.c.v. but not i.c. administration, suggests that it results from an action on periventricular structures and not from brainstem sites. A periventricular site of action for morphine is supported by the demonstration that in conscious rats microinjections into the hypothalamus of the μ-opioid agonist (D-Ala^2, MePhe^4, Gly^5-ol) enkephalin (DAGOL) increased plasma catecholamines and caused hypertension (Pfeiffer *et al.*, 1983).

The fall in HR after morphine appears to consist of two components. An initial fall concurrent with the rise in BP, that is probably baroceptor mediated, and a delayed fall similar to that after i.c. morphine. The bradycardia after i.c. morphine, which occurs in the absence of a significant rise in BP, may result from an increase in vagal tone due to stimulation of brainstem opioid receptors. Similar conclusions have been reached from studies in which i.c. administration of opiates caused bradycardia, although this was accompanied by hypotension probably because the studies were in anaesthetised animals (Laubie *et al.*, 1974; Feldberg & Wei, 1981). The tachycardia in response to naloxone, given 120 min after i.c.v. or i.c. morphine, is similar to that previously observed when naloxone was given after DAGOL (Pfeiffer *et al.*, 1983). This may result from antagonism by naloxone of an increase in vagal tone mediated by opioid receptors, probably in the brainstem as it occurs after i.c. as well as after i.c.v. morphine. The greater rise in the group treated with i.c.v. morphine may result from the positive chronotropic effects of the higher plasma levels of adrenaline in these animals at this time.

The cardiovascular effects of opiates could result from respiratory depression since hypoxia and hypercapnia increase sympatho-adrenal outflow (Korner & White, 1966). However, the differences in time course between the rapid increase in BP and the slowly developing respiratory depression argues against this as a mechanism. In addition, hypertension did not develop after i.c. morphine, although the degree of respiratory depression was similar, indicating that the initial increase in BP is independent of respiratory depression. However, the later rise in BP at 60 min after i.c.v. morphine, and the small rise in BP following i.c. morphine, may result from increased noradrenaline release secondary to the respiratory depression. The finding that i.c.v. morphine, given after naloxone pretreatment, caused hypertension in the absence of respiratory depression is further evidence that the morphine-induced increase in sympatho-adrenal outflow and rise in MAP are not caused by the hypoxia and hypercapnia.

In the present experiments the degree of hyperglycaemia was related to the increase in plasma adrenaline levels; plasma glucose was greater after i.c.v. than i.c. morphine and was reduced by naloxone pretreatment which reduced the rise in adrenaline. This agrees with the finding that in conscious rabbits the hyperglycaemic effect of i.v. morphine results from a centrally mediated increase in adrenaline secretion (May *et al.*, 1988; 1989). The greater rise after i.c.v. compared with i.c. morphine indicates an action on periventricular sites. However, in the cat the hyperglycaemic potency of i.c.v. morphine (0.75 mg) was similar to i.c. morphine (1.5 mg)

(Feldberg *et al.*, 1985). Hyperglycaemia was produced by injections into the subarachnoid space below the ventral surface of the brainstem which was proposed as the site of action of morphine. This species difference may be due to different localisation of μ-receptors in cat and rabbit or because the tissue distribution of morphine is different in the two species.

We have previously demonstrated that the hypertension following i.v. morphine results from increased sympatho-adrenal outflow (May *et al.*, 1989). The present findings indicate that this is also the mechanism by which i.c.v. morphine increases BP, as the increases in plasma catecholamines were similar and ganglionic blockade prevented the hypertension after i.v. and i.c.v. morphine. However, the central mechanisms leading to the increased sympatho-adrenal outflow appear to differ, as discussed below.

Naloxone, given i.v. ($1\,mg\,kg^{-1}$) or i.c.v. ($100\,\mu g\,kg^{-1}$), did not prevent the rise in plasma catecholamines or hypertension after i.c.v. morphine, although the respiratory depression and sedation were prevented. In contrast the effects of i.v. morphine are prevented by one tenth of these doses of naloxone, i.e. $100\,\mu g\,kg^{-1}$ given i.v. (unpublished) or $10\,\mu g\,kg^{-1}$ given i.c.v. (May *et al.*, 1989). These findings demonstrate that the increase in sympatho-adrenal outflow after i.c.v. morphine is naloxone-resistant which is consistent with previous observations. The hyperglycaemic responses to i.c.v. and i.c. morphine in conscious cats were not blocked by i.v. naloxone, although the effects of an equivalent dose of morphine given i.v. were blocked by the same dose of naloxone (Feldberg *et al.*, 1983). In conscious rats i.c.v. morphine increased BP and plasma catecholamines and these effects were not prevented by i.c.v. or i.v. naloxone (Conway *et al.*, 1983). These authors suggested that this could be explained by the different concentrations of agonists and antagonists at central receptor sites due to different routes of administration. However, this seems unlikely considering our demonstration that the hypertensive effects of i.c.v. morphine are not blocked by doses of naloxone, given either i.c.v. or i.v., which are 10 times greater than the doses needed to abolish the effects of i.v. morphine.

Naloxone can increase sympatho-adrenal outflow and blood pressure when given after various forms of shock (Holaday, 1983), which would obscure any inhibition of the effects of morphine. However, we have previously demonstrated that in unstressed conscious rabbits i.v. naloxone ($1\,mg\,kg^{-1}$) has no effect on systolic or diastolic blood pressure or on HR (May *et al.*, 1988). This is in agreement with other data demonstrating that opiate antagonism does not alter blood pressure or HR in conscious rabbits or rats (Holaday & Faden, 1978; Petty & Reid, 1982; Rutter *et al.*, 1987). The mechanism by which i.c.v. morphine increases sympatho-adrenal outflow awaits investigation but may involve central release of neurotransmitters or neuropeptides by morphine. Central administration of histamine elevates BP, probably by increasing sympathetic outflow as it is prevented by ganglionic blockade (Trendelenburg, 1957). In addition, several neuropeptides act centrally to alter sympatho-adrenal outflow (Brown & Fisher, 1984) which leads to selective cardiovascular changes.

In conclusion, these results indicate that the cardiovascular effects of centrally administered morphine result from actions on a number of sites. The increased sympatho-adrenal outflow appears to be mediated mainly via periventricular structures, but the mechanism appears to be different from that after i.v. morphine. The bradycardia is probably a combination of a reflex response and an increase in vagal tone, possibly mediated by brainstem opiate receptors. The respiratory depression is probably mediated by opiate receptors in the brainstem.

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Effects of adenosine on polymorphonuclear leucocyte function, cyclic 3′ : 5′-adenosine monophosphate, and intracellular calcium

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1 Inhibition of human polymorphonuclear leucocyte (PMN) function by adenosine was studied with respect to effects of adenosine on intracellular cyclic AMP and calcium during the PMN respiratory burst.

2 The adenosine analogue 5′-N-ethylcarboxamide-adenosine (NECA) and L-N^6-phenyl-isopropyl-adenosine (L-PIA) inhibited PMN oxygen metabolite generation with relative potencies (NECA > adenosine > L-PIA) characteristic of an A_2 receptor.

3 The respiratory burst was inhibited by adenosine when PMN were activated by calcium ionophore or chemotactic peptide but not when cells where activated by oleoyl-acetyl-glycerol (OAG).

4 Adenosine increased intracellular cyclic AMP during the PMN respiratory burst regardless of whether cells were stimulated by ionophore, chemotactic peptide or OAG.

5 To determine whether the differences in cell inhibition by adenosine were related to differences in intracellular calcium mobilization by each activating agent, calcium was evaluated with the fluorescent probe, indo-1. Adenosine suppressed the increase in intracellular calcium following PMN activation by calcium ionophore or chemotactic peptide. In contrast, calcium did not increase in PMN activated by OAG and adenosine did not affect intracellular calcium changes following this stimulus.

6 These results demonstrate that physiological concentrations of adenosine inhibit the PMN respiratory burst in association with an increase in intracellular cyclic AMP and reduction of intracellular calcium.

Introduction

Adenosine is an important regulator substance with potent effects on cardiovascular, pulmonary, endocrine and neuronal tissues (Daly, 1982; Berne *et al.*, 1982). Although adenosine has been shown to inhibit polymorphonuclear leucocyte (PMN) function (Cronstein *et al.*, 1983; 1987), the mechanisms of PMN regulation by adenosine are not well understood. The PMN generates reactive oxygen metabolites which are critical in host defence but also highly toxic to human tissues (Klebanoff, 1980; Babior, 1984). Consequently, regulation of PMN function by adenosine may be of physiological importance.

Both A_1 and A_2 adenosine receptors may be present on human PMN (Cronstein *et al.*, 1985; Marone *et al.*, 1985). The A_2 receptor subtype mediates adenylate cyclase stimulation while the A_1 subtype causes adenylate cyclase inhibition (Londos *et al.*, 1980). Because adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) may inhibit PMN function (Ignarro & Columbo, 1973; Busse & Sosman, 1984), effects of adenosine mediated by cyclic AMP may be relevant to the mechanism of PMN inhibition by adenosine.

Two partially distinct biochemical pathways of stimulus-response coupling have been identified in the PMN (McPhail & Snyderman, 1983). One pathway requires intracellular calcium mobilization while the other is associated with diacylglycerol activation of protein kinase C and is relatively calcium independent (Takenawa *et al.*, 1983; Nishizuka, 1984). Because only the calcium-dependent pathways of stimulus-response coupling may be inhibited by

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elevation of cyclic AMP (Mack *et al.*, 1986; Nielson, 1987), effects of adenosine on the respiratory burst were evaluated with respect to the mechanisms of cell activation.

Methods

Isolation of PMN

Blood samples from heathy volunteers, age 20 to 45 years, were drawn between 8 h 00 min and 9 h 00 min. All subjects were asked to abstain from methylxanthine-containing foods for one day before the study. No subject was using any medication. PMN were isolated from venous blood anticoagulated with 10 units ml^{-1} heparin. To sediment the red bloods cells, 30 ml of blood was added to 30 ml of 3% dextran containing 0.1% bovine serum albumin (BSA) and 1 mg ml^{-1} ethylenediaminetetraacetic acid. After 20 min the leucocyte-rich plasma was layered onto 3 ml Ficoll-Hypaque (6.4 g% Ficoll, 9.72 g% Diatriazoate, 0.1% BSA, SpG 1.077) (Boyum, 1968). PMN were isolated by centrifugation (200 *g*, 20 min) and hypotonic (10 s, distilled water) lysis of erythrocytes. PMN were stored in plasma from the same donor at 4°C until utilized (30 min or less). Immediately before each experiment PMN were removed from plasma by centrifugation at 200 *g* for 10 min, washed and resuspended in phosphate buffer. The final preparation was over 95% PMN and of these 95% were viable. Adenosine or adenosine analogues were introduced immediately before cell activation with calcium ionophore, chemotactic peptide or diacylglycerol.

Luminescence detection of oxygen metabolities

After cell suspension in phosphate buffer, PMN were stimulated by addition of N-formyl-methionyl-leucine-phenylalanine (FMLP, 1 μM), ionomycin (0.2 μM), A23187 (0.2 μM) or oleoyl-acetyl-glycerol (OAG, 1 μM). Luminescence response was measured using either a Beckman LS-7500 scintillation counter set in the out-of-coincidence mode or a Packard Instrument Model 6500 Picolight Luminometer. The temporal characteristics of PMN activation were determined with 10^4 PMN ml^{-1} and 100 μM lucigenin. This low cell concentration minimized effects of mediators generated during PMN activation and provided the most reproducible results. Concentration-effects of adenosine and adenosine analogue were evaluated in a second set of experiments using 5×10^4 PMN ml^{-1} and 1 μM lucigenin. Lucigenin (1 μM or 100 μM) did not alter cell viability as assessed by trypan blue exclusion. Lucigenin-dependent luminescence was completely inhibited by superoxide dismutase (100 u ml^{-1}) and therefore appeared to correlate with superoxide anion generation as previously demonstrated (Chari-Bitron *et al.*, 1983; Stevens & Hong, 1984). The stimulating agents and adenosine did not alter lucigenin-dependent luminescence in studies using xanthine-xanthine oxidase generation of superoxide anion (in the presence or absence of unactivated PMN).

Intracellular calcium measurement

The fluorescent probe indo-1/AM was used to evaluate intracellular calcium as described by Tsien and associates (Grynkiewicz *et al.*, 1985). PMN were incubated with 1 μM indo-1/AM in the subject's plasma for 60 min. Cells were then washed with buffer (without calcium), centrifuged at 200 *g* for 10 min and resuspended at a concentration of 1×10^4 PMN ml^{-1} in phosphate buffer with 1 mM calcium. Fluorescence using excitation at 355 nM was measured with a fluorescence spectrophotometer (Model LS-5, Perkin-Elmer, Oak Brook, Illinois). The ratio of 410 nM fluorescence (calcium-bound indo-l) to 485 nM fluorescence (calcium-free indo-1) was used as an index of intracellular calcium concentration. Measurements were performed after PMN incubation, after addition of adenosine and at 30 s intervals after cell activation. Calcium-dependent changes in indo-1 acid fluorescence in a cell free test system were not altered by adenosine, FMLP, ionomycin or OAG. Changes in intracellular calcium were evaluated as the change in fluorescence compared with measurements immediately before PMN stimulation. The initial fluorescence was therefore standardized to zero and subsequent positive ratios reflected an increase, while negative results indicated a decrease in calcium following activation. Evaluation of fluorescence in the presence of a high ionomycin concentration (100 μM) indicated that a 0.1 increase in indo-1 ratio was associated with a 67 nM increase in calcium (calcium concentration range of 100 nM–5 μM).

Cyclic AMP measurement

PMN (6×10^6 ml^{-1}) were suspended in buffer and activated by 1 μM FMLP, 0.2 μM A23187 or 1 μM OAG. Appropriate adenosine dilutions were added immediately before PMN activation. Aliquots of PMN (1×10^6) were removed for baseline and cyclic AMP measurement at appropriate times. Cyclic AMP was extracted by sonication of PMN in acidified ethanol (5 s at 35% maximum energy, Model 300, Fisher Sonic Dismembrator). The specimen was then centrifuged at 1000 *g* for 10 min and the supernatant decanted from the pellets of denatured protein. The pellet was resuspended in 50% ethanol,

centrifuged at 1000 *g* for 10 min, and the supernatant again decanted. The supernatants were dried under nitrogen and stored at −70°C. The specimens were resuspended in 0.05 M Tris, pH 7.5 with 4 mM EDTA and cyclic AMP was measured by a competitive protein binding assay originally described by Gilman (1970). Preliminary studies demonstrated a 90% decrease in detectable cyclic AMP following incubation of specimens with bovine heart phosphodiesterase in phosphate buffer at 37°C for 60 min. Effects of adenosine and PMN activators on cyclic AMP generation were studied in paired experiments using cells from the same donor isolated in a single procedure.

Data analysis

Luminescence was evaluated either as a function of time or was integrated over the interval 2–4 min after PMN activation. PMN activation was standardized either as a percentage of simultaneous PMN response using a sample not exposed to adenosine (adenosine dose-response analysis) or as a percentage of maximal luminescence achieved during 15 min following activation (temporal analysis of cell activation). Comparisons of drug effects were paired using PMN from the same blood sample, with activation and analysis performed simultaneously under each experimental condition. Experiments were usually repeated five times using specimens from different donors. Dose-response curves were evaluated with computer assisted parametric curve fitting to the logistic equation (DeLean *et al.*, 1978) for estimation of slope, drug concentration causing 50% maximal response (EC_{50}) and maximal response.

Reagents

Dulbecco's phosphate buffered saline was prepared with 1 mg ml^{-1} glucose, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The calcium ionophores A23187 (0.2 μM) and ionomycin (0.2 μM), the chemotactic peptide N-formyl-methionyl-leucine-phenylalanine (FMLP) (1 μM), and the synthetic diacylglycerol 1-oleoyl-2-acetyl-glycerol (OAG) (1 μM) were used to induce the PMN respiratory burst. A23187, FMLP and OAG were dissolved in dimethylsulphoxide and diluted in distilled water. Lucigenin (10,10′-dimethyl-bis-9,9′-biacridinium nitrate), a luminescent probe for superoxide anion, was dissolved in buffer. All diluents were included in all samples of any study at equal concentrations. The diluents had no effects on PMN function.

Indo-1/AM was obtained from Molecular Probes, Inc., Junction City, Oregon, U.S.A. A23187 was obtained from Behring-Calibiochem, La Jolla, CA, U.S.A. 5′-N-ethylcarboxamide-adenosine (NECA) was obtained from Research Biochemicals Inc., Wayland, MA, U.S.A. Materials used in cyclic AMP assays were obtained from Amersham Corporation, Arlington Heights, IL, U.S.A. All other materials were purchased from Sigma Chemical Co, St Louis, MO, U.S.A.

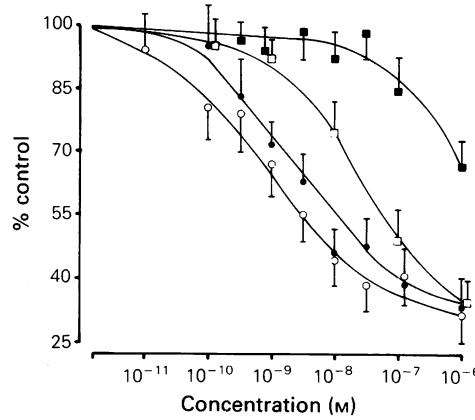

Figure 1 Effects of adenosine (●), 5′-N-ethylcarboxamide-adenosine (NECA, ○), L-N^6-phenylisopropyl-adenosine (L-PIA, □), and D-PIA (■) when introduced at the time of polymorphonuclear leucocyte (5×10^4 ml^{-1}) activation by 0.2 μM A23187. Lucigenin (1 μM)-dependent luminescence was integrated over 10 min following cell activation. The absolute magnitude of maximal luminescence was $103 \pm 23 \times 10^4$ c.p.m. Data represent means of specimens from 5 subjects; vertical lines show s.e.mean.

Results

Adenosine caused rapid, potent inhibition of PMN oxygen metabolite generation following stimulation with calcium ionophore (0.2 μM A23187) as measured using lucigenin (1 μM)-dependent luminescence (Figure 1). PMN stimulated by A23187 generated peak luminescence between 2 and 4 min following activation and the respiratory burst resolved over 20–30 min. Cells were inhibited by adenosine throughout the period of the respiratory burst. Adenosine inhibition was significant at concentrations as low as 1 nM (72% control, $P < 0.05$) and was maximal at concentrations over 100 nM.

To characterize the PMN receptor mediating adenosine effects, the adenosine analogues, 5′-N-ethylcarboxamide-adenosine (NECA) and N^6-phenyl-isopropyl-adenosine (PIA), were studied (Figure 1). The adenosine receptor which inhibits adenylate cyclase (A_1 receptor) is stimulated by adenosine analogues with relative affinities of L-

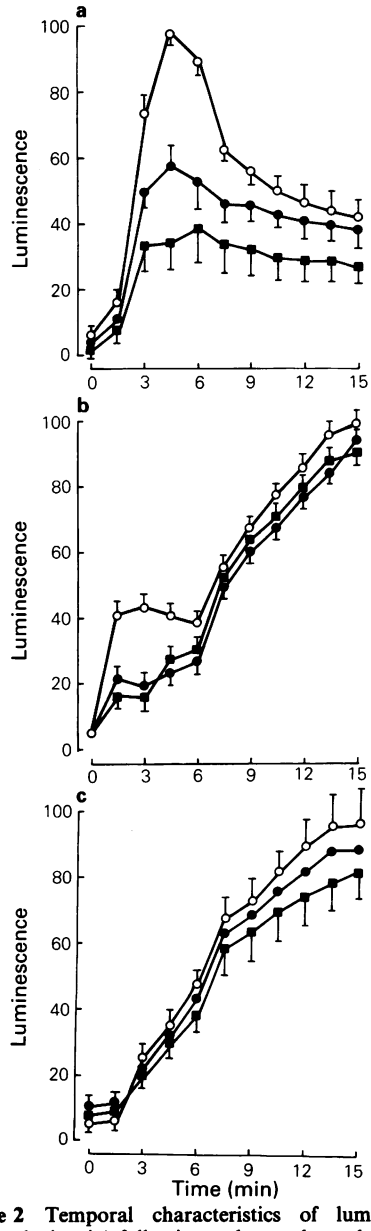

Figure 2 Temporal characteristics of luminescence (100 μM lucigenin) following polymorphonuclear leucocyte (PMN) (1×10^4 PMN per ml) activation by (a) 0.2 μM ionomycin, (b) N-formyl-methionyl-leucyl-phenylalanine 1 μM (FMLP), or (c) 1 μM oleoyl-acetyl-glycerol (OAG) in the presence of 1 nM adenosine (●), 100 nM adenosine (■) or no drug (○). PMN activation was standardized as % of maximal response achieved in control (activated without adenosine) specimen for each stimulus. The absolute magnitudes of the maximal response were 178 ± 35, 257 ± 58 and 134 ± 46 c.p.m. $\times 10^3$ using FMLP, A23187 and OAG, respectively. Data represent means of samples from 5 subjects; vertical lines show s.e.mean.

PIA > adenosine > NECA. Affinities of the analogues at the stimulatory (A_2) receptors are the reverse, NECA > adenosine > L-PIA. PMN inhibition by NECA was significant at 0.1 nM ($80 \pm 6\%$ control, $P < 0.05$) while effects of L-PIA were only significant at concentrations greater than 10 nM ($74 \pm 8\%$ control, $P < 0.05$). The concentrations of NECA, adenosine, and L-PIA required to cause 50% of maximal PMN inhibition were 1.0 ± 0.3 nM, 1.7 ± 0.2 nM and 26 ± 4 nM (means $\pm$ s.e.mean estimated from computer assisted curve fitting to the logistic equation) (DeLean *et al.*, 1978). Thus, the relative potencies of adenosine analogues were characteristic of an A_2 adenosine receptor response.

The respiratory burst may be induced by stimuli which elevate intracellular calcium (A23187 or ionomycin), activate phospholipase C (FMLP) or directly activate protein kinase C (OAG). To determine whether adenosine would cause similar inhibition of PMN stimulated through each biochemical pathway, the effects of adenosine were compared with cells activated by ionomycin, FMLP or OAG. Adenosine caused potent inhibition of PMN activated by ionomycin and inhibited the initial phase of the respiratory burst in PMN activated by chemotactic peptide (1 μM FMLP). In contrast, PMN activated by synthetic diacylglycerol (1 μM OAG) were minimally inhibited (Figure 2).

The A_2 adenosine receptor activates adenylate cyclase in many cell types (Daly, 1982). To determine whether cyclic AMP may mediate the effects of adenosine on PMN function, intracellular cyclic AMP was measured following adenosine exposure in both resting and activated PMN. Adenosine at concentrations of 1 nM and 100 nM rapidly (1 min) induced a modest cyclic AMP elevation in unactivated PMN and a marked increase in cyclic AMP during cell activation (Table 1). Although adenosine had a minimal effect on the respiratory burst when PMN were stimulated by OAG, cyclic AMP accumulation induced by adenosine was similar in PMN stimulated by either ionophore, FMLP or OAG. Additional measurements during the period of OAG-induced luminescence confirmed that an elevation of intracellular cyclic AMP was induced during this period despite the lack of inhibition. (Cyclic AMP was 1.70 ± 0.07 and 2.84 ± 0.03 pmol per 10^6 PMN in control specimens and PMN exposed to 100 nM adenosine respectively, at 8 min after OAG activation ($P < 0.05$)).

An elevation of intracellular calcium may be required for induction of the respiratory burst in PMN stimulated by calcium ionophore and FMLP (Simchowitz & Spilberg, 1979). Calcium mobilization may be less important in cells activated by agents which directly stimulate protein kinase C (Lehmeyer *et al.*, 1979). Because adenosine appeared to inhibit specifically PMN stimulation by agents which

Table 1 Cyclic AMP following cell exposure to adenosine

	PMN activating agent			
Adenosine	*None*	*A23187*	*FMLP*	*OAG*
0 nM	0.57 ± 0.03	1.21 ± 0.10	1.47 ± 0.05	0.83 ± 0.09
1 nM	0.97 ± 0.04*	2.67 ± 0.29*		
100 nM	1.56 ± 0.03*	3.22 ± 0.12*	2.06 ± 0.06*	2.33 ± 0.05*

Cyclic AMP (pmol per 10^6 polymorphonuclear leucocyte (PMN)) measured 1 min following addition of adenosine and/or cell activation by 0.2 μM A23187, 1 μM N-formyl-methionyl-leucyl-phenylalanine (FMLP), or 1 μM oleoyl-acetyl-glycerol (OAG). Data represent means ± s.e.mean using specimens from 5 subjects. *$P < 0.05$, in comparison with the sample not including adenosine.

induce calcium mobilization, intracellular calcium during cell activation was evaluated with the fluorescent probe indo-1 (Figure 3). Intracellular calcium increased in PMN activated by ionophore or FMLP but not cells activated by OAG. Adenosine blocked the increase in calcium induced by either ionophore or FMLP (Figure 3). In contrast, adenosine had an insignificant effect upon intracellular calcium in OAG-activated PMN.

Discussion

These results confirm previous findings that physiological concentrations of adenosine inhibit the PMN respiratory burst (Cronstein *et al.*, 1985; Schrier & Imre, 1986). Because adenosine concentrations of over 700 nM occur *in vivo* (Sanchez *et al.*, 1983), adenosine may have an important role in regulation of PMN function. Our studies further demonstrate that the effects of adenosine are dependent upon the biochemical pathway of PMN stimulus-response coupling.

The PMN respiratory burst may be induced by either protein kinase C stimulation or elevation of intracellular calcium (McPhail & Snyderman, 1983). Although the biochemical pathways are only partially distinct, induction of the respiratory burst by OAG is principally mediated by protein kinase C activation while effects of A23187 are mediated by elevation of intracellular calcium. The chemotactic peptide FMLP stimulates phospholipase C with resultant release of inositol tris-phosphate and diacylglycerol. Because inositol tris-phosphate mobilizes intracellular calcium and diacylglycerol activates protein kinase C, the respiratory burst when induced by FMLP is activated through both calcium and protein kinase C dependent pathways (Takenawa *et al.*, 1985). The two pathways of stimulus-response coupling may be important with respect to characteristics of cell response. The calcium-dependent pathway transduces rapid, transient responses while more prolonged responses are often initiated through the protein kinase C pathway (Rasmussen, 1986).

Our results suggest that adenosine selectively inhibits the calcium-dependent pathway of PMN stimulus-response coupling. This conclusion was supported both by the observation that adenosine inhibited PMN activated by A23187 or FMLP but not cells stimulated by OAG, and by studies with indo-1 which clearly demonstrated inhibition of calcium mobilization by adenosine. The biphasic respiratory burst in PMN stimulated by FMLP suggested that two pathways of stimulus-response coupling may have been activated. Although a second phase of the respiratory burst induced by FMLP has been related to cell surface contact (Dahinden *et al.*, 1983), the biochemical basis for a biphasic response is not well established. In our studies the initial phase of the respiratory burst was associated with an increase in intracellular calcium and was inhibited by adenosine. The second phase induced by FMLP was temporally similar to PMN activation following OAG stimulation and was minimally affected by adenosine. Because FMLP induces both calcium mobilization (inositol tris-phosphate) and protein kinase C activation (diacylglycerol), it is possible that the first phase of FMLP activation was induced by inositol tris-phosphate release and the second phase was induced by diacylglycerol release. Further studies of phosphatidylinositol hydrolysis and inositol tris-phosphate release would be required to confirm this hypothesis.

The studies of adenosine analogues demonstrated potencies of NECA, adenosine and PIA (NECA > adenosine > PIA) which were characteristic of an A_2 adenosine receptor (Londos *et al.*, 1980). The increase in PMN cyclic AMP induced by adenosine was consistent with adenylate cyclase stimulation by A_2 receptors as has been described in many cell types (Daly, 1982). Effects of cyclic AMP including reduction of intracellular calcium (Rasmussen, 1986) and inhibition of phosphatidylinositol hydrolysis (Della Bianca *et al.*, 1986) could be mechanisms of PMN inhibition by adenosine.

Because membrane transduction of FMLP stimulation requires phospholipase C (PLC) hydrolysis of phosphatidylinositol, an inhibitory effect of cyclic

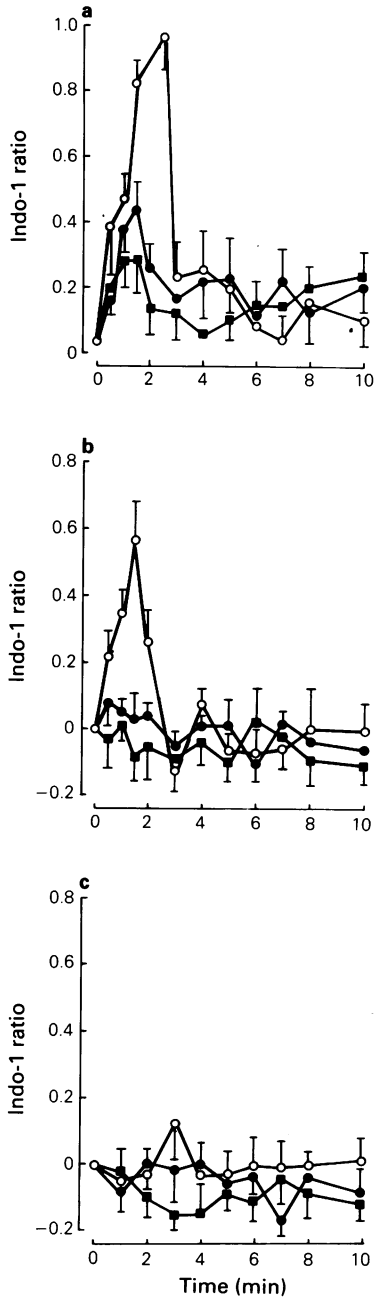

Figure 3 Change in intracellular calcium as indicated by indo-1 fluorescence ratio (410 nM/490 nM) following activation of 1×10^4 PMN ml^{-1} with (a) 0.2 μM ionomycin, (b) 1 μM FMLP or (c) 1 μM OAG in the presence of 100 nM adenosine (■), 1 nM adenosine (●) or no drug (○). Data represent means using samples from 5 subjects; vertical lines show s.e.mean. For abbreviations used see legend to Figure 2.

AMP on PLC could profoundly inhibit PMN stimulation by FMLP. However, inhibition of PLC would not provide a reason either for selective inhibition of the initial phase of FMLP activation or for a reduction in intracellular calcium in PMN stimulated by calcium ionophore. It is unlikely that adenosine or cyclic AMP would prevent calcium influx induced by ionophore, because calcium ionophore causes a passive diffusion of calcium across the plasma membrane. However, effects of cyclic AMP including calcium sequestration and stimulation of membrane channels which actively extrude calcium from the cell could reduce intracellular calcium (Rasmussen & Barrett, 1984). Such effects of cyclic AMP that reduce directly intracellular calcium would be consistent with our results showing (1) inhibition of PMN activated by calcium ionophore, (2) inhibition of an initial calcium-dependent phase of FMLP activation, and (3) a minimal effect of adenosine on PMN activated by OAG.

In summary adenosine inhibits the PMN respiratory burst, elevates intracellular cyclic AMP and reduces intracellular calcium mobilization. The importance of evaluation of each pathway of stimulus-response coupling in studies of PMN function is apparent, because the effects of adenosine were selective to PMN stimulated by chemotactic peptide or low concentrations of calcium ionophore. Our results are consistent with the hypothesis that adenosine inhibition of the PMN may be caused by cyclic AMP-mediated depression in intercellular calcium mobilization.

Note added in proof

Cronstein *et al.* (1988) recently reported less effect of adenosine on PMN cyclic AMP and calcium than we found. Many methods were different in the two studies. Potentially significant aspects of our methods include the use of EDTA in PMN isolation, cell resuspension in plasma, room temperature, PBS buffer and timing of adenosine addition.

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The ability of denbufylline to inhibit cyclic nucleotide phosphodiesterase and its affinity for adenosine receptors and the adenosine re-uptake site

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1 Denbufylline has been examined for its ability to inhibit cyclic nucleotide phosphodiesterase isoenzymes from rat cardiac ventricle and cerebrum, as well as for its affinity for adenosine A_1 and A_2 receptors and the re-uptake site. For comparison, SK&F 94120, theophylline and 3-isobutyl-1-methyl-xanthine (IBMX) were examined as phosphodiesterase inhibitors whilst N^6-cyclohexyladenosine, R(−)-N^6-(2-phenylisopropyl)-adenosine, 5'-N-ethylcarboxamido-adenosine, 2-nitrobenzylthioinosine, theophylline and IBMX were examined for their affinity for adenosine binding sites.

2 This investigation confirmed the presence of four phosphodiesterase activities in rat cardiac ventricle; in rat cerebrum only three were present.

3 Denbufylline selectively inhibited one form of Ca^{2+}-independent, low K_m cyclic AMP phosphodiesterase. The form inhibited was one of two present in cardiac ventricle and the sole one in cerebrum. This form was not inhibited by cyclic GMP. The inotropic agent SK&F 94120 selectively inhibited the form of cyclic AMP phosphodiesterase which was inhibited by cyclic GMP present in cardiac ventricle. Theophylline and IBMX were relatively non-selective phosphodiesterase inhibitors.

4 Denbufylline was a less potent inhibitor of ligand binding to adenosine receptors than of cyclic AMP phosphodiesterase. This contrasted with theophylline, which had a higher affinity for adenosine receptors, and IBMX which showed no marked selectivity. Denbufylline, theophylline and IBMX all had a low affinity for the adenosine re-uptake site.

5 Denbufylline is being developed as an agent for the therapy of multi-infarct dementia. The selective inhibition of a particular low K_m cyclic AMP phosphodiesterase may account for the activity of this compound.

Introduction

This investigation was carried out to characterise further the activity profile of the alkylxanthine denbufylline (1,3-di-n-butyl-7-(2'-oxopropyl)-xanthine). This compound has previously been shown to increase the oxygen tension (Po_2) and function of partially ischaemic skeletal muscle (Angersbach & Ochlich, 1984), to enhance the retention of information (Nicholson *et al.*, 1988) and to improve the flow properties of blood (Jukna & Nicholson, 1987). It is presently undergoing clinical evaluation in dementia (see existing data generated by O'Connolly *et al.*, 1986; 1988).

The ability of alkylxanthines to inhibit the effects of adenosine and cyclic nucleotide phosphodiesterase has been known since the work of Ther and collaborators (1957) and Sutherland and coworkers (Sutherland & Rall, 1958; Butcher & Sutherland, 1962), respectively. It has more recently become apparent that alkylxanthines are competitive antagonists at extracellular adenosine receptors (Londos *et al.*, 1978; Burnstock & Meghji, 1981; Fredholm & Persson, 1982; Nicholson, 1982; Schwabe *et al.*, 1983) and may inhibit the cellular re-uptake of adenosine (Stefanovich, 1983; Fredholm & Lindstrom, 1986). Many alkylxanthines are more potent adenosine receptor antagonists than inhibitors of cyclic nucleotide phosphodiesterase (Smellie *et al.*, 1979; Wu *et al.*, 1982).

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In this study, the ability of denbufylline to inhibit cyclic nucleotide phosphodiesterase from rat cerebral cortex and cardiac ventricle was examined. In mammalian tissue cells, several distinct molecular forms of cyclic nucleotide phosphodiesterase exist (Thompson & Appleman, 1971; Weishaar *et al.*, 1985; Reeves *et al.*, 1987). Accordingly, we examined the ability of denbufylline to inhibit separated phosphodiesterase isoenzymes. For comparison, theophylline, 3-isobutyl-1-methyl-xanthine (IBMX) and the inotropic agent SK&F 94120 (Gristwood *et al.*, 1986a) were also evaluated.

On the basis of their ability to mediate inhibition or activation of adenylate cyclase and agonist order of potency, extracellular adenosine receptors have been subdivided into A_1 and A_2 receptors (van Calker *et al.*, 1979; Bruns *et al.*, 1986). We have therefore determined the affinity of denbufylline for both A_1 and A_2 adenosine receptors as well as the adenosine re-uptake site, using ligand binding techniques. In these assays, theophylline, IBMX, R(−)-N^6-(2-phenylisopropyl)-adenosine (R-PIA), N^6-cyclohexyladenosine (CHA), 5'-N-ethylcarboxamidoadenosine (NECA) and 2-nitrobenzylthioinosine (NBI) were additionally examined. Part of this work was presented at the British Pharmacological Society (Nicholson & Wilke, 1987).

Methods

The study was performed using tissue obtained from adult male Wistar rats killed by cervical dislocation. Tissues were rapidly removed and placed in the relevant buffer; all preparative procedures were performed at 4°C.

Separation of cyclic nucleotide phosphodiesterase activities

Cyclic nucleotide phosphodiesterase activities were separated by use of a modification of the methods of Reeves *et al.* (1987). Rat cerebral cortex and cardiac ventricle were homogenized, with a Potter S homogenizer, in 8–10 volumes of buffer (composition in mM: Bis-Tris 20, 2-mercaptoethanol 5, benzamidine 2, EDTA 2, phenylmethanesulphonylfluoride (PMSF) 0.05, sodium acetate 50; pH 6.5). Genapol × 0.15% (v/v) was included in the cerebral cortex assays to aid extraction of membrane bound phosphodiesterase (Strada *et al.*, 1984). The cerebral homogenates were filtered through glass wool, sonicated three times for 20 s at 100 W and filtered through a Millex GS 0.22 μm filter unit. This step was omitted for the ventricular homogenates. The homogenates were then centrifuged (15,000 *g* for 15 min cardiac tissue; 100,000 *g* for 60 min cerebral tissue) and the supernatants applied to columns (17 cm × 1.6 cm) of Q sepharose Fast Flow (Pharmacia) pre-equilibrated with homogenization buffer. The flow rate was 70 ml h^{-1}. The column was washed with 100 ml of buffer and phosphodiesterase activities eluted with a linear gradient of 0.05–1.0 M sodium acetate in buffer. The eluate was collected in 7 ml fractions and analysed for phosphodiesterase activity. For storage, ethylene glycol was added to a final concentration of 30% (v/v) and fractions were stored at −20°C.

Assay of phosphodiesterase activity

Phosphodiesterase activity was assayed by the radiochemical procedure described by Arch & Newsholme (1976). The incubation medium consisted of 50 mM Tris/HCl, 6 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.05 mg ml^{-1} 5'nucleotidase, 0.23 mg ml^{-1} bovine serum albumin and the relevant concentrations of [^{3}H]-adenosine 3':5'-cyclic monophosphate ([^{3}H]-cyclic AMP), [^{3}H]-guanosine 3':5'-cyclic monophosphate ([^{3}H]-cyclic GMP), cyclic GMP, calmodulin and Ca^{2+}; pH 7.5. The reaction was initiated by the addition of 30 μl of enzyme preparation and tubes were incubated for 10 min at 30°C. The reaction was terminated by the addition of 1.2 ml of an anion-exchange resin slurry (BIO-RAD AG 1 × 8, 200–400 mesh). The tubes were centrifuged (200 *g* for 15 min) and a portion (0.25 ml) of supernatant was added to 5 ml of scintillation fluid (Aqualuma). Radioactivity was measured in a liquid scintillation counter.

The total radioactivity in the substrate at zero time was measured in the supernatant when all the substrate had been converted into product by addition of commercial phosphodiesterase (10 μg). Assays were conducted in the linear reaction range, where less than 50% of the initial substrate was hydrolysed. All assays were repeated at least three times.

Determination of V_{max} and K_m for the phosphodiesterase activities and K_i values for inhibitors

For determination of V_{max} and K_m the concentration of label ([^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP) was kept constant in the presence of increasing amounts of unlabelled substrate. K_i values were obtained by using substrate and inhibition concentrations in the range of K_m and K_i values, respectively. Data were analysed by least squares linear regression analysis. Results are expressed as means (± s.e.mean).

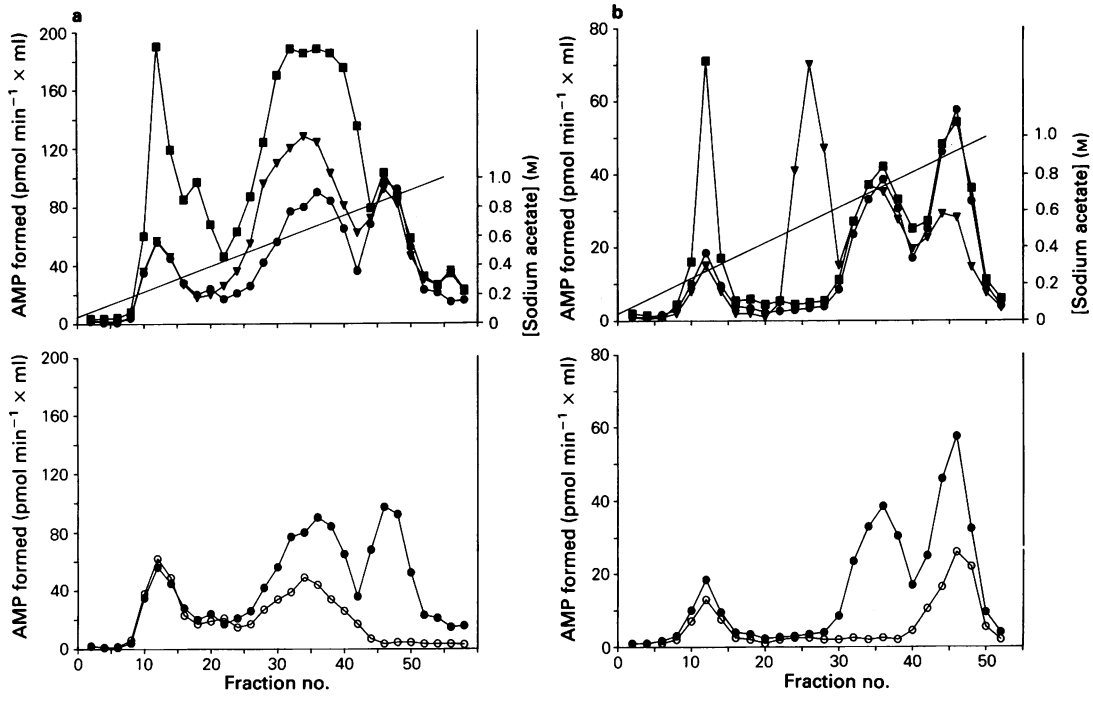

Figure 1 Separation profiles of cyclic nucleotide phosphodiesterase activities from rat cerebral cortex (a) and cardiac ventricle (b). Fractions were assayed with 1 μM cyclic AMP (●), 1 μM cyclic AMP and Ca^{2+}/calmodulin (0.1 mM per 75 u) (■), 1 μM cyclic AMP and 1 μM cyclic GMP (▼) and 1 μM cyclic AMP and 100 μM denbufylline (○).

Adenosine receptor and re-uptake site ligand binding assays

The A_1, [^{3}H]-CHA, binding assays and the A_2, [^{3}H]-NECA, binding assays were performed as described by Bruns *et al.* (1986). The [^{3}H]-NBI binding assays to the adenosine re-uptake site were carried out according to Marangos *et al.* (1982).

Brains were removed and one gram of the relevant tissues (whole brain minus cerebellum, for the [^{3}H]-CHA and [^{3}H]-NBI assays, and striata, for the [^{3}H]-NECA assays) were homogenized in 50 mM Tris-HCl (pH 7.4) supplemented with 10 μM PMSF for 30 s with a Potter S homogenizer, 10 strokes at 1500 r.p.m. The homogenate was diluted and centrifuged at 42,000 *g* for 18 min. The pellet was re-

Table 1 Kinetic properties of cyclic nucleotide phosphodiesterases from rat cerebrum and cardiac ventricle

Tissue	*PDE activity*	K_m *cyclic AMP* (μM)	K_m *cyclic GMP* (μM)	*Effector*
Cerebrum	Peak I	35	5	Ca^{2+}/calmodulin
	Peak II	40	4	Ca^{2+}/calmodulin, cyclic GMP
	Peak III	2	50	None
Cardiac ventricle	Peak I	2	2	Ca^{2+}/calmodulin
	Peak II	50*	40*	Cyclic GMP stimulated
	Peak III	1.6	>50	None
	Peak IV	0.8	5	Cyclic GMP inhibited

* Positive cooperativity.

suspended with the Potter S homogenizer in Tris-HCl buffer and the centrifugation step repeated. The pellet was then resuspended in Tris-HCl buffer, which contained 1 mM EDTA, and stored in plastic vials in liquid nitrogen. The membranes were used within one week.

All incubations were performed in triplicate in 12 × 75 mm polycarbonate tubes in a shaking water bath. Tubes contained 1 ml of 50 mM Tris-HCl (pH 7.4) and 2.5 or 0.1 units ml^{-1} of adenosine deaminase in the [^{3}H]-CHA and [^{3}H]-NECA assays, respectively. Adenosine deaminase was omitted from the [^{3}H]-NBI assays. Membranes obtained from 2 mg of tissue in 400 μl buffer were used in the [^{3}H]-CHA assay whilst for the [^{3}H]-NECA or [^{3}H]-NBI assays the membranes from 5 mg of original tissue were used suspended in 400 μl of buffer, respectively. The radiolabelled ligand concentrations were 2.5 nM [^{3}H]-CHA, 4 nM [^{3}H]-NECA and 0.6 nM [^{3}H]-NBI. Non-specific binding was determined by the addition of 50 μM PIA, 100 μM N^6-cyclopentyladenosine (CPA) and 5 μM NBI in the A_1, A_2 and re-uptake site assays, respectively. In the [^{3}H]-NECA assays, 50 nM CPA was included in all assays to eliminate A_1 binding (Bruns *et al.*, 1986; this was confirmed by us in preliminary experiments).

Incubations were for 90 min at 37°C for the [^{3}H]-CHA assays and at 25°C for 60 min and 10 min for [^{3}H]-NECA and [^{3}H]-NBI assays, respectively. Preliminary experiments had shown that incubation times were sufficient to ensure an equilibrium in receptor occupation. Incubations were terminated by the addition of 3.8 ml ice cold buffer (50 mM Tris-HCl, 10 mM $MgCl_2$; pH 7.4) followed by rapid filtration through 2.4 cm GF/B filters under reduced pressure using a Millipore 1225 sampling manifold. Filters were washed with three 3.8 ml portions of buffer. The damp filters were placed in scintillation vials and 5 ml of Aqualuma added. The vials were left overnight, shaken and radioactivity counted in a liquid scintillation counter. All assays were repeated at least three times.

Data analysis

Specific receptor binding was defined as total binding minus non-specific binding. Composite K_D values for receptor sites occupied by radioligand were calculated from IC_{50} values for displacement of unlabelled ligand using the equation: $K_D = IC_{50} - L$ where L = ^{3}H-ligand concentration. Kinetic constants for the inhibition of ligand binding to A_1 and A_2 receptors and the re-uptake site were determend as described by Bruns *et al.* (1986). Results are expressed as means (± s.e.mean).

Materials

Denbufylline was synthesized in these laboratories by Dr J. Göring and SK&F 94120 ((5-acetimidophenyl)pyrazin-(1H)-one) was a gift from Smith Kline & French. The sources for the remaining compounds were as follows: [^{3}H]-CHA and [^{3}H]-NBI (New England Nuclear); [^{3}H]-NECA (Amersham/Buchler); CHA (Boehringer, Mannheim); CPA (Research Biochemicals); R-PIA, NECA, NBI, IBMX, theophylline, adenosine deaminase (Type IV from bovine spleen) and PMSF (Sigma). GF/B filters were from Whatman and the Aqualuma scintillation fluid was from J.T. Baker.

Apart from NBI, all compounds were dissolved in buffer. NBI was dissolved in DMSO, appropriate quantities of the vehicle were added to the binding assay controls in these experiments.

Results

The phosphodiesterase activity profile obtained from rat cerebral and cardiac ventricle homogenates is shown in Figure 1. The kinetic properties of the separated activities and their modulation by Ca^{2+}/calmodulin and cyclic GMP are shown in Table 1.

Three activities were separated from cerebral tissue. The first two peaks eluted were Ca^{2+}/calmodulin-dependent and had a greater affinity for cyclic GMP than cyclic AMP. Hydrolysis of cyclic AMP by the second peak was activated by cyclic GMP. The third peak isolated from rat cerebral tissue had a lower K_m for cyclic AMP than for cyclic GMP and was not inhibited by cyclic GMP. The hydrolysis of cyclic nucleotides by all phosphodiesterase activities from cerebral tissue followed simple Michaelis-Menten kinetics.

Four peaks of phosphodiesterase activity were isolated from cardiac tissue. In contrast to cerebral tissue, only peak one was Ca^{2+}/calmodulin-dependent. In cardiac tissue, this form of phosphodiesterase had high and equal affinity for cyclic AMP and cyclic GMP. The second peak was dependent upon cyclic GMP for activation, showed positive cooperativity and had relatively low affinity for both cyclic GMP and cyclic AMP. Peaks III and IV both had a higher affinity for cyclic AMP than cyclic GMP, although the selectivity was much greater for peak III than peak IV. The hydrolysis of cyclic AMP by peak IV was inhibited by cyclic GMP. Peaks I, III and IV followed linear Michaelis-Menten kinetics.

The inhibition of the phosphodiesterase activities is shown in Table 2. Theophylline and IBMX were relatively non-selective inhibitors of all phosphodiesterase (PDE) activities, IBMX being more potent

Table 2 Inhibition constants of inhibitors of cyclic nucleotide phosphodiesterases from rat cerebrum and cardiac ventricle

Tissue	*PDE activity*	*Denbufylline* K_i (μM)	*SK&F 94120* K_i (μM)	*IBMX* K_i (μM)	*Theophylline* K_i (μM)
Cerebrum	Peak I	N.I.	N.I.	44 ± 3	442 ± 28
	Peak II	(20)	N.I.	18 ± 5	240 ± 10
	Peak III	0.7 ± 0.2	N.I.	14 ± 6	142 ± 13
Cardiac ventricle	Peak I	(30)	N.I.	7 ± 2	108 ± 22
	Peak II	N.I.	N.I.	11 ± 2	220 ± 34
	Peak III	0.8 ± 0.3	N.I.	12 ± 4	176 ± 20
	Peak IV	(40)	1.5 ± 0.5	3.4 ± 0.8	58 ± 9

Data shown are mean ± s.e.mean (n = 3). (% inhibition) at 100 μM. N.I. = no inhibition up to 100 μM.

than theophylline. Denbufylline was a potent and selective inhibitor of peak III (cyclic AMP-specific PDE) from both tissues. The competitive nature of the inhibition of denbufylline is shown in Figure 2. SK&F 94120 selectively inhibited peak IV (cyclic GMP-inhibited PDE) eluted from cardiac tissue. This low K_m cyclic AMP phosphodiesterase was only weakly inhibited by denbufylline; it was absent in cerebral tissue.

The inhibition of [^{3}H]-CHA, [^{3}H]-NECA and [^{3}H]-NBI binding is shown in Figure 3. The relative potencies of CHA, NECA, R-PIA and NBI served to characterize the [^{3}H]-CHA as the A_1 receptor, the [^{3}H]-NECA site as the A_2 receptor and the [^{3}H]-NBI site as the re-uptake site. The orders of potency were CHA ≥ R-PIA > NECA for the A_1 site, NECA > R-PIA > CHA for A_2 binding and NBI ⩾ R-PIA, CHA and NECA for the re-uptake site. Denbufylline displaced [^{3}H]-CHA from A_1 and

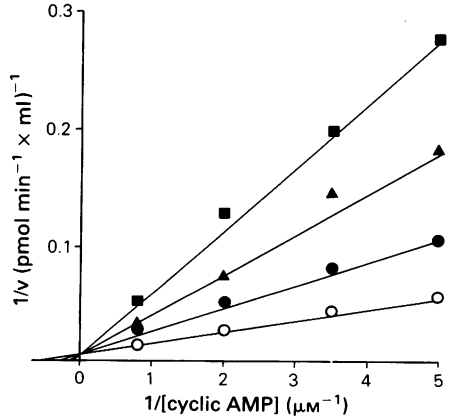

Figure 2 Competitive inhibition of fraction III cyclic nucleotide phosphodiesterase from rat cerebral cortex by denbufylline 1 (●), 3 (▲) and 10 (■) μM. (○) Control in absence of denbufylline.

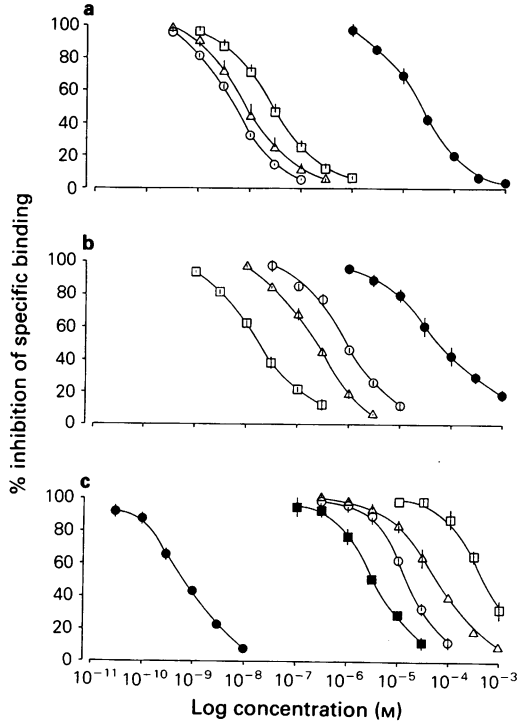

Figure 3 The inhibition of [^{3}H]-N^6-cyclohexyladenosine ([^{3}H]-CHA) (a) and [^{3}H]-2-nitrobenzylthioinosine ([^{3}H]-NBI) (c) binding to rat cortical membranes and of [^{3}H]-5'-N-ethylcarboxamidoadenosine ([^{3}H]-NECA) (b) binding to rat striatal membranes. Compounds examined as inhibitors of [^{3}H]-CHA and [^{3}H]-NECA binding were CHA (○), R(−)-N^6-(2-phenylisopropyl)adenosine (△), NECA (□) and NBI (●). These compounds and additionally dipyridamole (■) were examined as inhibitors of [^{3}H]-NBI binding. Results are presented as means with vertical lines showing s.e.mean ($n \geq 3$).

Table 3 The affinity of alkylxanthines for extracellular adenosine receptors and the adenosine re-uptake site (rat brain)

Compound	A_1 [^3H]-CHA K_i (μM)	A_2 [^3H]-NECA K_i (μM)	Uptake [^3H]-NBI K_i (μM)
Denbufylline	20 ± 5	46 ± 2	200 ± 22
IBMX	1.8 ± 0.7	10 ± 3	690 ± 82
Theophylline	14 ± 1	24 ± 2	1100 ± 57

[^{3}H]-NECA from A_2 receptors (Table 3). However, the affinity of denbufylline for the adenosine receptors was markedly lower than for cerebral low K_m cyclic AMP phosphodiesterase (Table 2). IBMX had a greater affinity for adenosine receptors than denbufylline, its affinity for A_1 receptors being greater than for A_2. IBMX was a less effective inhibitor of phosphodiesterase than of ligand binding to adenosine A_1 receptors. Theophylline had greater affinity for A_1 receptors than for A_2 receptors and for both extracellular adenosine receptors than for cyclic nucleotide phosphodiesterase. All three xanthines had a low affinity for the adenosine re-uptake site.

Discussion

The present study has demonstrated the presence of four cyclic nucleotide phosphodiesterase isoenzymes in rat heart. As judged by their kinetic properties and their sensitivity to the modulators Ca^{2+}/calmodulin and cyclic GMP and the selective inhibitor SK&F 94120, the phosphodiesterase activities in rat myocardium appear similar to those previously found in human and guinea-pig myocardium (Reeves *et al.*, 1987). The previous study of Reeves *et al.* (1987) was the first demonstration of two forms of Ca^{2+}-independent cyclic nucleotide phosphodiesterase with a high affinity for cyclic AMP in cardiac muscle. These two isoenzymes may, however, be present in many tissues. Two such low K_m cyclic AMP phosphodiesterases have previously been separated from calf liver homogenates (Yamamoto *et al.*, 1984) and adipocytes (Weber & Appleman, 1982). Their detection has been made possible by the improved separation properties of modern chromatographic medium (Reeves *et al.*, 1987). The nomenclature used for the classification of phosphodiesterase isoenzymes varies between groups. In this paper, we have based our descriptions of the phosphodiesterase isoenzymes upon their order of elution by QAE-Sepharose with an increasing salt gradient, their substrate affinities, effector characteristics and the activity of inhibitors. Thus in our hands, cardiac peak III is a Ca^{2+}-independent low K_m cyclic AMP phosphodiesterase which is not inhibited by cyclic GMP. This is similar to the third elution peak obtained by Reeves *et al.* (1987) from guinea-pig heart and the fourth elution peak obtained by this group from human heart. Reeves *et al.* (1987) based their nomenclature on data obtained from human heart and termed this phosphodiesterase PDE IV. The cardiac peak IV isoenzyme, in the present study, was termed PDE III by Reeves *et al.* (1987), because of its order of elution from human tissue. In our opinion, the most useful classification of phosphodiesterase isoenzymes presently available is that recently described by Beavo (1988). This classification assumes there are families of phosphodiesterase isoenzymes. According to this nomenclature, cardiac peak I phosphodiesterase and cerebral peak I and II phosphodiesterase belong to the calmodulin-stimulated family. Cardiac peak II phosphodiesterase is a cyclic GMP-stimulated phosphodiesterase. Cerebral peak III and cardiac peak III belong to the cyclic AMP-specific phosphodiesterase family. This is the isoenzyme that most investigators have referred to in the past as the 'low K_m' cyclic AMP PDE. It is selectively inhibited by Ro 20-1724 and by rolipram and, as shown in this study, by denbufylline. Cardiac peak IV is a member of the cyclic GMP-inhibited phosphodiesterase family. This isoenzyme is selectively inhibited by SK&F 94120 and by a number of positive inotropic agents (Weishaar *et al.*, 1985).

The present investigation has shown that rat cerebral tissue, in contrast to cardiac ventricle, contains not more than three phosphodiesterases, only one of which is a Ca^{2+}-independent isoenzyme with a high affinity for cyclic AMP. The detection of two forms of Ca^{2+}/calmodulin-stimulated phosphodiesterase in cerebral tissue confirms previous findings (Kincaid *et al.*, 1984; Strada *et al.*, 1984). Both Ca^{2+}/calmodulin-stimulated phosphodiesterases have low affinity for cyclic AMP. This contrasts with the cardiac ventricle, where the Ca^{2+}/calmodulin-stimulated enzyme, as shown in the present study and previously by others (Reeves *et al.*, 1987) has a high affinity for cyclic AMP.

With the presently available separation techniques it is not possible to ensure that all forms of phosphodiesterase will be resolved, that the isoenzymes will be separated in their physiological state or that the relative recovery of the different phosphodiesterase isoenzymes will be identical between tissues (see Appleman *et al.*, 1982, for further discussion of these points). However, there is strong evidence that two Ca^{2+}-independent phosphodiesterases with a high affinity for cyclic AMP do co-exist in tissues and that they are not simply separation artefacts. The two such cyclic AMP low K_m isoenzymes identified by Reeves *et al.* (1987) and ourselves display simple

Michaelis-Menten kinetics, suggesting that the hyperbolic kinetics for cardiac Ca^{2+}-independent low K_m cyclic AMP phosphodiesterase observed previously (Weishaar *et al.*, 1985; 1986) were due to the incomplete separation of two isoenzymes. This suggestion is supported by the findings of ourselves and others (Yamamoto *et al.*, 1984; Reeves *et al.*, 1987) that selective inhibitors of the two cyclic AMP phosphodiesterase subtypes can be identified. The presence of two forms of cyclic AMP phosphodiesterase, which differ in their tissue distribution and which can be selectively inhibited, suggests that compounds can be developed to influence selectively tissue function.

In contrast to theophylline and IBMX, which are non-selective inhibitors of phosphodiesterase isoenzymes (Davis, 1984; Lugnier *et al.*, 1986; Weishaar *et al.*, 1986), denbufylline selectively inhibits one form of Ca^{2+}-independent cyclic AMP phosphodiesterase in both rat heart and brain. This is the first account of an alkylxanthine selectively inhibiting a low K_m cyclic AMP phosphodiesterase. Interestingly, denbufylline has weak inotropic activity (Nicholson, unpublished information) and little effect on arterial blood pressure (Angersbach & Ochlich, 1984; Nicholson & Angersbach, 1986). In contrast, compounds such as SK&F 94120, which selectively inhibit the denbufylline insensitive, cyclic GMP inhibited low K_m cyclic AMP phosphodiesterase, produce positive inotropy (Gristwood *et al.*, 1986b) and have marked vasodilator activity (Gristwood *et al.*, 1986a). These data support the hypothesis that selective inhibitors of the different Ca^{2+}-independent low K_m cyclic AMP phosphodiesterases can selectively affect tissue function. Denbufylline improves recollection of information in mice and gerbils in which a learning or memory deficit has been induced by forebrain ischaemia (Nicholson *et al.*, 1988) and, in this regard, cyclic AMP has been postulated to influence synaptic efficacy and memory formation (Goelet *et al.*, 1986; Gray & Johnston, 1987). Consequently, selective inhibition of a cerebral cyclic AMP phosphodiesterase may account for the enhancing effect of denbufylline on learning and memory.

In addition to their ability to inhibit the hydrolysis of cyclic nucleotides, alkylxanthines are potent adenosine receptor antagonists (Londons *et al*, 1978; Burnstock & Meghji, 1981; Schwabe *et al.*, 1985). The present study has shown that denbufylline does bind to adenosine A_1 and A_2 receptors. However, it is a more potent inhibitor of cerebral, high-affinity cyclic AMP phosphodiesterase than of ligand binding to adenosine receptors. Selective adenosine receptor antagonists, such as theophylline and caffeine, reduce cerebral blood flow (Mathew *et al.*, 1983; Grome & Stefanovich, 1986). Denbufylline, on the other hand, increases the blood flow and oxygen tension of the oligaemic cortex (Nicholson & Angersbach, 1986) at doses which enhance retention of information (Nicholson *et al.*, 1988), which indicates that it is not effectively antagonising the effects of adenosine at these doses. Further evidence for this is that denbufylline, at doses greater than those which enhance learning or memory formation, neither potentiates nor attenuates ischaemia-induced neuronal damage (Jukna & Nicholson, unpublished information). Adenosine antagonists and uptake inhibitors potentiate (Jarrott & Domer, 1980; Rudolphi *et al.*, 1987a) and reduce (Rudolphi *et al.*, 1987b) ischaemic neuronal damage, respectively. The lack of an effect of denbufylline is presumably a reflection of the relatively low affinity of denbufylline for neuronal adenosine receptors and the re-uptake site.

In summary, this investigation has confirmed previous findings (Reeves *et al.*, 1987) of four cyclic nucleotide phosphodiesterase isoenzymes in cardiac tissue. The same separation techniques reveal only three isoenzymes in cerebral tissue. Denbufylline is an alkylxanthine which selectively inhibits one form of Ca^{2+}-independent low K_m cyclic AMP phosphodiesterase. This is one of two such isoenzymes in cardiac tissue and the sole one in the cerebrum. Denbufylline is a weak inhibitor of other forms of cyclic nucleotide phosphodiesterase and has a relatively low affinity for extracellular adenosine receptors and the re-uptake site. The selective inhibition of a cyclic AMP-specific phosphodiesterase may largely be responsible for the pharmacological effects of this compound, particularly as regards the increase in nutrition and metabolism of the oligaemic cortex and the enhancement of the retention of information.

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Differential release of histamine and eicosanoids from human skin mast cells activated by IgE-dependent and non-immunological stimuli

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1 Cells were dispersed from human foreskin using a mixture of collagenase and hyaluronidase and separated into mast cell-depleted (<1%) or enriched (>75%) preparations by density-gradient centrifugation.

2 Challenge of gradient fractions with ε-chain-specific anti-human IgE stimulated the release of histamine, prostaglandin D_2 (PGD_2) and leukotriene C_4 (LTC_4). The release of eicosanoids was significantly correlated with that of histamine, suggesting that they are derived from the mast cell population of the dispersate. In highly purified (76.2 ± 4.2%) mast cell preparations, maximum net release of histamine, PGD_2 and LTC_4 was 3432 ± 725, 84.9 ± 10.8 and 6.6 ± 1.2 pmol/10^6 nucleated cells.

3 The non-immunological stimuli substance P, vasoactive intestinal peptide (VIP), somatostatin, compound 48/80, morphine and poly-L-lysine released similar amounts of histamine to anti-IgE, but 12 to 21 fold less PGD_2 and LTC_4.

4 These studies suggest that IgE-dependent and non-immunological stimuli activate human skin mast cells by different secretory mechanisms, a hypothesis supported by our previous findings of differences in Ca^{2+} requirements and time-course of histamine release. Activation by the non-immunological mechanism may be of importance *in vivo* due to the close anatomical association between skin mast cells and dermal nerve-terminals containing neuropeptides.

Introduction

There is increasing evidence that inflammatory mediators released from skin mast cells are involved in a variety of pathological processes, including adverse reactions to drugs, allergic dermatitis and various forms of urticaria. The efficacy of histamine H_1-receptor antagonists in the treatment of these disorders suggests that this amine plays an important role in their underlying pathology. Intradermal injection of histamine mimics the wealing (plasma extravasation) and flaring (vasodilatation) frequently observed in skin disorders (Lewis, 1927) and in the early response of sensitised individuals injected intradermally with allergen (Prausnitz & Kustner, 1962).

The work of Ishizaka & Ishizaka (1968) gave a direct demonstration of the ability of skin mast cells to be activated *in vivo* by cross-linkage of their IgE-receptors. However, this is not the only mechanism by which these cells may be activated; recent studies have shown that mast cells dispersed from human skin differ from those dispersed from human lung, tonsil, adenoid and intestine in secreting histamine in response to a variety of non-immunological secretagogues including compound 48/80, poly-L-lysine, substance P, vasoactive intestinal peptide (VIP) and somatostatin (Church *et al.*, 1982; Benyon *et al.*, 1986; 1987a; Lowman *et al.*, 1988a, b; Rees *et al.*, 1988). Histamine secretion induced by these stimuli differs from that triggered by anti-IgE in being only partially dependent on extracellular Ca^{2+} and rapid in time-course, suggesting that the two types of stimuli utilise different mechanisms of cell activation. However, possible differences in eicosanoid release by these stimuli have not yet been investigated.

Human mast cells are capable of generating a variety of pre-formed and newly-generated mediators in addition to histamine. *In vitro* studies show that mast cells dispersed from human lung parenchyma (Holgate *et al.*, 1984; Peters *et al.*, 1984) or skin (Benyon *et al.*, 1987b; Robinson *et al.*, 1989) release both prostaglandin D_2 (PGD_2) and leukotriene C_4

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(LTC_4) after activation with anti-IgE or calcium ionophore A23187. When injected intradermally, these eicosanoids induce wealing and flaring and promote leucocyte accumulation (Bisgaard *et al.*, 1982; Camp *et al.*, 1983; Soter *et al.*, 1983) and, therefore, if liberated in sufficient quantities by mast cells within the dermis, may be important mediators of skin inflammation.

In this study, we have continued our investigations of the response of human dispersed skin mast cells to non-immunological stimuli. We provide the first demonstration that IgE-dependent and non-immunological stimuli differ in their capacity to activate the release of eicosanoids from human skin mast cells, which supports the concept that these stimuli utilise different secretory mechanisms.

Methods

Human skin mast cell dispersion

Mast cells were dispersed from human foreskin as previously described (Benyon *et al.*, 1987a). Briefly, foreskin, obtained by circumcision of patients aged 1–9 years, was chopped finely with scissors into 1–2 mm^3 fragments which were washed twice by centrifugation (500 *g*, 30 s, 25°C) in MEM containing 1·5% v/v FCS (MEM/FCS). Mast cells were passively-sensitised by incubation of the fragments for 2 h at 37°C in 5 ml of MEM/FCS containing 2 $\mu g\,ml^{-1}$ of human myeloma IgE (generously donated by Dr Teruko Ishizaka, Johns Hopkins Medical School, Baltimore, MD, U.S.A.). Fragments were washed and then digested for two intervals of 60 min at 37°C in MEM containing collagenase, 1.5 $mg\,ml^{-1}$, hyaluronidase, 0.75 $mg\,ml^{-1}$ and bovine serum albumin, 3.5% w/v. Dispersed cells were washed three times by centrifugation in MEM/FCS (500 *g*, 10 min, 25°C) and resuspended in 1 ml of MEM/FCS. Mast cell numbers and purity were assessed by optical microscopy after unfixed cell suspensions had been stained with Kimura's metachromatic stain (Kimura *et al.*, 1973).

Density-gradient separation of mast cells

Cell preparations depleted or enriched in mast cell content were obtained by density-gradient centrifugation as previously described (Benyon *et al.*, 1987a). Briefly, cells dispersed by the above enzymatic method and containing 5–8% mast cells were resuspended in 1 ml of MEM/FCS and layered over a five-step discontinuous gradient of 40–80% isotonic Percoll (density 1.051–1.100 $g\,ml^{-1}$). After centrifugation (500 *g*, 10 min, 25°C), cells which collected at the interfaces between layers of different density were aspirated and washed twice in MEM/FCS, before assessment of mast cell numbers and purity as described above. Lymphocytes constituted >90% of the cells contaminating mast cell-enriched preparations collected from high density (>70% Percoll) fractions of gradients.

Mast cell activation

Cells were washed once by centrifugation in HBSS and then resuspended in this buffer to a density of 5–10 $\times 10^4$ mast cells ml^{-1}. Aliquots of 360 μl were added to Eppendorf tubes and warmed at 37°C for 10 min before challenge with 40 μl of secretagogue. Aliquots of cells challenged with a relevant concentration of non-immune IgG or dimethylsulphoxide were included as controls for mediator release by anti-IgE or ionophore, respectively. After a 30 min incubation at 37°C, tubes were centrifuged (10,000 *g*, 30 s, 25°C) and aliquots of supernatant removed for storage at −20°C before measurement of eicosanoid and histamine content.

Assay of mediators

Radioimmunoassay of PGD_2 was performed as described previously (Holgate *et al.*, 1984) using a rabbit antiserum generously supplied by Dr L. Levine (Brandeis University, MA, U.S.A.). Immunoreactive LTC_4 was measured in unextracted incubation supernatants using an antiserum from Amersham International PLC, Buckinghamshire, with a claimed cross-reactivity of <1.6% with other leukotrienes and <0.15% with other eicosanoids. By use of high performance liquid chromatography we have established that PGD_2 and LTC_4 are the major eicosanoids released from human skin mast cells activated by anti-IgE or calcium ionophore (Robinson *et al.*, 1989). Supernatant and cell-associated histamine was measured by an automated spectrophotofluorimetric method.

Data presentation

Data are shown as mean ± s.e.mean of the number of experiments (*n*) indicated. Mediator secretion is expressed as the net release calculated after subtraction of unstimulated release occurring in the absence of stimulus. Significance of the difference between mean values was calculated by use of Student's *t* test for paired data. Correlation coefficients were calculated by unweighted least-squares linear regression analysis.

Materials

Collagenase (type 1), hyaluronidase (type 1), deoxyribonuclease (bovine pancreas), calcium ionophore

A23187 (dissolved as a 1 mM stock solution in dimethyl sulphoxide), compound 48/80, poly-L-lysine (average molecular weight 45,000–55,000 Daltons), substance P (human, synthetic), vasoactive intestinal peptide (VIP, porcine, synthetic), somatostatin (human, synthetic), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), gelatin and bovine serum albumin were from Sigma Chemical Company, Poole, Dorset. Foetal calf serum (FCS) and Eagle's minimum essential medium (MEM) containing 25 mM HEPES and L-glutamine were purchased from Gibco Europe Ltd., Paisley, Scotland. Percoll density gradient medium was from Pharmacia, Milton Keynes, Buckinghamshire. Morphine sulphate was from Evans Medical Ltd., Greenford, Middlesex. ε-Chain-specific goat anti-human IgE and non-immune IgG were prepared as described (Holgate *et al.*, 1984). HEPES-buffered salts solution (HBSS), pH 7.2, comprised (mM): NaCl 137, HEPES 20, D-glucose 5, KCl 2.7, NaH_2PO_4 0.4, $MgCl_2$ 0.5, $CaCl_2$ 1.8 and gelatin 0.1% w/v. All reagents in this buffer were of analytical grade.

Results

Separation of enzymatically-dispersed skin cells according to buoyant density yielded cell preparations containing from 1% to 86% mast cells (Table 1). In four experiments in which highly-purified (73.2 ± 2.4%) preparations of skin mast cells collected from the higher density (70/80% and >80% Percoll) fractions of the gradient were activated with anti-IgE, there was a concentration-dependent release of histamine, PGD_2 and LTC_4 (Table 2). Release of all three mediators was significantly ($P < 0.05$) greater than spontaneous release over the range 0.83–25 $\mu g\,ml^{-1}$ anti-IgE and reached maxima of 3432 ± 725, 84.9 ± 10.8 and 6.6 ± 1.2 pmol/10^6 nucleated cells respectively at the highest concentration of anti-IgE used. Over the whole concentration-response curve of 0.25 to 25 $\mu g\,ml^{-1}$ anti-IgE, there were significant correlations between the net release of PGD_2 and histamine ($r = 0.871$, $P < 0.001$, $n = 20$) and LTC_4 and histamine ($r = 0.686$, $P < 0.001$, $n = 20$).

Table 1 Preparation of mast cell-enriched or depleted fractions by density-gradient centrifugation

Percoll concentration (%)	*Mast cell purity* (%)
Unseparated	9.5 ± 1.5
<40	1.0 ± 0.1
40/50	2.5 ± 0.1
50/60	20.1 ± 4.2
60/70	61.5 ± 7.3
70/80	86.0 ± 2.9
>80	86.8 ± 2.2

Enzymatically-dispersed skin cells comprising 9.5 ± 1.5% mast cells were fractionated on five-step discontinuous gradients of 40–80% Percoll as described. Results shown are from 8 separate gradients.

Activation of gradient fractions with anti-IgE, 25 $\mu g\,ml^{-1}$, stimulated the release of PGD_2, LTC_4 and histamine. When all gradient fractions in five experiments were assessed, the release of PGD_2 was significantly correlated with both histamine release ($r = 0.498$, $P < 0.01$, $n = 38$) (Figure 1a) and with numbers of mast cells ($r = 0.454$, $P < 0.01$, $n = 35$). Release by anti-IgE of LTC_4, was also significantly correlated with that of histamine ($r = 0.712$, $P < 0.001$, $n = 25$) (Figure 1b) and with numbers of mast cells ($r = 0.773$, $P < 0.001$, $n = 21$). These correlation studies suggest that activated skin mast cells are likely to be the major source of both eicosanoids in the skin dispersates.

In the same experiments, PGD_2 and histamine release induced by substance P were compared to

Table 2 Anti-IgE-induced release of mediators from human purified skin mast cells

	Net release of mediator (pmol/10^6 cells)			
Anti-IgE ($\mu g\,ml^{-1}$)	*PGD_2*	*LTC_4*	*Histamine*	*Histamine release* (%)
0.25	13.0 ± 2.7	0.2 ± 0.1	269 ± 62	1.1 ± 0.5
0.83	21.2 ± 3.9	0.8 ± 0.2	522 ± 149	2.3 ± 0.6
2.5	57.8 ± 10.7	3.2 ± 0.7	1310 ± 347	5.4 ± 1.2
8.3	66.7 ± 11.2	5.7 ± 1.0	2647 ± 735	12.0 ± 3.7
25	85.0 ± 10.8	6.6 ± 1.2	3430 ± 725	16.4 ± 4.3

Results are from 4 experiments in which mast cell purity was 73.2 ± 2.4%. Spontaneous releases of prostaglandin D_2 (PGD_2), leukotriene C_4 (LTC_4) and histamine were 4.4 ± 1.9, 0.9 ± 0.3 and 957 ± 336 pmol/10^6 nucleated cells respectively, the latter corresponding to 4.8 ± 1.1% of total histamine.

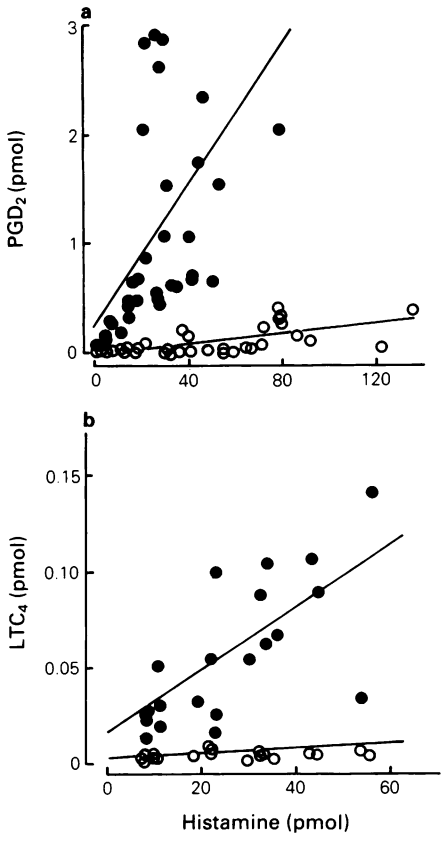

Figure 1 Relationship between net release of (a) prostaglandin D_2 (PGD_2) and histamine, (b) leukotriene (LTC_4) and histamine in skin cells separated by density-gradient centrifugation. Preparations containing 0.8–95.1% mast cells were activated with anti-IgE, 25 $\mu g ml^{-1}$ (●), or substance P, 30 μM (○). Release of PGD_2 and LTC_4 was measured in fractions from 5 and 3 density-gradients respectively.

that by anti-IgE (Figure 1a). Although this stimulus activated PGD_2 release which was significantly correlated with that of histamine ($r = 0.529$, $P < 0.01$), the shallower gradient of the correlation line demonstrated that the peptide was less effective than anti-IgE in stimulating release of this newly-generated mediator. Similar results were obtained when release of LTC_4 and histamine were compared for each stimulus (Figure 1b). Again, there was a significant correlation between release of the two mediators ($r = 0.441$, $P < 0.05$), but the greater effectiveness of the immunological stimulus in generating LTC_4 was demonstrated by the shallower gradient of the correlation line for substance P. The data from these experiments are summarised in Table 3. Whilst both stimuli induced the release of similar amounts of histamine per mast cell, as expected from the correlation analysis, substance P was less effective than anti-IgE in stimulating release of eicosanoids, the peptide releasing 21 fold less PGD_2 and 18 fold less LTC_4 than the immunological stimulus.

Several other non-immunological agents, which we have previously demonstrated to activate histamine release from skin mast cells (Benyon *et al.*, 1987a; Lowman *et al.*, 1988b), were also tested for their capacity to stimulate release of eicosanoids. In four experiments with skin cell preparations containing 76.2 ± 4.2% mast cells, comparisons were made of histamine and eicosanoid release following cell activation with anti-IgE or with substance P, VIP, somatostatin, compound 48/80, morphine and poly-L-lysine (Figure 2). Although histamine release was similar with each secretagogue, being in the range 3500–4500 $pmol/10^6$ nucleated cells, only the IgE-dependent stimulus released significant quantities of PGD_2 and LTC_4. In the same preparations, calcium ionophore A23187, like anti-IgE, stimulated release of all three mediators, a 1 μM concentration of this stimulus releasing 6882 ± 1065, 71.0 ± 6.6 and 7.7 ± 1.7 $pmol/10^6$ nucleated cells of histamine, PGD_2 and LTC_4, respectively.

Discussion

Mast cells dispersed from human skin resemble those of lung parenchyma in that both PGD_2 and LTC_4 are released in addition to histamine following

Table 3 Mediator release from density gradient-separated skin cells activated with anti-IgE (25 $\mu g ml^{-1}$) or substance P (30 μM)

	Net release (pmol/10^6 mast cells)		*Molar ratio of release*	
	Anti-IgE	*Substance P*	*Anti-IgE*	*Substance P*
Histamine	2897 ± 368	4132 ± 347	1000	1000
PGD_2	107 ± 15	7.1 ± 1.3	36.9	1.7
LTC_4	8.0 ± 0.6	0.7 ± 0.1	2.8	0.2

Results shown are from 20–35 fractions of 4–7 gradients. Net release of histamine by anti-IgE and substance P was 12.8 ± 2.1% and 17.9 ± 3.7%, respectively.

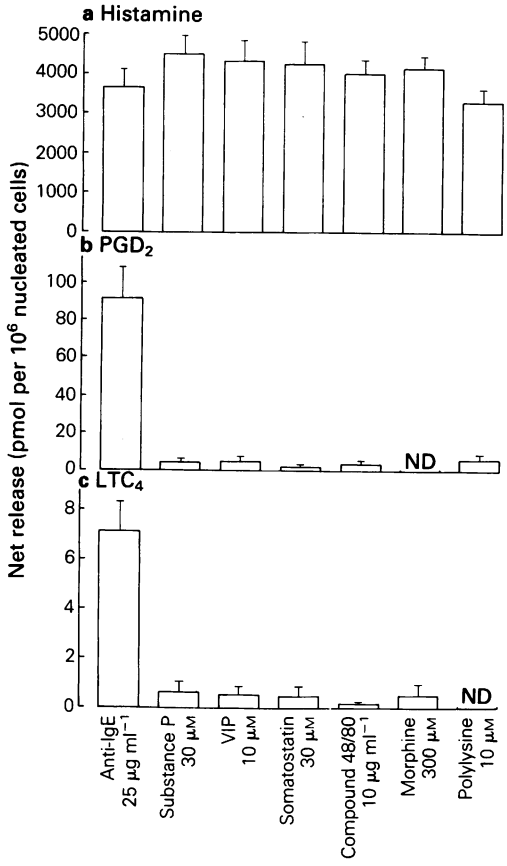

Figure 2 Comparison of the release of (a) histamine, (b) prostaglandin D_2 (PGD_2) and (c) leukotriene C_4 (LTC_4) from purified mast cells activated with anti-IgE or non-immunological stimuli. Results are from 4 experiments in which mast cell purity was $76.2 \pm 4.2\%$ and spontaneous releases of PGD_2, LTC_4 and histamine were 4.8 ± 0.7, 0.8 ± 0.4 and 910 ± 90 pmol/10^6 nucleated cells, respectively. Net release of histamine by each stimulus was: anti-IgE, $13.1 \pm 1.8\%$; substance P, $16.9 \pm 3.2\%$; vasoactive intestinal peptide (VIP), $15.8 \pm 2.9\%$; somatostatin, $17.2 \pm 4.1\%$; compound 48/80, $14.7 \pm 2.5\%$; morphine, $16.1 \pm 2.8\%$; poly-L-lysine, $13.3 \pm 3.2\%$. ND = not determined.

stimulation with anti-IgE. However, skin mast cells also release histamine after challenge with a variety of non-immunological stimuli to which lung mast cells, like those of tonsil, adenoid and intestine, are refractory (Lowman *et al.*, 1988a). These studies demonstrate that non-immunological stimuli differ from anti-IgE in being relatively poor activators of the release of PGD_2 and LTC_4 from human dispersed skin mast cells, despite inducing the release of similar amounts of histamine to the immunological stimulus.

Differential release of histamine and LTC_4 has been observed in human basophils activated with the complement fragment C5a (Schulman *et al.*, 1988) or hyperosmolar stimuli (Findlay *et al.*, 1981), although, to our knowledge, differential release of pre-formed and newly-generated mediators by IgE-dependent and non-immunological stimuli has not previously been demonstrated in human mast cells. Mast cells of human dispersed lung (Holgate *et al.*, 1984) and from patients with spleen mastocytosis (Robinson *et al.*, 1988) activated with anti-IgE release approximately twice as much PGD_2 per unit amount of released histamine compared to those stimulated by calcium ionophore A23187, but these differences are small compared to those between anti-IgE and non-immunological stimuli obtained in the present study. Our findings of large differences between anti-IgE and non-immunological stimuli in their ability to activate eicosanoid release from human skin mast cells, when considered together with their differing Ca^{2+}-dependency and time course of histamine release, would suggest that these two types of stimuli activate these cells by distinct biochemical mechanisms. Non-immunological stimuli may, therefore, be poor activators of mast cell cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism. As the activity of these pathways is primarily dependent on the supply of free arachidonic acid (Vonkeman & Van Dorp, 1968; Van den Bosch, 1980), it is possible that the two types of stimuli differ in their ability to activate the phospholipase A_2 enzymes responsible for the liberation of this fatty acid from membrane phospholipids.

The physiological implications of these findings with skin mast cells are uncertain but are worthy of consideration, as non-immunological stimuli, in the form of the substance P, VIP and somatostatin, have each been identified by histochemical techniques in mammalian dermal nerve endings (Hökfelt *et al.*, 1975; 1976; Hartschuh *et al.*, 1983; Johansson, 1986) and have been observed in close anatomical association with mast cells (Wiesner-Menzel *et al.*, 1981; Newson *et al.*, 1983; Skotfisch *et al.*, 1985). Ever since the proposal by Langley (1900) of an axon-reflex mechanism to explain the neurogenic spread of the flare component of the triple response, a large body of evidence has accumulated supporting the hypothesis that neuropeptides can increase dermal microvasculature blood-flow and, to a lesser extent, blood vessel permeability by activating mast cell histamine secretion (reviewed by Foreman *et al.*, 1987). The ability of skin mast cells to respond in different ways to IgE-dependent and non-immunological stimuli may allow them to have bi-functional roles in the dermis. The mounting of an effective tissue response to an allergic stimulus may require the release of the whole spectrum of mast cell mediators,

with their various chemotactic and vasoactive properties. For example, PGD_2 has several activities relevant to inflammation by potentiating IgE-dependent histamine release from basophils (Peters *et al.*, 1984) and stimulating leukocyte migration (Goetzl *et al.*, 1976). In contrast, neuropeptides appear to stimulate rapid release of a smaller variety of mediators including histamine, presumably in conjunction with other pre-formed mediators such as heparin and neutral proteases, which may have homeostatic rather than defensive functions such as control of blood flow (Schayer, 1962), angiogenesis (Marks *et al.*, 1986) or fibroblast proliferation (Pillarisetti *et al.*, 1983).

In conclusion, we have demonstrated that in human skin mast cells non-immunological stimuli, which include a variety of dermal neuropeptides, are of similar effectiveness to anti-IgE in releasing histamine but generate 10 to 20 fold less PGD_2 and LTC_4 from these cells. In combination with other differences such as their Ca^{2+}-dependency and time-course of histamine secretion (Benyon *et al.*, 1987a; Lowman *et al.*, 1988b), these findings suggest that these two types of stimulus have different mechanisms of mast cell activation. As activation of skin mast cells by neuropeptides may be involved in physiological and pathological processes, their detailed mechanism of mast cell activation would be a subject worth studying further.

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Block of sodium channels by psychotropic drugs in single guinea-pig cardiac myocytes

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1 Effects of imipramine and haloperidol on voltage-gated sodium channels were investigated in guinea-pig isolated ventricular myocytes by the whole-cell patch clamp technique. Some additional experiments were also performed with chlorpromazine for the purpose of comparison.

2 All test drugs in micromolar concentrations suppressed the amplitude of peak sodium current associated with step depolarization from a holding potential of −140 mV in a reversible manner. The order of potency was chlorpromazine > imipramine > haloperidol.

3 Dose-response curves obtained with a holding potential of −140 mV were best fitted by 2:1 stoichiometry in all three drugs and were shifted in the direction of lower concentrations when a holding potential of −90 mV was used.

4 The drug-induced block was not associated with any change in the time courses of sodium current activation and inactivation.

5 Steady-state sodium channel inactivation curve was shifted in the direction of more negative potentials by the drugs.

6 All three drugs also produced marked use-dependent block as demonstrated by a cumulative increase in the block during a train of depolarizing pulses.

7 The use dependence was due to a higher affinity of the drugs for the inactivated state of sodium channels than the resting state and to a very slow repriming of the drug-bound sodium channels from inactivation.

8 The steady-state and use-dependent block of voltage-gated sodium channels by psychotropic drugs may contribute to their cardiotoxic and perhaps antiarrhythmic effect.

Introduction

Tricyclic antidepressants are used in the treatment of affective disorders, major depression in particular. Neuroleptic or antipsychotic drugs are widely used in the treatment of psychiatric disorders. Their antidepressant and antischizophrenic actions are thought to involve changes in amine function in the brain (Matthysse & Lipinski, 1975; Seeman, 1981). One of the major side effects of tricyclic antidepressants and neuroleptic drugs is cardiotoxicity. Cholinolytic, sympatholytic, and direct 'quinidine-like' membrane actions (Baldessarini, 1985; Risch *et al.*, 1981a,b; Stimmel, 1979) appear to interplay in the heart. Increased heart rate is believed to be due primarily to the antiacetylcholine action, whereas electrocardiographic changes and conduction disturbances largely result from the direct membrane effect (Frommer *et al.*, 1987).

The quinidine-like effect is thought to be caused by slowing of Na^+ flux into cells, resulting in altered configuration of action potential and slowing of conduction. However, the detailed cellular mechanism underlying the quinidine-like action remains to be elucidated. Since sodium channels play a pivotal role in causing various cardiac arrhythmias, the quinidine-like effects of tricyclic antidepressants and neuroleptic drugs on cardiac cells appear to be caused by their ability to block the sodium channels. In our previous study, it was indeed found that the neuroleptic chlorpromazine had a potent sodium channel blocking action (Ogata *et al.*, 1989). Since tricyclic antidepressants are chemically different from chlorpromazine, a phenothiazine neuroleptic, the

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quinidine-like effect of the former might be exerted by a mechanism different from that of chlorpromazine. In the present study, we investigated the action of imipramine, a tricyclic antidepressant, on the gating mechanism of sodium channels of cardiac myocytes by using the whole-cell patch clamp technique (Hamill *et al.*, 1981). The effect of haloperidol, a non-phenothiazine neuroleptic, was also studied for comparison. It was found that despite the considerable differences in chemical structure, imipramine and haloperidol blocked the sodium channels in a manner similar to that of chlorpromazine. These drugs had a greater affinity for the inactivated state of the sodium channel than the resting and activated states.

Methods

The materials and methods used in the present study were similar to those described in a previous paper (Ogata *et al.*, 1989). In brief, adult guinea-pigs of either sex (300–500 g) were anaesthetized with pentobarbitone (40 mg kg^{-1}, i.p.), and cardiac myocytes were isolated by a method modified from that described by Mitra & Morad (1985). The heart was excised and retrogradely perfused for 5 min with an oxygenated Ca^{2+}-free modified Tyrode solution with the following composition (mM): NaCl 135, KCl 5.4, $MgCl_2$ 1, NaH_2PO_4 0.33, HEPES 10, and glucose 11, with pH adjusted to 7.4 with NaOH.

The heart was then perfused for 7.5 min with the oxygenated, Ca^{2+}-free modified Tyrode solution containing 1.5 mg ml^{-1} collagenase type 1 (Sigma, St. Louise, MO), 0.2 mg ml^{-1} protease type 14 (Sigma), and 1 mg ml^{-1} bovine serum albumin (Sigma) at a temperature of 37 ± 1°C. Then the heart was perfused for 3 min with Krebs bicarbonate (KB) medium of the following composition (mM): KCl 25, KH_2PO_4 10, KOH 116, EGTA 0.5, glutamic acid 80, taurine 10, oxalic acid 14, glucose 11, and HEPES 10, with pH adjusted to 7.4 with KOH. The left ventricle was minced with fine scissors and cells were strained through a 200 μm nylon mesh and stored in the KB medium for 0.5 to 10 h. Only Ca^{2+}-tolerant, rod shaped cells with evenly spaced sarcomeres were selected for the experiment.

The external perfusate used was a low Na^+ HEPES buffered solution of the following composition (mM): tetramethylammonium chloride (TMA-Cl) 90, NaCl 50, $CaCl_2$ 1.8, CsCl 5, $MgCl_2$ 1, glucose 25, HEPES 5, and pH titrated to 7.4 with 1 M TMA hydrochloride. In some experiments, NaCl concentration was increased to 100 mM or reduced to 25 mM (substituted by TMA-Cl). The solution inside the suction-pipette contained (mM): CsF 130, NaF 20, HEPES 5, and pH titrated to 7.0 with 1 M CsOH. Test drugs were diluted to desired concentrations from a 10 mM stock solution.

Patch clamp electrodes were fire-polished borosilicate glass capillaries (0.8–1.1 mm i.d.; Kimble, Vineland, NJ, U.S.A.). Membrane currents were recorded by a current-to-voltage converter, and stored on disk using a PDP11/23 computer (Digital Equipment, Pittsburgh, PA). Each pulse protocol was applied at intervals longer than 30 s to avoid the use-dependent effects of drugs.

As described in our previous paper (Ogata *et al.*, 1989), the space- and voltage-clamp control was not satisfactory in the conditions that generated sodium currents larger than approximately 5 nA. In order to avoid such artifacts, we used patch electrodes with resistances less than 0.5 megohm and external Na^+ concentration at 50 or 25 mM. The experiments in which the peak current exceeded 5 nA were discarded.

The series resistance arising from the pipette tip and the cell interior was carefully compensated (Marty & Neher, 1983; Matteson & Armstrong, 1984). Capacitive and leakage currents were subtracted digitally by the P-P/4 procedure (Bezanilla & Armstrong, 1977). Exponential fits and fits to a simple Boltzmann distribution were determined by computer using a non-linear sum of the least squares fitting routine. The data were compensated for the liquid junction potential between internal and external solutions (about 3 mV). Experiments were carried out at room temperature (22°C). Results are expressed as the mean ± s.e.mean and Student's *t* test was used to estimate the significance of differences.

Results

Block of sodium current

At a concentration of 5 μM, imipramine suppressed the sodium current. Figure 1a illustrates changes in sodium current before (0 min) and during (1, 2 and 3 min) superfusion of the test solution. The peak sodium current decreased to approximately one-third of control value, and attained a steady state within 5 min. Washing with drug-free solution restored the sodium current, and a complete recovery was observed in 8–10 min. The sodium currents recorded with a faster time scale revealed no change in the time course of current during imipramine-induced suppression (Figure 1b). These results were confirmed by 30 experiments.

Similar results were obtained with chlorpromazine and haloperidol ($n = 10$ for each drug). Both chlorpromazine (2–5 μM) and haloperidol (5–10 μM) reversibly reduced the peak amplitude of sodium

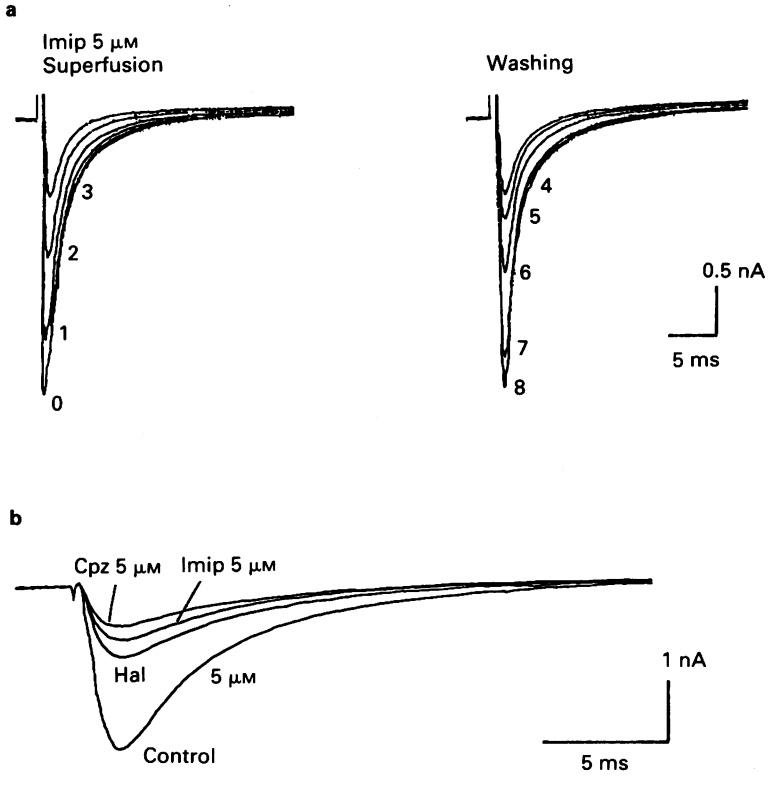

Figure 1 Effects of psychotropic drugs on sodium current in guinea-pig ventricular cells. (a) Sodium currents associated with step depolarizations to −10 mV from a holding potential of −140 mV before, during and after superfusion of 5 μM imipramine (Imip). The left panel illustrates successive recordings obtained 0, 1, 2 and 3 min after the start of superfusion of imipramine-containing solution. The right panel illustrates successive recordings obtained 4, 5, 6, 7 and 8 min after the start of superfusion with drug-free solution. (b) Sodium currents obtained in control, 5 μM imipramine, 5 μM chlorpromazine (Cpz) and 5 μM haloperidol (Hal) solutions.

current without affecting its time course (Figure 1b). The order of potency as measured in the same cell was chlorpromazine > imipramine > haloperidol. Detailed comparison of the potency of the three drugs in blocking sodium current will be given later.

The current-voltage relationships for the peak sodium current before and during application of 3 μM imipramine are shown in Figure 2. The membrane was held at −140 mV, and step depolarizing pulses were applied to various potential levels at a frequency of 0.05 Hz to record sodium currents. The current amplitude was suppressed by imipramine to the same extent at all membrane potentials tested. Thus there was no change in the voltage dependence of sodium channel activation. These results were confirmed by three experiments. Similar results were obtained with 7 μM haloperidol and 2 μM chlorpromazine (3 experiments for each drug).

The dose-response relationships for sodium current block caused by imipramine, haloperidol and chlorpromazine are illustrated in Figure 3. Step depolarizing pulses to −10 mV were applied at a frequency of less than 1 min^{-1} from a holding potential of −140 mV. The peak amplitude of sodium current was normalized to that of control sodium current, and is plotted against the drug concentration. The

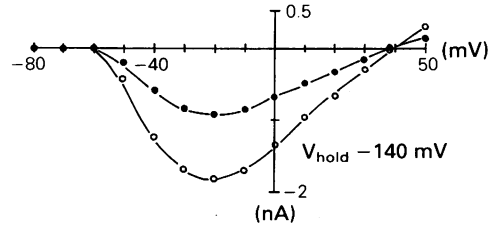

Figure 2 Current-voltage relationships of sodium current before (○) and after application of 3 μM imipramine (●).

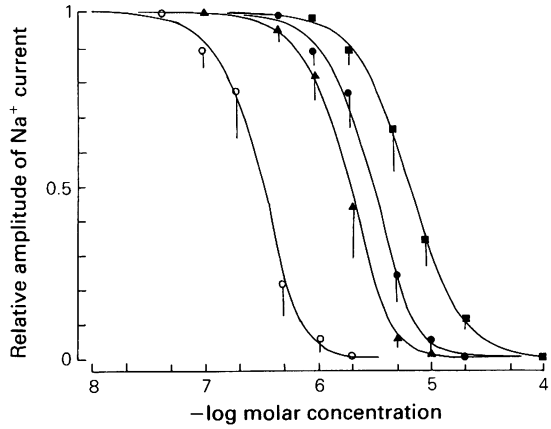

Figure 3 Dose-response relationships for the block of sodium current. The amplitude of peak sodium current elicited by a 20 ms depolarizing pulses to −10 mV from a holding potential of −140 mV in the presence of imipramine (●), chlorpromazine (▲) and haloperidol (■) and that from a holding potential of −90 mV in the presence of imipramine (○) were normalized to the control amplitude measured in the absence of drugs. Each point represents the mean (with s.e.mean shown by vertical lines) from six experiments. Theoretical curves are drawn by equation (1) for the value of $n = 2$.

measurements for each drug are fit by a sigmoid curve calculated by the following equation

$$I_{Na} = \frac{1}{1 + ([C]/K_d)^n} \quad (1)$$

in which I_{Na}, [C] and K_d represent sodium current amplitude, drug concentration, and the dissociation constant of drug binding reaction, and the exponent n is assumed to be 2. The exponent n = 1 and n = 3 gave less steep and steeper curves, respectively, than the measurements. Therefore, it was concluded that two drug molecules bind to one receptor in the sodium channel to exert the blocking action. The apparent dissociation constants for sodium current block were estimated to be 3.5 ± 0.74, 7.0 ± 0.86 and 2.1 ± 0.4 μM for imipramine, haloperidol and chlorpromazine, respectively ($n = 6$).

The degree of imipramine block was dependent upon the holding potential as is shown by the dose-response curves with holding potentials of −90 mV and −140 mV (Figure 3). At a holding potential of −90 mV, the apparent dissociation constant was estimated to be 0.35 ± 0.09 μM ($n = 3$), representing a 10 fold increase in the blocking potency compared with the data at a holding potential of −140 mV. The apparent dissociation constants for haloperidol and chlorpromazine were also decreased by changing the holding potential from −140 mV to −90 mV; to 0.65 ± 0.06 μM ($n = 3$) and 0.18 ± 0.03 μM ($n = 3$), respectively, representing an 11 fold increase in potency in both cases. Therefore, it was concluded that the block of sodium current by these three drugs is highly voltage-dependent, being augmented by membrane hyperpolarization.

Effects on sodium current kinetics

The currents illustrated in Figure 1b show that imipramine, haloperidol and chlorpromazine did not change the time course of sodium current. In order to substantiate the observation in a more quantitative manner, the time courses of sodium channel activation and inactivation were analyzed by Hodgkin-Huxley (1952) formulation as shown in Figure 4. The time course of sodium current activation is described by the equation

$$e^{-t/\tau_m} = 1 - (I(t)/I'(t))^{1/n} \quad (2)$$

where τ_m is the time constant of activation, and I(t) and I′(t) represent the recorded current amplitude and the current amplitude estimated by the extrapolation of the falling phase of current at time t, respectively, and n is the exponent.

The sodium currents recorded before and during imipramine (5 μM) application are superimposed in Figure 4a. The current in imipramine multiplied by 3.3 completely overlaps the control current. The extrapolation of the falling phase of sodium current is also shown. The time courses of activation as calculated by equation 2 in both control and imipramine fall on a straight line when n is assumed to be 3, indicating that the activation process is described by m^3 kinetics as it is in the sodium channel of squid giant axons (Hodgkin & Huxley, 1952). Imipramine (2–5 μM) had no effect on τ_m ($P > 0.05$, $n = 5$).

The analysis of the time course of sodium channel inactivation is shown in Figure 4b. The sodium current in the presence of 3 μM imipramine was suppressed to 50% of control and mimicked the control current when multiplied 2 fold. The time course of current decay is expressed by two exponential functions with time constants of 1.7 ms (τ_{fast}) and 12.3 ms (τ_{slow}) in both control and imipramine for the experiment shown in Figure 4b. The values for mean ± s.e.mean are 2.0 ± 0.9 ms and 13.7 ± 1.4 ms ($n = 6$) for the fast and slow time constants, respectively, in both control and imipramine (2–5 μM).

Steady-state sodium channel inactivation

The steady-state sodium channel inactivation curve was greatly shifted in the direction of hyperpolarization after application of 5 μM imipramine (Figure 5). As illustrated in the inset of the figure, 5 s after a control step depolarizing pulse (−10 mV, 20 ms), a

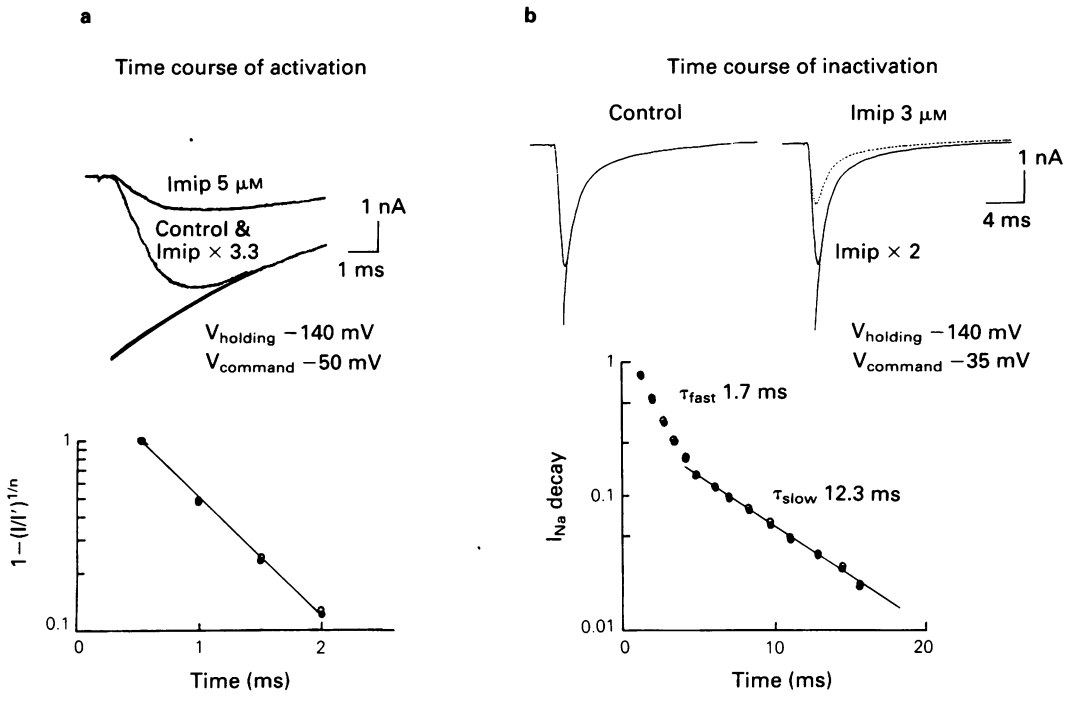

Figure 4 Effects of imipramine on the activation and inactivation of sodium current. (a) The activation time course. The sodium currents in control and imipramine (5 μM) are shown. When multiplied 3.3 fold, the current in imipramine completely overlaps the control current. The time course of sodium current activation was assessed by equation (2), and the measurements fall on a straight line with $n = 3$. (b) The time course of the falling phase of the sodium current. The current in 3 μM imipramine, when multiplied 2 fold, mimics the control current. The decay phases of currents before and during application of imipramine are plotted on a semi-logarithmic scale. In both (a) and (b): (●) control; (○) imipramine.

conditioning pulse (1 s duration) was applied to various potential levels, which in turn, with a 2 ms gap at the holding potential level (−120 mV), was followed by a test depolarizing pulse (−10 mV, 20 ms). The 2 ms interval allowed the capacitive current to decay without significant recovery of sodium current before the test pulse was applied. The sodium current associated with the test pulse, in a value relative to the current associated with the control pulse, is plotted as a function of the conditioning voltage. The sodium currents in imipramine were suppressed. When normalized to the control sodium currents obtained before application of imipramine, the inactivation curve is seen to be shifted by 19 mV in the direction of more negative potentials without change in the slope factor. The mean ± s.e.mean for the shift was estimated as 18.0 ± 2.4 mV ($n = 4$). Similar shifts were observed after application of haloperidol (7 μM) and chlorpromazine (2 μM) in amounts of 21.0 ± 0.5 mV ($n = 3$) and 17.5 ± 3.0 mV ($n = 4$), respectively. The slope factor was not affected by these two drugs. The results indicate that these drugs have a high affinity for the inactivated state of sodium channel.

Use-dependent block

The suppression of sodium current by imipramine was greatly enhanced by applying repetitive stimulations. An example of such an experiment is illustrated in Figure 6a. Step depolarizing pulses (−10 mV, 30 ms) were applied at an interval of 100 ms. Whereas the sodium current amplitude remained constant during repetitive stimuli in control experiments, drastic decreases were observed in the presence of imipramine. The time course of the use-dependent block is more clearly seen in Figure 6b in which the current amplitude associated with each pulse (I_n) is plotted as a function of pulse number. The use-dependent block was accelerated by shortening the pulse interval, and an interval of 5 s was required to avoid the use-dependent block in the presence of imipramine (not shown). Similar results were obtained in all ten cells examined.

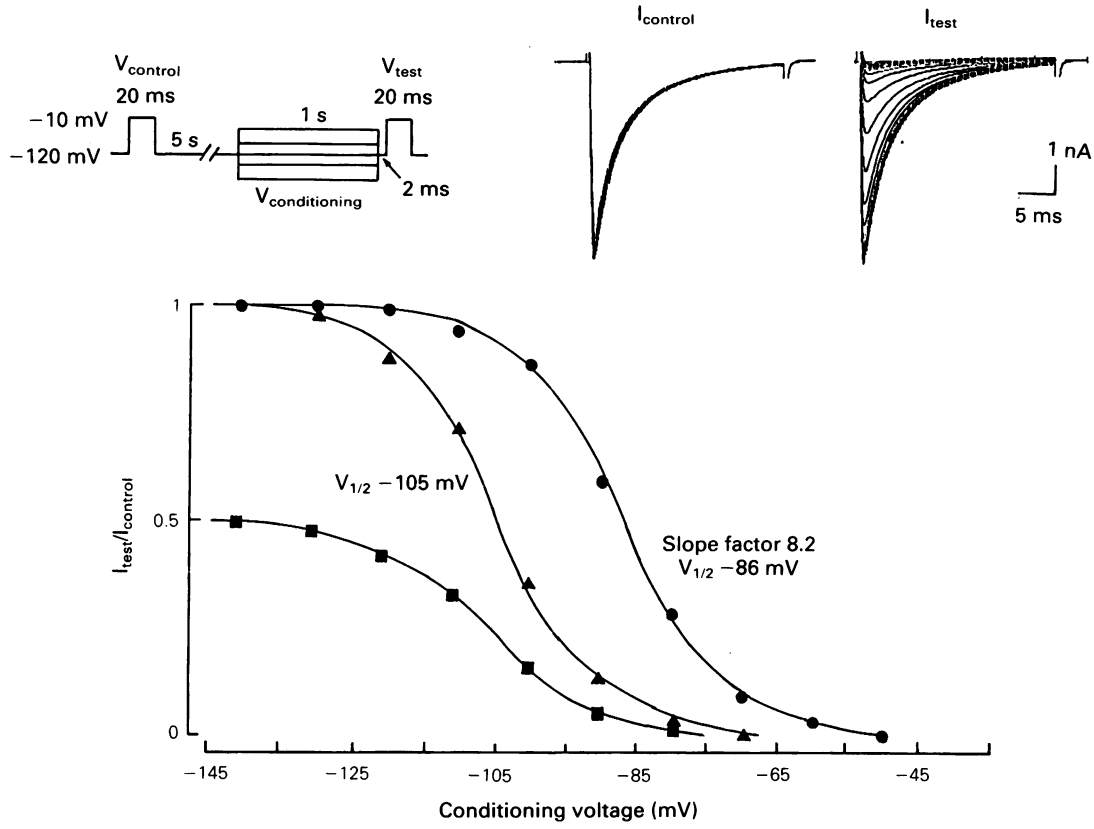

Figure 5 Steady-state inactivation curves for sodium current in the presence and absence of 5 μM imipramine. Measurements were made using a standard two-pulse protocol (see inset). The amplitude of peak current associated with a test pulse was normalized to the control current amplitude measured 5 s before the conditioning pulse, and is plotted as a function of the conditioning potential: (●) control; (■) imipramine 5 μM; (▲) imipramine normalized to control.

However, the use-dependent block was not observed with very brief pulse intervals (2–4 ms) in any of three cells tested during application of 5 μM imipramine. Similar use-dependent block was also observed in the presence of 7 μM haloperidol or 2 μM chlorpromazine (Figure 6b).

Dissociation of drugs from inactivated channels

Figure 7a illustrates the pulse protocol used for assessing the recovery from drug-induced block. The control sodium current was evoked by a 20 ms depolarizing pulse to −10 mV 10 s before the conditioning pulse. The conditioning depolarizing pulse (5 s) to −10 mV was followed by various recovery periods, and then by a test pulse to −10 mV. Each pulse sequence was given at a 30 s interval. Figure 7b illustrates a series of superimposed traces of control and test currents in the absence or presence of 1 μM imipramine. The peak current for each test pulse was normalized to that for the control pulse, and is plotted as a function of the recovery period in Figure 7c. The test currents in drug-free solution recovered within 1 s. In the presence of 100 nM or 1 μM imipramine, however, it took more than 2 s for complete recovery. As shown in Figure 7d, the recovery time course in control solution is expressed by two exponential functions (time constants of 43 ms and 192 ms for the fast and slow components, respectively). In the presence of 100 nM imipramine, the time course of recovery was markedly slowed, and a time longer than 2 s was required for complete recovery. The recovery time course is again expressed by two exponential functions (time constants of 210 ms and 1.6 s for the fast and slow components, respectively). The fast time constant approximated the slow component in the control. Thus, it appears that the slow component of the sodium current recovery in imi-

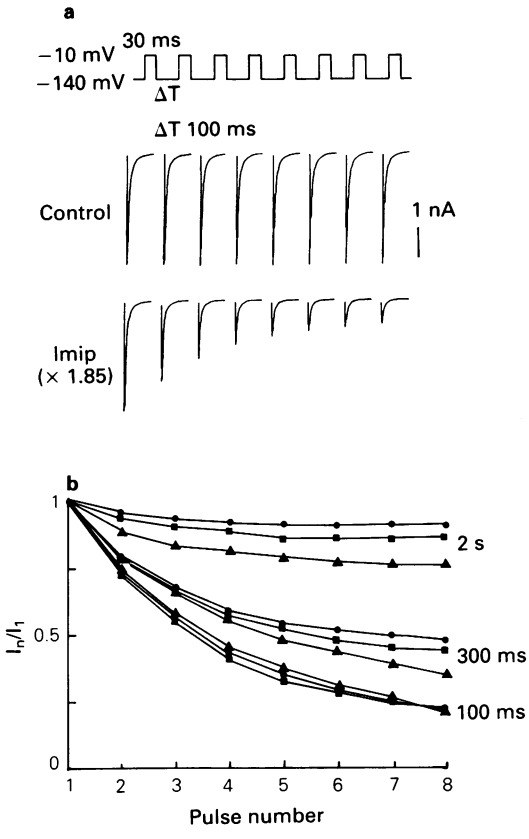

Figure 6 Use-dependent block of sodium current by 3 μM imipramine (■), 2 μM chlorpromazine (●) and 7 μM haloperidol (▲). Eight consecutive pulses to −10 mV were delivered at 100 ms, 300 ms and 2 s intervals from a holding potential of −140 mV. (a) Pulse protocol and successive current traces obtained in the control and imipramine (Imip)-containing solutions. The first current in imipramine was normalized to that of control. (b) The peak current amplitude for each pulse (I_n) in the train was normalized to that of the first pulse (I_1), and is plotted as a function of the pulse number.

pramine reflects the time course of drug dissociation from the inactivated sodium channel, whereas the fast component reflects the recovery of drug-unbound channels from inactivation. The extremely slow recovery of the drug-bound channels from inactivation is consistent with the observation that imipramine produced profound use-dependent block in a wide range of frequencies used (Figure 6). The recovery time constants after applications of imipramine (1 μM), chlorpromazine (1 μM) and haloperidol (3 μM) were 201 ± 37, 185 ± 42, and 220 ± 31 ms, respectively, for the fast components, and 1.8 ± 0.27, 1.7 ± 0.39, and 2.4 ± 0.23 s, respectively, for the slow components.

Discussion

Despite the considerable differences in chemical structure among imipramine, haloperidol and chlorpromazine, the characteristics of sodium channel block are very similar to each other. The present results with imipramine and haloperidol are in general agreement with those for chlorpromazine (Ogata *et al.*, 1989) in many respects. The sodium current is blocked without any change in the activation or inactivation kinetics. The resting or steady-state block is voltage-dependent, and intensified by shifting the holding potential to less negative values. The drugs have high affinities for the inactivated state of sodium channels as the steady-state sodium channel inactivation curve is shifted in the hyperpolarizing direction (Hondeghem & Katzung, 1977). There is a use-dependent component of block, as repetitive depolarizing pulses greatly accelerate the block. The recovery from the drug-induced block is very slow, indicating a slow dissociation of drug molecules from the inactivated sodium channels.

The apparent dissociation constant for imipramine block of sodium current is estimated to be 0.35 μM when measured at a holding potential of −90 mV which is close to the normal resting potential of cardiac cell. The plasma concentration for therapeutic antidepressant effects is in the range of 0.35 to 0.7 μM (Baldessarini, 1985). Therefore, the observed sodium channel block by imipramine is likely to cause alterations of cardiac function, including direct depression of the myocardium and amelioration of arrythmias. The potency will be high due to cardiac beat which will augment the use-dependent block.

The use-dependent block of sodium channels is important in interpreting the action of psychotropic drugs, because the sodium channels of the cardiac myocyte are continuously modulated by rhythmic activity which is intrinsic to the cell or even by incoming synaptic bombardments, and also because rhythmic activity of the heart is modulated by various pathological conditions.

A single or a train of brief conditioning pulses (2–4 ms), which were long enough for the sodium channels to open but short enough to avoid inactivation, produced little or no use-dependent block by imipramine. Therefore, in contrast to the relatively high affinity for the resting and inactivated channels, the drug appears to have only minimal effects on the sodium channel in the activated state. The absence of drug effect on the time course of sodium current decay (Figure 4b) is consistent with this notion, since drugs that bind to the open state of the channel have

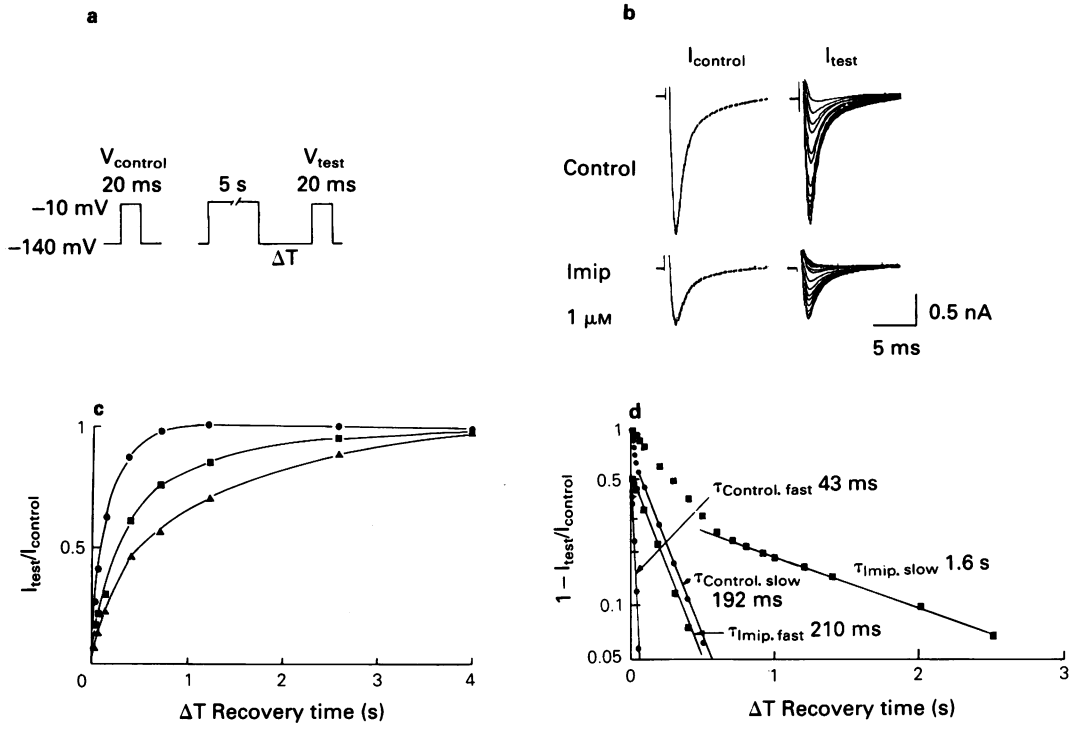

Figure 7 Recovery from the imipramine block as assessed by using the protocol shown in (a). (b) Sodium currents associated with the control pulse and the test pulse before and during application of 1 μM imipramine (Imip). (c) Recovery from the block caused by a 5 s conditioning pulse before (●) and during application of 100 nM (■) and 1 μM (▲) imipramine. The peak current for each test pulse was normalized to the current associated with control pulse, and is plotted as a function of the recovery time. (d) The recovery time courses plotted on a semilogarithmic scale for control (●) and 100 nM imipramine (■) data.

been shown to accelerate the falling phase of current (Colquhoun & Hawkes, 1983). The observation that imipramine had no effect on the current-voltage relationship for sodium channel activation (Figure 2) further rules out the possibility of the open-channel block. Drug binding to the inactivated state is probably much slower than channel gating (opening and closing).

As in the case of chlorpromazine (Ogata *et al.*, 1989), imipramine and haloperidol require the cooperation of two drug molecules for one channel site (Figure 3), whereas the binding of antiarrhythmic agents so far examined are well described by 1:1 stoichiometry (e.g. Bean *et al.*, 1983; Yatani & Akaike, 1984). This indicates that the dose-response relation will be steeper for the psychotropic drugs than for the antiarrhythmic drugs. Therefore, administration of these psychotropic drugs may tend to lead to 'overdose' intoxication. The dose may be high enough to cause toxic action through cardiac sodium channels. It is possible that the use-dependent effect of the psychotropic drugs and their binding stoichiometry are useful for the treatment of cardiac arrhythmias when properly used. In fact, tricyclic antidepressants are known to ameliorate considerably paroxysmal ventricular tachycardia (Manoach *et al.*, 1979; Frommer *et al.*, 1987).

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PC12 phaeochromocytoma cells contain an atypical muscarinic receptor binding site

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1 Kinetic, saturation and competition binding studies were conducted on the muscarinic receptor binding site labelled by [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) in intact PC12 cells and cell membranes. Similar studies were conducted on M_1 receptors of rat cortex labelled with [^{3}H]-pirenzepine and M_2 and M_3 receptors present in rat heart and submaxillary gland respectively, and labelled with [^{3}H]-NMS.

2 The dissociation of [^{3}H]-NMS from muscarinic receptors in PC12 cells was slower than dissociation from both M_2 and M_3 muscarinic receptors.

3 The K_d of [^{3}H]-NMS in the PC12 cells was significantly lower than that obtained at the M_2 and M_3 receptor.

4 In competition studies the affinity data for pirenzepine, hexahydroadiphenine and 4-diphenylacetoxy-N-methylpiperidine methiodide were consistent with the presence of an M_3 receptor in the PC12 cells. However, for AF-DX 116, cyclohexylphenyl(2-piperidinoethyl)silanol and methoctramine affinity estimates in PC12 cells were 3–6 fold lower than at the M_3 receptor.

5 On the basis of these data we conclude that the muscarinic receptor present in the PC12 cells differs from the M_1, M_2 and M_3 subtypes already described.

Introduction

The PC12 cell line is a phaeochromocytoma cell line derived from the rat adrenal medulla (Greene & Tischler, 1976). These cells are used extensively in studies on nerve growth factor (NGF) owing to the ability of this neurotrophic hormone to differentiate the cells into a neuronal cell morphology (Greene & Tischler, 1976). In addition PC12 cells have been used extensively to study the effects of acetylcholine receptor agonists on the release of neurotransmitters from storage granules in the undifferentiated cell line (Greene & Rein, 1977). The mechanisms behind this effect of cholinoceptor agonists are not clearly defined and at present evidence for both a nicotinic (Ritchie, 1979) and muscarinic (Rabe *et al.*, 1987) component to this response have been described.

In terms of the muscarinic response of the PC12 cells, ligand binding studies have identified a muscarinic receptor in membrane preparations of PC12 cells (Jumblatt & Tischler, 1982; Cross *et al.*, 1984). In functional studies muscarinic receptors have been shown to increase inositol phosphate (IP) accumulation (Vincentini *et al.*, 1985), mobilize calcium (Vincentini *et al.*, 1985) and release noradrenaline form the intact PC12 cells (Rabe *et al.*, 1987).

There is now increasing evidence for the presence of multiple subtypes of muscarinic receptors, termed M_1, M_2 and M_3 (De Jonge *et al.*, 1986; Mihm & Wetzel, 1987; Hammer *et al.*, 1987), which can interact with a diverse range of second messengers (Harden *et al.*, 1986). At present there have been few studies which have characterized the subtype of muscarinic receptor present in the PC12 cells. Initial studies (Cross *et al.*, 1984) classified the receptor as belonging to the M_2 subtype, as defined by Hammer & Giachetti (1982), based upon its low affinity for pirenzepine. Given the anomalous findings that the muscarinic receptor of PC12 cells stimulates IP accumulation, yet appears to be of the M_2 subtype which is defined as coupling to adenylate cyclase (Hammer & Giachetti, 1982) we conducted a re-evaluation of the muscarinic receptor subtype present in PC12 cells.

To this end we have compared the pharmacology of the PC12 cell muscarinic receptor with that of the putative M_1 receptor of rat cortex labelled with [^{3}H]-pirenzepine, the M_2 receptor of rat heart labelled with [^{3}H]-N-methylscopolamine and the

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M_3 receptor of rat submaxillary gland also labelled with [^{3}H]-NMS (Delmendo *et al.*, 1989).

A preliminary account of these data has been presented at the Dublin meeting of the British Pharmacological society (Michel *et al.*, 1988).

Methods

Cell culture

Rat phaeochromocytoma (PC12) cells were provided by Dr E. Shooter, Stanford, Ca., U.S.A. Cells were cultured at 37°C in a humidified atmosphere (10% CO_2) as a monolayer culture in DMEM (J.R Scientific) supplemented with 7% non heat inactivated donor horse serum (Hyclone) and 7% foetal calf serum (Hyclone). Cells were seeded at a density of 4×10^4 cells ml^{-1} and grown until confluent (usually 5 days). After 3 days the culture media was removed from the cells and fresh media added. At confluence the cells were dislodged from the flasks by vigorous shaking. When cells were grown in 150 cm^2 flasks the yield of cells from 1 flask was sufficient to provide between 20–40 assay tubes.

Membrane preparations

EDTA washed membrane fractions were prepared as described previously (Michel & Whiting, 1988; Kunysz *et al.*, 1988). Briefly, rat cerebral cortices, hearts and submaxillary glands obtained from Pel-Freez (Arkansas) and PC12 cells (harvested as described above) were separately homogenized in 50 mM Tris, 5 mM Na_2EDTA buffer (pH 7.4 at 4°C) with a polytron P10 tissue disrupter (setting 10; 2×10 s bursts). The cardiac and glandular homogenates were centrifuged at 500 *g* for 10 min and the supernatant retained. The PC12 and cerebrocortical homogenates and the cardiac and glandular supernatants were centrifuged at 48,000 *g* for 15 min. The pellets obtained were washed, by resuspension and centrifugation, once in homogenizing buffer and twice in 50 mM Tris, 0.5 mM EDTA buffer (pH 7.4 at 4°C). Membranes were stored under liquid nitrogen until required.

Binding assays

Binding assays were conducted as described previously (Michel & Whiting, 1988) with minor modifications. In all studies a Tris-Krebs assay buffer of the following composition was used (mM): NaCl 144, KCl 4.7, KH_2PO_4 1.7, $CaCl_2.(H_2O)_6$ 2.5, $MgCl_2$ 1.1, glucose 10 and Tris 10, pH 7.4 at 32°C. Assays were conducted at 32°C in a final volume of 1 ml of assay buffer. In all studies atropine (1 μM) was used to define non-specific radioligand binding (NSB). In studies conducted on membranes from rat heart, gland and PC12 cells and in studies with intact PC12 cells the muscarinic receptors were labelled with the hydrophilic radioligand [^{3}H]-N-methylscopolamine ([^{3}H]-NMS), usually at a fixed concentration of 0.1 nM. In competition experiments with cerebrocortical membranes a fixed concentration of 0.5 nM [^{3}H]-pirenzepine was used.

Incubations were for 2 h at 32°C and were terminated by vacuum filtration over 0.1% polyethyleneimine pretreated Whatman GF/B glass fibre using a Brandel 48 well cell harvester. After filtration the filters were washed for 10 s with ice cold water. Radioactivity retained on the filters was determined by liquid scintillation counting.

Data analysis

All data were analyzed by iterative curve fitting techniques as described previously (Michel & Whiting, 1988).

Materials

[^{3}H]-NMS (specific activity 73.8 Ci mmol^{-1}) and [^{3}H]-pirenzepine (specific activity 87 Ci mmol^{-1}) were obtained from New England Nuclear. Pirenzepine hydrochloride was obtained from Boehringer Ingelheim. Hexahydroadiphenine was obtained from Ciba-Geigy. Atropine sulphate, carbachol and hexamethonium were purchased from Sigma Chemical Company as were all chemicals and reagents used. 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide) was obtained from Research Biochemical Inc. AF-DX 116 (11-[[2-(diethylamino)-methyl]-1-piperidinyl]-acetyl-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one), CPPS (cyclohexylphenyl-(2-piperidinoethyl)silanol), and methoctramine (N,N'-bis[6-[(2-methoxybenzyl)amino]-hexyl]-1,8-octanediamine tetrahydrochloride) were synthesized by J. Berger, Dr R. Clark and D. Repke (IOC, Syntex, Palo Alto).

Results

In the intact PC12 cells [^{3}H]-NMS could be used to label muscarinic receptors. It was not possible to detect binding of [^{3}H]-pirenzepine (10–30 nM) to PC12 cells ($1–4 \times 10^6$ cell) under the assay conditions employed.

Although the lipohilic radioligand [^{3}H]-quinuclidinyl benzilate could also label muscarinic receptors in PC12 cell membranes there are problems associated with its use in intact cells (Galper *et al.*, 1982) and consequently we chose to use the more hydrophilic ligand [^{3}H]-NMS in our present characterization of the PC12 muscarinic receptor.

In order to characterize the muscarinic receptors present in the PC12 cells the data were compared to results obtained at the three subtypes of muscarinic receptor that can be identified at present. These were the M_1 receptor in rat cortex which can be selectively labelled with [^{3}H]-pirenzepine, and the [^{3}H]-NMS binding sites in rat heart and submaxillary gland which, respectively, contain homogeneous populations of M_2 and M_3 muscarinic receptors (De Jonge *et al.*, 1986; Delmendo *et al.*, 1989).

Kinetic experiments

Radioligand dissociation rates provide a convenient technique for differentiating subtypes of muscarinic receptor (Waelbroeck *et al.*, 1986). In the PC12 cells, dissociation of [^{3}H]-NMS induced by addition of atropine to a final assay concentration of 1 μM was slow with a half life of 26 ± 2.2 min ($n = 3$). This contrasted both with the more rapid dissociation rate of [^{3}H]-NMS obtained at the M_2 receptor present in cardiac membranes (half life = 1.6 ± 0.2 min; $n = 3$) and with the slow dissociation rate of [^{3}H]-NMS (half life = 20 ± 1.7 min; $n = 3$) obtained at the M_3 muscarinic receptor present in glandular tissue. In Figure 1 data from a single representative experiment are presented in a graphical form. It should be noted that in all cases dissociation was monophasic for at least 3 half lives.

We do not possess a preparation containing a homogeneous population of M_1 muscarinic receptors that can be labelled with [^{3}H]-NMS and consequently were unable to determine the half life of the M_1 receptor-[^{3}H]-NMS complex.

Saturation experiments

In the PC12 cells the equilibrium binding of [^{3}H]-NMS was of high affinity (Table 1). Analysis of both

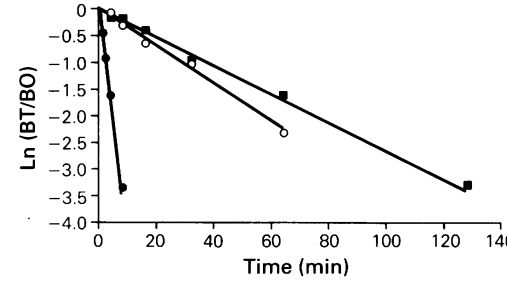

Figure 1 Dissociation of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) from rat cardiac (●), rat submaxillary gland (○) and PC12 cell membranes (■). After a 90 min equilibration period, dissociation of [^{3}H]-NMS was initiated by adding atropine to a final concentration of 1 μM. At the indicated time points specific binding was determined by vacuum filtration. BT refers to specific binding at the indicated time point after initiating dissociation and BO represents specific binding measured before initiating dissociation. The lines were drawn by hand.

specific and total binding isotherms by the programme LIGAND (Munson & Rodbard, 1980) indicated that binding was to an apparently homogeneous population of sites. The K_d of [^{3}H]-NMS in intact PC12 cells and membrane fractions of PC12 cells was significantly lower than the K_d of [^{3}H]-NMS at both the M_2 receptor in the heart and the M_3 receptor present in the gland (Table 1 and Figure 2). Because of the aforementioned problems associated with the lack of a preparation containing a homogeneous population of M_1 muscarinic receptor binding sites we do not have a K_d for the non-selective ligand [^{3}H]-NMS at the M_1 receptor. It should be noted, however, that the K_d of [^{3}H]-NMS in PC12 cells and membranes was similar to the

Table 1 Binding parameters for muscarinic receptor radioligands in rat cortical, cardiac and glandular membranes and in PC12 cells

Radioligand	*Tissue*	K_d (mol litre^{-1}) ($\times 10^{-10}$)	B_{max} (fmol mg^{-1} protein)
[^{3}H]-Pir	Cortex membrane	175 ± 19	1420 ± 99
[^{3}H]-NMS	Cortex membrane	0.51 ± 0.11	2741 ± 310
[^{3}H]-NMS	Heart membrane	1.91 ± 0.02*	402 ± 35
[^{3}H]-NMS	Gland membrane	1.08 ± 0.07*	725 ± 60
[^{3}H]-NMS	PC12 cells	0.67 ± 0.09	45 ± 9.8
[^{3}H]-NMS	PC12 membrane	0.48 ± 0.06	61 ± 12.8

Binding parameters were determined in a Tris-Krebs assay buffer.
The values shown are the mean ± s.e.mean. The data in all cases could only be fitted to a model describing an interaction of the radioligand with a single homogeneous population of binding sites. [^{3}H]-Pir = [^{3}H]-pirenzipine; [^{3}H]-NMS = [^{3}H]-N-methylscopolamine.
* K_d value for [^{3}H]-NMS significantly different ($P < 0.05$; Students 't' test) from that obtained in the PC12 cell membranes or intact cells.

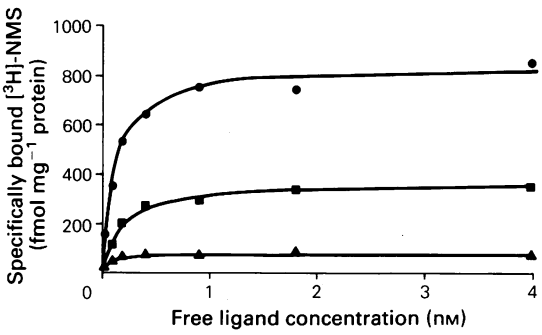

Figure 2 Equilibrium binding of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) in rat cardiac (■), rat submaxillary gland (●) and PC12 cell membranes (▲). The figure shows specific binding of [^{3}H]-NMS plotted as a function of ligand concentration. In the experiment shown the B_{max} and K_d values for [^{3}H]-NMS in rat submaxillary gland, rat heart and PC12 cells were 840, 370 and 74 fmol mg^{-1} protein, respectively, while K_d values in these tissues were 0.10, 0.19, and 0.06 nM, respectively.

overall K_d value for this ligand in rat cortex (Table 1).

Competition binding studies

Affinity estimates for a range of muscarinic receptor subtype selective compounds are shown in Table 2. Also included in Table 2 are affinity data obtained at the M_1, M_2 and M_3 receptors.

Most of the antagonists studied could clearly differentiate between the three putative muscarinic receptor binding sites. Despite the considerable degree of selectivity of these ligands, the Hill coefficients (nH) for all of the compounds studied were close to unity in the PC12 cells indicating that [^{3}H]-NMS was probably labelling a homogeneous population of binding sites in this preparation.

Comparing the profile of the muscarinic receptors present in the PC12 cells with that obtained at the three other binding sites it can be seen that for pirenzepine, 4-DAMP and hexahydroadiphenine, affinity estimates were in close agreement with those obtained at the M_3 receptor. However, for AF-DX 116, CPPS and methoctramine the affinity estimates in the PC12 cells were similar to those obtained at the M_1 receptor.

Discussion

The major aim of the present study was to determine the subtype of the muscarinic receptor present in the PC12 cell line. To this end we conducted kinetic, saturation and competition binding studies.

The use of a kinetic approach, especially the measurement of radioligand dissociation rates, in classifying muscarinic receptors has been described previously (Waelbroeck *et al.*, 1986). In cardiac tissue, [^{3}H]-NMS dissociates rapidly from M_2 muscarinic receptors (Waelbroeck *et al.*, 1986) whereas in submaxillary glands the dissociation rate of this ligand from the M_3 receptor was considerably slower. The considerable differences in dissociation

Table 2 Binding parameters of muscarinic antagonists at the M_1, M_2, M_3 and PC12 cell muscarinic receptors.

	Membrane preparations			
	Cortex (M_1)	*Heart* (M_2)	*Submaxillary* (M_3) *gland*	*PC12 cells*
Ligand	pKi/nH	pKi/nH	pKi/nH	pKi/nH
AF-DX 116	6.59(0.08)	7.02(0.04)*	5.99(0.06)*	6.75(0.05)
	1.05(0.03)	0.92(0.10)	1.04(0.04)	1.03(0.05)
CPPS	8.36(0.06)	7.56(0.08)*	7.70(0.07)*	8.27(0.06)
	1.00(0.04)	0.92(0.07)	1.11(0.04)	1.09(0.04)
4-DAMP	8.51(0.09)*	7.97(0.04)*	8.74(0.05)	8.91(0.11)
	0.89(0.06)	0.95(0.06)	1.02(0.05)	0.99(0.03)
Hexahydroadiphenine	8.64(0.06)*	7.35(0.08)*	7.97(0.03)	8.11(0.05)
	0.94(0.04)	1.04(0.05)	0.95(0.03)	1.03(0.06)
Methoctramine	6.86(0.11)	7.76(0.07)*	6.29(0.01)*	6.83(0.05)
	0.97(0.04)	0.91(0.04)	1.02(0.03)	0.91(0.06)
Pirenzepine	7.72(0.09)*	6.38(0.02)*	6.79(0.06)	6.89(0.05)
	0.92(0.04)	0.91(0.06)	1.11(0.04)	0.89(0.05)

The radioligand used was [^{3}H]-N-methylscopolamine (0.1 nM) except in the M_1 assay where [^{3}H]-pirenzepine (0.5 nM) was used. The values shown are the mean (± s.e.mean; $n = 4$) for the negative logarithm of the K_i (pKi) and the hill coefficient (nH). The nH values shown are not significantly different ($P < 0.05$) from unity.

* Value significantly different ($P < 0.05$; Students t test) to that obtained in the PC12 cells.

rates enabled a clear distinction to be made between M_2 and M_3 muscarinic receptor subtypes. In the PC12 cells the dissociation rate of [^{3}H]-NMS was markedly different from that expected of an M_2 receptor and was also significantly different from that obtained at the M_3 receptor. On the basis of these data it would appear that the muscarinic receptors labelled by [^{3}H]-NMS in the PC12 cells were not of either the M_2 or M_3 subtype. Whether the dissociation rate for [^{3}H]-NMS observed in the PC12 cells was consistent with the presence of an M_1 receptor cannot be determined at present since there are no preparations available that express a homogeneous population of M_1 muscarinic receptors.

In saturation studies the affinity estimates obtained for [^{3}H]-NMS also indicated that the receptors present in the PC12 cells differed from the M_2 and M_3 receptors present, respectively, in cardiac tissue and in salivary glands. Thus, the K_d values for [^{3}H]-NMS binding to the M_2 and M_3 receptor were significantly higher than those obtained in the PC12 cells. As in the case of the kinetic studies, the absence of preparations expressing homogeneous populations of M_1 receptors prevented us from determining the affinity of [^{3}H]-NMS for the M_1 receptor although it should be noted that in rat cortex where between 65% (Hammer *et al.*, 1980) and 52% (Kunysz *et al.*, 1988) of the muscarinic receptors are of the M_1 subtype, the K_d of [^{3}H]-NMS was similar to that observed in the PC12 cells.

The most commonly used means of classifying the muscarinic receptor subtypes identified in functional and ligand binding studies involves the use of subtype selective ligands. In recent years a range of selective ligands have been described including pirenzepine (Hammer *et al.*, 1980), hexahydroadiphenine (Delmendo *et al.*, 1989) and CPPS (Delmendo *et al.*, 1989) which are selective for M_1 as opposed to M_2 receptors. AF-DX 116 (Hammer *et al.*, 1986) and methoctramine (Melchiorre *et al.*, 1987, Michel & Whiting, 1988) display the converse selectivity and possess higher potency at M_2 than at M_1 receptors. These M_2 selective antagonists also display higher affinity for M_2 than for M_3 receptors. There are no selective M_3 receptor antagonists available at present although in some studies 4-DAMP has been claimed to display a higher affinity for the M_3 than for the M_1 receptor (De Jonge *et al.*, 1986) and hexahydrosiladiphenidol has been described as a selective ligand for the muscarinic receptor subtype encoded by the m3 gene (Akiba *et al.*, 1988).

In the present study pirenzepine and methoctramine were able to clearly distinguish between the M_1, M_2 and M_3 subtypes of the muscarinic receptor. On the basis of the pirenzepine affinity estimates the muscarinic receptor of the PC12 cells was of the M_3 subtype. This conclusion was also supported by the affinity estimates obtained for hexahydroadiphenine and 4-DAMP.

In contrast to these data, however, affinity estimates for AF-DX 116, CPPS and methoctramine were not consistent with the presence of an M_3 receptor in the PC12 cells. The affinity estimates for these compounds in PC12 cells were similar to those obtained at the M_1 receptor and were between 0.54 and 0.76 log units higher than should have been obtained if the receptors present in the PC12 cells were of the M_3 subtype.

On the basis of the competition binding data it is clear that the receptors present in the PC12 cells cannot be considered as belonging to the M_2 subtype. In addition, the muscarinic receptors present in the PC12 cells did not appear to correspond to either the M_1 or M_3 receptor subtype although it may have been possible that the PC12 cells contained a heterogeneous population of both M_1 and M_3 receptors.

The kinetic studies may not have detected this since we are uncertain of the rate of dissociation of [^{3}H]-NMS from the M_1 receptor. However, in the competition studies all compounds produced mass action displacement isotherms in the PC12 cells and membranes indicating that the receptors in PC12 cells were homogeneous. It should of course be noted that a subtype of muscarinic receptor that comprised less than 10% of the total population of binding sites would not be observed in competition studies, although such a contribution would not have significantly affected the overall affinity estimates of the compounds.

The major factor arguing against the presence of M_1 receptors in the PC12 cells was the failure to label directly muscarinic receptors in the PC12 cells with [^{3}H]-pirenzepine at a concentration of 30 nM which was higher than the apparent K_d of pirenzepine for M_1 receptors in rat cortex.

On the basis of the kinetic, saturation and competition data obtained in the present study, it would appear that the muscarinic receptor present in the PC12 cells is different from the M_1, M_2 and M_3 muscarinic receptor subtypes that have been defined to date (De Jonge *et al.*, 1986; Mihm & Wetzel, 1987). It is not known whether the PC12 muscarinic receptor represents a physiologically expressed muscarinic receptor subtype or has arisen as a result of the transformed nature of the cell line. Studies with rat adrenal medulla from which the PC12 cell line was derived may help to resolve this issue. With respect to the transformed nature of the cell line it should be noted that in a wide range of other transformed cell lines, muscarinic receptors with the pharmacology of the M_1, M_2 and M_3 muscarinic

detail at present. When the pharmacology of these lines include the 1321 N1 human astrocytoma (Kunysz *et al.*, 1988) and SK-N-SH neuroblastoma (Fisher & Heacock, 1988) cell lines which contain M_3 receptors, and the NIE 115 (Lai & El-Fakahany, 1986) and SH-SY5Y (Sera *et al.*, 1988) neuroblastoma cell lines which contain both M_1 and M_2 receptors.

With regard to our current knowledge of the muscarinic receptor subtypes it should be noted that molecular biology studies have indicated at least four muscarinic receptor genes in the human genome (Peralta *et al.*, 1988) while five muscarinic receptor genes are present in the rat (Bonner *et al.*, 1987; 1988). The five genes encoding muscarinic receptors have been termed m1, m2, m3, m4 and m5 by Bonner *et al.* (1987, 1988).

The m1 and m2 genes encode the M_1 and M_2 receptors, respectively, identified in ligand binding and functional studies (Kubo *et al.*, 1986; Peralta *et al.*, 1987). The m3 gene may encode the M_3 receptor since the gene product has been predicted, although not directly demonstrated, to possess a MW of 90,000 (Kerlavage *et al.*, 1987) which is identical to that of the M_3 receptor identified in binding studies (Fisher & Heacock, 1988).

The pharmacology of the muscarinic receptors encoded by the m4 and m5 genes is not known in receptor subtypes have been detected. These cell receptors is better known it will be interesting to compare them with the PC12 cell muscarinic receptors. In this respect it should be noted that the pharmacology of the muscarinic receptor present in the PC12 cells is identical to that of the muscarinic receptors expressed by the NG108-15 cell line (Michel *et al.*, 1989). In Northern blots, Peralta *et al.* (1987) have demonstrated that NG108-15 cells express messenger RNA for the HM_3 muscarinic receptor, which is homologous to the m4 receptor described by Bonner *et al.* (1987). Given the similar pharmacology of the muscarinic receptors in the PC12 and NG108-15 cells it is possible that PC12 cells express the muscarinic receptor encoded by the m4 gene. The use of the cDNA probes for the 5 muscarinic receptor genes may help to delineate the subtype of muscarinic receptor present in the PC12 cell.

In conclusion then, the present data have demonstrated that based upon kinetic, saturation and competition ligand binding studies the muscarinic receptor present in the PC12 cells is unlike the M_2 muscarinic receptor and also demonstrates significant differences from both the M_1 and the M_3 receptors. Further study will be required to elucidate the exact nature of this receptor in order to determine if it represents an M_4 receptor.

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5-HT_1-like receptors requiring functional cyclo-oxygenase and 5-HT_2 receptors independent of cyclo-oxygenase mediate contraction of the human umbilical artery

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1 The interactions between 5-hydroxytryptamine (5-HT) and the antagonists ketanserin, methysergide and phentolamine were studied in isolated preparations of human umbilical artery (HUA) at physiological oxygen tension ($Po_2 \sim 15$ mmHg) and at high Po_2 (~ 120 mmHg).

2 At physiological Po_2 ketanserin, methysergide and phentolamine behaved as silent competitive antagonists of the 5-HT-induced contraction of HUA. pA_2 values calculated by Schild analysis were 8.92, 8.52 and 6.37, respectively.

3 At high Po_2, 5-HT-induced contractions were antagonised in a biphasic manner by ketanserin (0.1 μM); the response to low but not to high concentrations of 5-HT was resistant to blockade by ketanserin. The ketanserin-resistant component was abolished following cyclo-oxygenase inhibition by indomethacin (1 μM).

4 At high Po_2, methysergide behaved as a partial agonist. Methysergide-induced contractions were inhibited but not abolished by indomethacin, and resistant to 5-HT_2 receptor and α_1-adrenoceptor blockade.

5 At high Po_2 the component of the response to 5-HT mediated by the ketanserin-resistant receptor was mimicked by the selective 5-HT_1-like receptor agonist 5-carboxamidotryptamine (5-CT): 5-CT was 7 fold more potent than 5-HT.

6 At high Po_2 the component of the response to 5-HT mediated by the ketanserin-resistant receptor was antagonised by phentolamine and the selective α_2-adrenoceptor antagonist Wy 26703.

7 These results suggest that (i) at physiological Po_2 5-HT_2 receptors almost exclusively mediate contractions induced by 5-HT, and (ii) at high Po_2 the agonist potency order of 5-CT > 5-HT > methysergide suggests that ketanserin-resistant responses are mediated by 5-HT_1-like receptors which require functional cyclo-oxygenase.

Introduction

We have previously shown that, *in vitro*, under conditions which mimic the arterial blood-gas status of the human umbilical artery (HUA) *in utero*, 5-hydroxytryptamine (5-HT) causes contraction via a receptor which could be described as 5-HT_2 (McGrath *et al.*, 1985). The criteria for this classification relied upon rather non-selective antagonists: methysergide which is non-selective between 5-HT receptor sub-types, and phentolamine which, besides being a non-selective α-adrenoceptor antagonist has micromolar affinity for both peripheral 5-HT_2 receptors (e.g. rabbit aorta, Apperley *et al.*, 1976) and 5-HT_1-like receptors (e.g. dog saphenous vein, Humphrey, 1978). A further examination of the receptor in the HUA was therefore necessary in order to address the guidelines for 5-HT receptor classification, which have more recently been published (Bradley *et al.*, 1986a), central to which are the use of the selective 5-HT_1 receptor agonist 5-carboxamidotryptamine, and the selective 5-HT_2 receptor antagonist ketanserin.

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A second question we wished to address relates to how varying the oxygen tension (Po_2) of the solution bathing the isolated arterial strips might influence the reactivity of the smooth muscle. Under the normally low level of oxygen ($Po_2 \sim 15$ mmHg; Wulf, 1964) the receptor population appears to be homogeneous (McGrath *et al.*, 1985). We have since established that increasing the Po_2 induces the isolated HUA to contract by cyclo-oxygenase products (McGrath *et al.*, 1986; MacLennan *et al.*, 1988b); therefore, we wished to examine how cyclo-oxygenase products might influence the expression of the 5-HT_2 receptor. In the present study we showed that increasing the Po_2 above the physiological level had no apparent effect on 5-HT_2 receptor expression in the HUA. However, on elevating the Po_2 a second population of 5-HT receptors was 'revealed' which could be classified as 5-HT_1-like.

Some of these results have been presented previously at meetings of the British Pharmacological Society (McGrath & MacLennan, 1986; MacLennan & McGrath, 1986).

Methods

Lengths of umbilical cord (5–30 cm) were cut from the placental portion as soon as practically possible after delivery but normally within 45 min. The cords were placed in de-oxygenated Krebs-bicarbonate saline (composition, mM: NaCl 119, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.1) at 4°C for up to 48 h, before being used. The saline was de-oxygenated by pre-gassing with 8% CO_2 in nitrogen.

Arteries were dissected free of the surrounding Wharton's jelly in de-oxygenated Krebs solution, to reduce the oxygen tension as far as possible. Longitudinal strips of artery, length 1–1.5 cm, were suspended within 40 ml organ baths containing Krebs saline at 37°C under a force of 1 g. Isometric force was monitored using Grass FT03c transducers and a Grass model 7 polygraph. Unpublished studies suggest the endothelium is not functional in this tissue.

In all experiments the tissues were equilibrated for 2–2.5 h before any experimentation, gassed with 2.5% O_2, 8% CO_2, balance N_2, to mimic the gas tensions and pH of umbilical arterial blood, *in utero*, i.e. Po_2, 15 mmHg; Pco_2 50 mmHg; pH 7.28 (Wulf, 1964; Pearson, 1976). Pco_2 and pH of the organ baths were monitored by withdrawing samples and analysing them on a blood-gas analyser (Instrumentation Laboratories, model 1302). Po_2 was continuously monitored by use of an oxygen electrode (Instrumentation Laboratories, model 1302) and meter (Strathkelvin Instruments, model 781). The oxygen electrode was incorporated into a specially designed organ bath via a side port. It was established that the Po_2 measured in one bath was within ± 3 mmHg of the Po_2 found for the Krebs solution in any of the three other baths used (when aerated with the same gas mixture, and at a similar rate), throughout the range tested −0 to 600 mmHg.

Agonists and antagonists were studied at two levels of Po_2: (i) physiological Po_2, ~15 mmHg (2.5% O_2), (ii) high Po_2, ~120 mmHg (17% O_2). The values in parentheses are the % O_2 composition of the gas mixtures used to aerate the Krebs solution. The remaining gas was composed of 8% CO_2/balance N_2. The gas mixtures were made up in Douglas bags using the rotameters from an anaesthetics trolley. The Krebs solution was gassed with these mixtures by a small aquarium pump.

Concentration-response curves (CRCs) to agonists were constructed cumulatively, by increasing the bathing solution concentration by $0.5 \log_{10}$ increments at intervals when the preceding response had reached a plateau – this was at approximately 3 min intervals for all agonists. Response (% of maximum response, g tension or % of a prior 50 mM KCl contraction) was plotted against log(agonist concentration). pD_2 values were calculated as $-\log(EC_{50})$, where EC_{50} is the concentration of agonist which gives 50% of the maximum response.

The potency of test agonists was compared to that of 5-HT by first constructing a CRC to 5-HT followed by one for the test agonist in the same preparation. The potency of the test agonist was calculated as the ratio of EC_x (test agonist), over EC_x (5-HT), where x is the level of response, e.g. EC_{50}, EC_{75}, as given in the text. Where the potency of 5-carboxamidotryptamine (5-CT) relative to 5-HT was examined, the calculated potency was corrected for the time-related change in sensitivity of the tissue by division by the concentration-ratio of a control preparation in which two successive CRCs to 5-HT were constructed. The relative potencies are given as the geometric mean and 95% confidence limits.

Antagonist experiments

Low Po_2, *~15 mmHg* Four preparations from the one artery were used. In each preparation CRCs to 5-HT or 5-CT were constructed as already described. After washout of the drug (60 min) different concentrations of antagonist were added to three preparations and allowed to equilibrate for 30 min before again constructing a CRC to 5-HT (or 5-CT). One preparation therefore acted as a control to assess the change in sensitivity (with time), which was approximately two fold. Response was calculated as the % of the 1st curve's maximum response and plotted

Table 1 Parameters of the Schild plot for the interaction of antagonists with 5-hydroxytryptamine (5-HT) and 5-carboxamidotryptamine (5-CT) in the human isolated umbilical artery at physiological Po_2 (~15 mmHg)

Antagonist	*Agonist*	pA_2	*Slope*	n
Methysergide	5-HT	8.52 (8.32–8.72)	0.94 (0.76–1.12)	6
Phentolamine	5-HT	6.37 (5.88–6.86)	1.04 (0.82–1.26)	6
Ketanserin	5-HT	8.92 (8.70–9.14)	0.91 (0.60–1.22)	6
	5-CT	8.77 (8.39–9.15)	1.19 (0.68–1.70)	5
Ketanserin (+1 μM indomethacin)	5-HT	8.94 (8.22–9.66)	0.99 (0.66–1.32)	4
	5-CT	9.05 (8.58–9.52)	1.00 (0.75–1.25)	4

Values are the mean (95% confidence limits) of *n* estimates.

against log(5-HT concentration) (log[5-HT]). For each preparation the concentration-ratio (CR) was calculated as the ratio of the EC_{50} of 5-HT (+ antagonist), over the EC_{50} for 5-HT from the control (1st) curve. The CR was corrected for time-related change in sensitivity by division by the CR of the control preparation. A Schild plot was constructed with the CRs: log(CR − 1) (ordinate scale) was plotted against log(antagonist concentration) (abscissa scale) (Arunlakshana & Schild, 1959). A line of best-fit was found for the points by linear regression (least squares) which gave the slope and an estimate of the pA_2 as the intercept of the regression line with the line, log (CR − 1) = 0. In each estimate of a pA_2, 4 to 6 preparations were used. The mean values of the slope of the regression line and estimated pA_2 are presented together with their respective 95% confidence limits.

High Po_2, *~120 mmHg* In any one preparation a single concentration of antagonist was studied at higher than physiological Po_2 (~120 mmHg) as follows: CRC to 5-HT was constructed at Po_2 ~ 15 mmHg. After washout (60 min) the Po_2 was increased to ~120 mmHg which sometimes caused a contraction, but which was always transient. The antagonist was added to the bath for 30 min before a 2nd CRC to 5-HT was constructed. A third CRC to 5-HT was constructed in the presence of the same antagonist concentration plus indomethacin (1 μM, 30 min). Response (% of 1st (low Po_2) maximum response) was plotted against log[5-HT]. Estimates of antagonist potency were made by calculating the log agonist concentration-ratio as described above. Log agonist CRs were calculated at the EC_{25} and EC_{75} levels in the presence and absence of indomethacin.

Methysergide was an agonist at high Po_2 but due to tachyphylaxis two successive CRCs (before and in the presence of the antagonist) could not be constructed. Antagonists were studied as follows: one strip acted as a control and antagonist(s) were added to other strips from the same artery. CRCs to the agonist were constructed simultaneously on all strips.

Drugs

The following drugs were used (source in parentheses): 5-hydroxytryptamine creatinine sulphate (Sigma); indomethacin (Sigma); methysergide bimaleate (Sandoz); (±)-pindolol (Sandoz); phentolamine mesylate (Ciba); prazosin hydrochloride (Pfizer); ketanserin tartrate (Janssen); 5-carboxamidotryptamine (Glaxo); buspirone hydrochloride (Bristol-Myers); Wy 26703 (N-methyl-N-(1,3,4,6,7,11b-hexahydro-2H-benzo-[a]-quinolizin-2-yl)-i-butanesulphonamide HCl, Wyeth). All drugs, with the exception of indomethacin, were dissolved in distilled water. Indomethacin was initially dissolved in absolute ethyl alcohol and diluted further in distilled water. The concentration of vehicle in the organ bath (0.8 mM) did not influence tissue responsiveness.

Statistics

Statistical comparisons of the means of groups of data were made by use of Student's *t* test for paired or unpaired data, where appropriate. A level of probability of $P < 0.05$ was taken to indicate statistical significance.

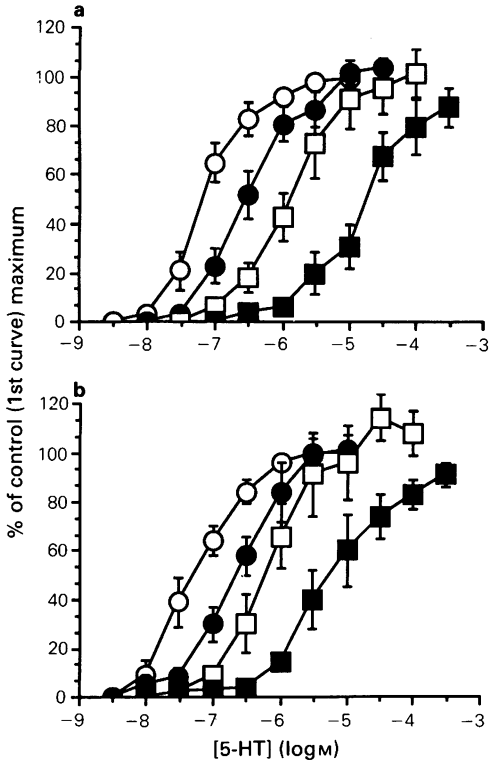

Figure 1 Log concentration-response curves (CRCs) to 5-hydroxytryptamine (5-HT) at low Po_2 in the presence of (a) methysergide ($n = 6$), and (b) phentolamine ($n = 6$). CRCs to 5-HT were constructed twice in each of four preparations from the same artery. In three of the four preparations the second CRC was repeated in the presence of one concentration of antagonist. In the fourth (control) preparation the 1st (○) and second CRCs were constructed without antagonist in order to assess the change in sensitivity to 5-HT with time, which was in (a) 2.0 ± 0.7 fold and in (b) 1.8 ± 0.5 fold. (a) [Methysergide] (●) 10 nM; (□) 100 nM; (■) 1000 nM; $Po_2 = 11 \pm 2$ mmHg. (b) [Phentolamine] (●) 1 μM; (□) 10 μM; (■) 100 μM; $Po_2 = 16 \pm 2$ mmHg. Each point represents the mean and vertical lines show s.e.mean.

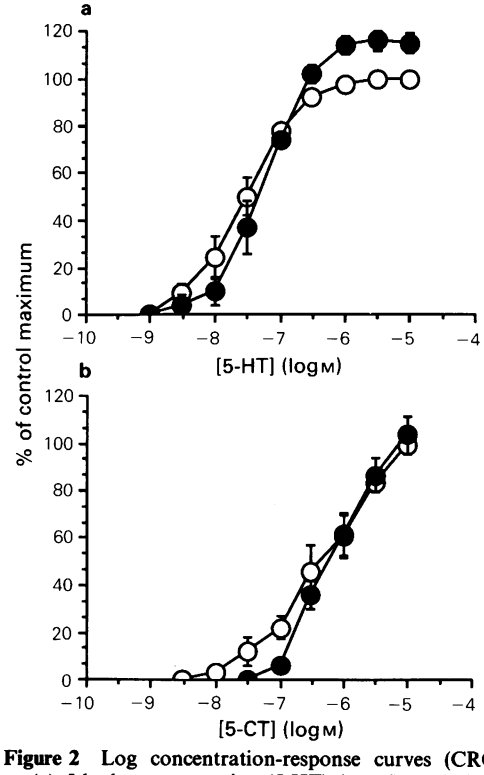

Figure 2 Log concentration-response curves (CRCs) to (a) 5-hydroxytryptamine (5-HT) ($n = 6$), and (b) 5-carboxamidotryptamine (5-CT) ($n = 5$), at low Po_2 in the presence (●) and absence (○) of indomethacin (1 μM). The protocol was to construct a CRC to either 5-HT or 5-CT before and after exposure (30 min) to indomethacin. Indomethacin had no effect on baseline tension. (a) $Po_2 = 16 \pm 2$ mmHg, (b) $Po_2 = 13 \pm 2$ mmHg.

Results

Antagonist studies at physiological Po_2

The parameters of the Schild plots obtained from the interaction between the antagonists employed in this study, and the agonists 5-HT and 5-CT, are summatised in Table 1.

Methysergide and phentolamine were found to be silent competitive antagonists of the 5-HT-induced contraction of the HUA, since antagonism was surmountable and the slopes of the Schild plots were not significantly different from unity (Figure 1). pA_2 values for methysergide and phentolamine were 8.52 and 6.37, respectively (mean values). The concentration-response curves appeared, in a qualitative manner, to be displaced in parallel fashion by these antagonists. This was confirmed quantitatively since the log(agonist concentration-ratios) calculated at the level of the EC_{25} and EC_{75} at each antagonist concentration, were not significantly different; for example, in the presence of methysergide (1 μM) the log agonist CR at the EC_{25} and EC_{75} levels were 2.26 ± 0.2 and 2.62 ± 0.28, respectively (mean ± s.e.mean). In the presence of phentolamine (100 μM) the log agonist CR at the EC_{25} and EC_{75} levels were 2.41 ± 0.24 and 2.50 ± 0.28, respectively.

The possible role of cyclo-oxygenase products was investigated. Indomethacin (1 μM) alone did not shift the concentration-response curves to 5-HT or 5-CT

(Figure 2). The control concentration-response curve to 5-CT appeared to be slightly biphasic and the responses to lower concentrations of 5-CT were blocked by indomethacin: the 5-CT concentration-response curve thus became monophasic in the presence of indomethacin with a resulting steepening of the concentration-response curve. At higher concentrations, the maximum response to 5-HT was significantly increased by $18 \pm 4\%$ in the presence of indomethacin. However, in paired control tissues not exposed to indomethacin, a smaller but significant increase of the maximum of $7 \pm 3\%$ was obtained (not shown). Indomethacin (1 μM) caused no change of the resting baseline tension.

The interaction between ketanserin and both 5-HT and 5-CT was investigated in the absence and presence of indomethacin (1 μM), (Figure 3 and Table 1).

5-Hydroxytryptamine Without indomethacin present ketanserin caused a rightward shift of the 5-HT concentration-response curve that was parallel only at concentrations (of 5-HT) which produced greater than (approximately) 30% of the maximum response (Figure 3a), i.e. at physiological Po_2 there was a small component of the response to 5-HT that was resistant to ketanserin. Log agonist CRs were calculated from 0.1 μM ketanserin. At the EC_{25} the mean log agonist CR (1.33 ± 0.38) was smaller than at either the EC_{50} (1.90 ± 0.14) or EC_{75} (1.89 ± 0.23), but the difference was not significant. The Schild plot, which is based on concentration-ratios calculated at the EC_{50}, indicated that ketanserin was a competitive antagonist of 5-HT at physiological Po_2 since the slope of the regression line (0.91, mean) was not significantly different from unity. The estimated pA_2 for ketanserin was 8.92 (mean).

In the presence of indomethacin (1 μM) ketanserin displaced the 5-HT concentration-response curve in a parallel manner at all concentrations (Figure 3b). The Schild plot had a slope of 0.99 (mean) and the estimated pA_2 for ketanserin against 5-HT was 8.94 which was not significantly different from the pA_2 obtained in the absence of indomethacin, 8.92. The broken line of Figure 3a (which is the concentration-response curve to 5-HT in the presence of 0.1 μM ketanserin plus 1 μM indomethacin – from Figure 3b) highlights the small component of the response to 5-HT that was resistant to 0.1 μM ketanserin but sensitive to indomethacin.

5-Carboxamidotryptamine Ketanserin (3 nM–30 nM) caused biphasic shifts of the 5-CT concentration-response curve similar to those described for 5-HT, i.e. the responses to the lowest concentrations of 5-CT were resistant to blockade by ketanserin but were sensitive to indomethacin. Nevertheless the

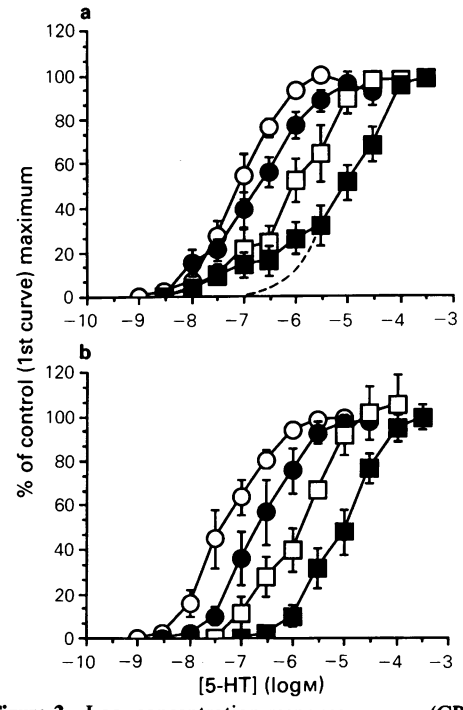

Figure 3 Log concentration-response curves (CRCs) to 5-hydroxytryptamine (5-HT) at low Po_2 (14 ± 2 mmHg), (a) in the presence of ketanserin ($n = 6$), and (b) in the presence of ketanserin plus indomethacin (1 μM) ($n = 4$). CRCs to 5-HT were constructed twice in each of four preparations from the same artery. In three of the four preparations the second CRC was repeated in the presence of one concentration of ketanserin; (●) 1 nM; (□) 10 nM; (■) 100 nM. In the fourth (control) preparation the 1st (○) and second CRCs were constructed without antagonist in order to assess the change in sensitivity to 5-HT with time, which was in (a) 1.8 ± 0.3 fold and in (b) 1.5 ± 0.5 fold. The broken line in (a) is the CRC to 5-HT in the presence of 100 nM ketanserin plus 1 μM indomethacin (from b). Each point represents the mean and vertical lines show s.e.mean.

Schild plot, based on concentration-ratios calculated at the EC_{50}, had a slope (1.19) not significantly different from unity. In this set of experiments the slopes of two of five of the individual Schild plot regression lines were considerably greater than unity (1.57 and 1.78), which was due to the rather low concentration-ratios obtained at the lowest antagonist concentration. In the presence of indomethacin (1 μM), ketanserin (3–30 nM) caused a parallel shift of the 5-CT concentration-response curve. The estimated pA_2 values for ketanserin against 5-CT were not significantly different in the presence (9.05) (mean) or absence (8.77) of indomethacin. These pA_2 values were not significantly different from the

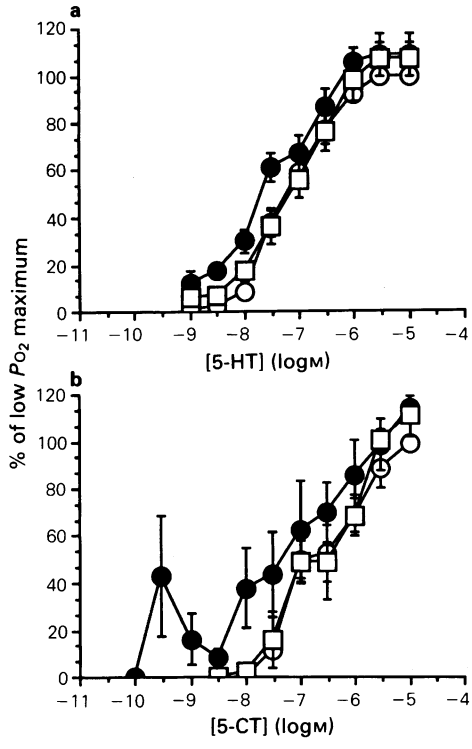

Figure 4 Log concentration-response curves (CRCs) to (a) 5-hydroxytryptamine (5-HT) ($n = 7$) and (b) 5-carboxamidotryptamine (5-CT) ($n = 5$) at low Po_2 (□), and at high Po_2 in the presence (○) and absence (●) of indomethacin (1 μM). The protocol was to construct a CRC to either 5-HT or 5-CT at low Po_2. Following washout the Po_2 was increased and a 2nd CRC constructed in the presence or absence of indomethacin. In those tissues exposed to indomethacin, the drug was added 30 min before raising the Po_2. Response (ordinate scale) is expressed as a % of the maximum response to 5-HT or 5-CT at low Po_2. Each point represents the mean and vertical lines show s.e.mean. (a) Low $Po_2 = 8 \pm 3$ mmHg, high $Po_2 = 118 \pm 3$ mmHg. (b) Low $Po_2 = 12 \pm 3$ mmHg, high $Po_2 = 119 \pm 4$ mmHg.

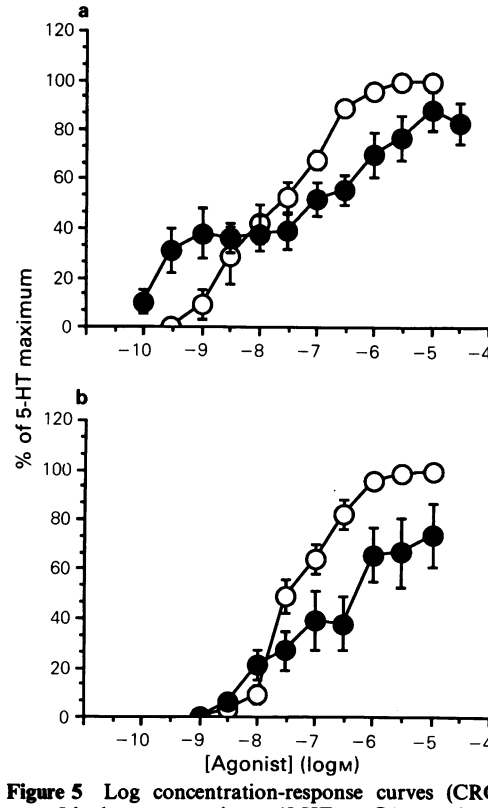

Figure 5 Log concentration-response curves (CRCs) to 5-hydroxytryptamine (5-HT, ○) and 5-carboxamidotryptamine (5-CT, ●) at (a) low Po_2 (13 ± 1 mmHg) ($n = 5$); (b) high Po_2 (123 ± 1 mmHg) ($n = 6$). The protocol was to construct a CRC to 5-HT followed by one to 5-CT in each preparation. Response (ordinate scale) is expressed as a % of the maximum response to 5-HT. Each point represents the mean and vertical lines show s.e.mean.

respective values (8.94 and 8.92) in the presence and absence of indomethacin, with 5-HT as the agonist (Table 1).

Effect of raising the Po_2

The effects, *per se*, of increasing the Po_2 from the physiological level (~15 mmHg) to high Po_2 (~120 mmHg) on the response to 5-HT and 5-CT were investigated (Figure 4). Concentration-response curves were first constructed at physiological Po_2 then re-constructed at high Po_2 in the presence or absence of indomethacin (1 μM). In the absence of indomethacin increasing the Po_2 contracted the HUA. This contraction was $35 \pm 10\%$ of the maximum contraction to 5-HT but was not maintained and the change in baseline tension before the construction of a 2nd concentration-response curve was $4 \pm 3\%$, which was not significant. Raising the Po_2 failed to induce a contraction in the presence of indomethacin.

5-Hydroxytryptamine Increasing the Po_2 caused a significant leftward shift of the 5-HT concentration-response curve. Assessed at the EC_{50} level the shift was 3.23 fold (geometric mean, 95% confidence limits 1.1–5.3). In paired preparations incubated with indomethacin increasing the Po_2 did not significantly increase (or decrease) the potency of 5-HT.

5-Carboxamidotryptamine Increasing the Po_2 had a significant facilitating effect on the response to 5-CT, which, at high Po_2, was found to be distinctly biphasic. The 1st phase of the response lay between 0.1 nM and 10 nM, and the 2nd phase between 10 nM and 10 μM. The 1st phase of the 5-CT response was very variable between different preparations, both in terms of the magnitude of the response and the degree of fade of the response. At the different levels of Po_2 the 5-CT concentration-ratios were calculated at the EC_{25} and EC_{75} levels which were taken as parameters of the 1st and 2nd phases respectively. Increasing the Po_2 caused a significant leftward shift: at the EC_{25} level this was by 24.5 (6.8–89.1) fold and at the EC_{75} level the shift was 6.9 fold (1.9–25.1) which was smaller than at the EC_{25} but was still significant. As was found for 5-HT, increasing the Po_2 in the presence of indomethacin had no significant effect on the concentration-response curve to 5-CT.

The potency of 5-CT relative to 5-HT was examined at both physiological Po_2 and at high Po_2 by first constructing a concentration-response curve to 5-HT followed by one to 5-CT. The experiments at the two levels of Po_2 were examined in two separate series of experiments (Figure 5 and Table 2). The relative potencies of 5-HT and 5-CT were calculated at the EC_{25} and EC_{75}. As described above, the predominant difference between the two levels of Po_2 was the distinctly biphasic nature of the 5-CT concentration-response curve at high Po_2. These experiments showed that (i) at physiological Po_2 5-CT was approximately equipotent to 5-HT at the EC_{25} but 5 fold less potent at the EC_{75}, (ii) at high Po_2 5-CT was 7 fold more potent than 5-HT at the EC_{25} but 4 fold less potent at the EC_{75}.

Antagonist studies at high Po_2 (*~ 120 mmHg*)

The interactions between 5-HT and the different receptor antagonists, in the presence and absence of indomethacin (1 μM) was investigated by employing only a single concentration of each of the antagonists (Figure 6). Log agonist CRs were calculated at the level of the EC_{25} and EC_{75}. These levels were chosen as parameters of the ketanserin-resistant and ketanserin-sensitive components of the concentration-response curve to 5-HT at high Po_2. Log agonist CRs in the absence and presence of indomethacin were calculated with reference to the following control concentration-response curves: in the absence of indomethacin the control curve was that for 5-HT at high Po_2 (non-paired preparations, from Figure 4a); in the presence of indomethacin the control curve was to 5-HT at physiological Po_2 (same preparation). The rationale for the latter calculation was that the concentration-response curves to 5-HT at physiological Po_2, and at high Po_2 in the presence of indomethacin were not significantly different – there was no significant difference between the two curves at the level of the EC_{25} or EC_{75} (Figure 4a).

Ketanserin (0.1 μM) caused a distinctly biphasic shift of the 5-HT concentration-response curve at high Po_2. The log agonist CR calculated at the EC_{25} (0.62 ± 0.12) was significantly lower than at the EC_{75} (1.67 ± 0.18), indicating that at high Po_2 the response to lower concentrations of 5-HT was mediated by a ketanserin-resistant mechanism. The log agonist CR at the EC_{75} (1.67) was not significantly different from that calculated at physiological Po_2 (1.89 ± 0.23), indicating that at high Po_2 the response to higher concentrations of 5-HT was mediated by a ketanserin-sensitive mechanism. In the presence of indomethacin, ketanserin caused a parallel rightward displacement of the 5-HT concentration-response curve: the log agonist CRs at the EC_{25} (2.12 ± 0.14) and EC_{75} (1.97 ± 0.10) levels were not significantly different. Thus the ketanserin-resistant phase was mediated by an indomethacin-sensitive mechanism (Figure 6a).

At high Po_2 methysergide contracted the HUA. At 0.1 μM this contraction was $16 \pm 8\%$ of the maximum response to 5-HT. This contrasts with the lack of agonist action of methysergide at physiological Po_2. Methysergide (0.1 μM) caused a shift of the

Table 2 Comparison of the activities of 5-carboxamidotryptamine (5-CT) and 5-hydroxytryptamine (5-HT) at different oxygen tensions (Po_2) in the human isolated umbilical artery

	5-CT			*5-HT*		$\frac{EC_x\ 5\text{-}CT}{EC_x\ 5\text{-}HT}$		
Po_2 (mmHg)	EC_{25} (nM)	EC_{75} (μM)	*% of 5-HT maximum*	EC_{25} (nM)	EC_{75} (μM)	EC_{25}	EC_{75}	n
13 ± 1	13.2 (1.5–115)	1.1 (0.26–4.4)	81 ± 10	17.8 (12.6–25.1)	0.22 (0.09–0.51)	0.64 (0.04–9.1)	5.4 (1.3–21.4)	5
123 ± 1	0.25 (0.06–1.0)	0.58 (0.1–2.6)	88 ± 8	3.3 (0.9–12.3)	0.1 (0.05–0.19)	0.15 (0.02–1.4)	3.5 (1.6–7.4)	6

Values are geometric mean (95% confidence limits) and mean $\pm$ s.e.mean, from *n* estimates.
Relative potencies were calculated at the EC_{25} and EC_{75}.

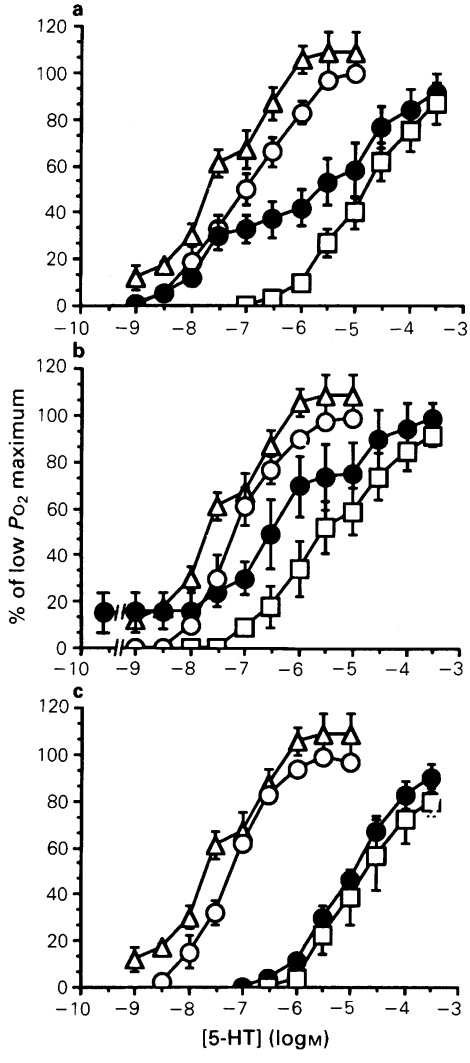

Figure 6 Interaction between 5-hydroxytryptamine (5-HT) and (a) ketanserin (0.1 μM), (b) methysergide (0.1 μM) and (c) phentolamine (100 μM) at high Po_2 in the presence and absence of indomethacin (1 μM). In each experiment (a, b and c) a CRC to 5-HT at low Po_2 ($\sim$15 mmHg), was first constructed (○). Following washout the Po_2 was then increased (to $\sim$120 mmHg), antagonist was added and a CRC to 5-HT repeated (●). A third CRC to 5-HT was then constructed in the presence of antagonist plus indomethacin (1 μM) at the high Po_2 (□). A control CRC to 5-HT at high Po_2 (△), (from non-paired experiments, Figure 4a) is shown in each figure. The extreme left hand symbol (Figure 4b) indicates the tone induced by methysergide. Responses were calculated as a % of the maximum response to 5-HT at low Po_2. (a) [Ketanserin] = 0.1 μM; low Po_2 = 13 $\pm$ 2 mmHg; high Po_2 = 117 $\pm$ 1 mmHg (n = 8). (b) [Methysergide] = 0.1 μM; low Po_2 = 14 $\pm$ 2 mmHg; high Po_2 = 123 $\pm$ 3 mmHg (n = 6). (c) [Phentolamine] = 100 μM; low Po_2 = 14 $\pm$ 2 mmHg; high Po_2 = 127 $\pm$ 2 mmHg (n = 4). Each point represents the mean and vertical lines show s.e.mean. For clarity, error bars are omitted at some points.

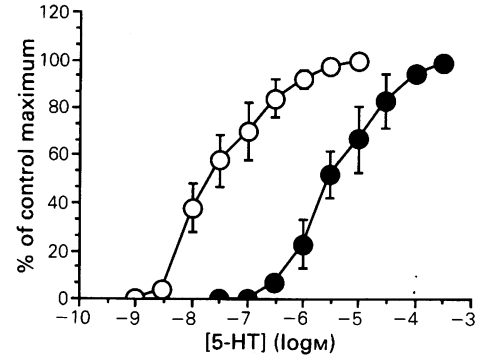

Figure 7 Log concentration-response curves (CRCs) to 5-hydroxytryptamine (5-HT) (n = 4) at high Po_2 (119 $\pm$ 1 mmHg) before (○) and after (●) exposure to Wy 26703 (10 μM). Each point represents the mean and vertical lines show s.e.mean when these are greater than the height of the symbols.

5-HT concentration-response curve which, in descriptive terms, was biphasic. However, log agonist CRs at the EC_{25} (1.65 $\pm$ 0.51) and EC_{75} (1.58 $\pm$ 0.49) were not significantly different. After incubation with indomethacin, methysergide (0.1 μM) failed to contract the HUA and the displacement of the 5-HT concentration-response curve was parallel; the log agonist CR at the EC_{25} (1.48 $\pm$ 0.23) and EC_{75} (1.90 $\pm$ 0.18) levels were not significantly different (Figure 6b).

The agonist action of methysergide at high Po_2 was investigated further. Cumulative concentration-response curves were constructed to methysergide. The pD_2 was 6.82 $\pm$ 0.12. The pD_2 for 5-HT at high Po_2 was 8.01 $\pm$ 0.26 (non-paired preparations, from Figure 5b). Thus 5-HT was approximately 15 fold more potent than methysergide. The maximum responses to methysergide and 5-HT (relative to a prior contraction induced by 50 mM KCl) were 64 $\pm$ 18% and 222 $\pm$ 26%, respectively. Neither ketanserin (30 nM) nor the selective α_1-adrenoceptor antagonist prazosin (30 nM) antagonised the response to methysergide. Prazosin (30 nM) did not antagonise either the 1st or 2nd phase of the 5-HT concentration-response curve at high Po_2. Indomethacin (1 μM) significantly reduced the maximum response to methysergide from 64 $\pm$ 18% to 43 $\pm$ 11% (relative to a prior contraction induced by 50 mM KCl).

The non-selective α-adrenoceptor antagonist phentolamine (100 μM) displaced the 5-HT

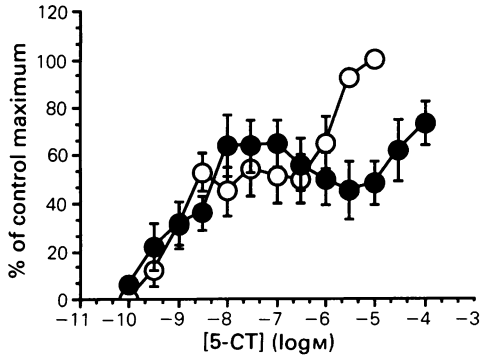

Figure 8 Log concentration-response curves to 5-carboxamidotryptamine (5-CT) ($n = 5$) at high Po_2 (118 ± 4 mmHg) before (○) and after (●) exposure to ketanserin (0.1 μM). Each point represents the mean and vertical lines show s.e.mean.

concentration-response curve in a parallel manner, either in the absence or presence of indomethacin (Figure 6c). In the absence of indomethacin the log agonist CRs at the EC_{25} and EC_{75} were 2.69 ± 0.10 and 2.57 ± 0.22 respectively. In the presence of indomethacin the log agonist CRs at the EC_{25} and EC_{75} were 2.57 ± 0.39 and 2.57 ± 0.33 respectively.

In the absence of indomethacin, the selective α_2-adrenoceptor antagonist Wy 26703 (10 μM) caused a parallel shift of the 5-HT concentration-response curve (Figure 7): log agonist CRs at the EC_{25} (2.16 ± 0.24) and EC_{75} (1.96 ± 0.33) were not significantly different. Thus the ketanserin-resistant phase of the 5-HT concentration-response curve could be antagonised by the α_1/α_2-adrenoceptor antagonist phentolamine or by the selective α_2-antagonist Wy 26703.

The interaction between 5-CT and ketanserin at high Po_2 was investigated. Ketanserin (0.1 μM) antagonised the 2nd phase (log agonist CR 2.30) but not the 1st phase (log agonist CR 0.2 ± 0.3) of the 5-CT concentration-response curve (Figure 8). At the EC_{75} log agonist CR was calculated only from the mean EC_{75} value, since in the presence of ketanserin contractions in some preparations did not reach the level of the EC_{75}.

The ligands buspirone (0.1 μM) and (±)-pindolol (3 μM) had no affinity (at the given concentrations) for either receptor mediating the response to 5-CT at high Po_2; at these concentrations neither ligand contracted the HUA or displaced the 1st or 2nd phase of the 5-CT concentration-response curve.

Discussion

Under conditions of physiological Po_2 the antagonists ketanserin, methysergide and phentolamine interact with 5-HT in a simple, competitive manner suggesting that the 5-HT-induced contraction of the HUA is mediated by a homogeneous population of receptors. At higher Po_2, however, the nature of the interaction of these antagonists with 5-HT suggests that a heterogeneous population of 5-HT receptors exists.

It is appropriate at this point to rule out some possible mechanisms of action of 5-HT in the HUA. In other vascular smooth muscle tissues 5-HT can act directly (Apperley *et al.*, 1976) or indirectly (Innes, 1962; Humphrey, 1978; Humphrey *et al.*, 1983) at α-adrenoceptors. In the HUA there is only a small functional population of prazosin-sensitive, α_1-adrenoceptors that require a higher than physiological Po_2 for their expression (MacLennan, 1986). However, the effects of 5-HT at high Po_2 were not antagonised by prazosin. The general consensus of opinion is that the HUA is not innervated (Spivack, 1943; Roach, 1972), so that we doubt that 5-HT could possibly displace stored noradrenaline. This evidence rules out a direct or indirect action of 5-HT at α-adrenoceptors.

We have not examined a possible contribution of 5-HT_3 receptors in the 5-HT-induced contraction of the HUA but, as described above, the HUA is not innervated and as yet peripheral 5-HT_3 receptors have been described only on neurones (see Bradley *et al.*, 1986a; Humphrey & Feniuk, 1987). An involvement of 5-HT_3 receptors is therefore discounted.

At physiological Po_2 ketanserin, methysergide and phentolamine acted as simple competitive antagonists. A comparison of the affinities of these antagonists at the 5-HT_2 recognition site in radiolabelled ligand binding studies (Hoyer, 1988) and the pA_2 values from the present study in HUA are given in Table 3. It is clear that there is a marked similarity of the affinities of the antagonists for the 5-HT_2 recognition site in the CNS and for the receptor in the HUA, suggesting that they are the same. Hence, the receptor for 5-HT in the HUA at physiological Po_2 can be classified as a 5-HT_2 receptor and confirms our previous study (McGrath *et al.*, 1985).

Further (if somewhat indirect) evidence for describing the receptor as 5-HT_2 comes from the similarity of the receptor in the HUA and that in the rabbit aorta, since other studies have shown an identity between the 5-HT receptor in the rabbit aorta and the 5-HT_2 recognition site in binding studies (Humphrey *et al.*, 1982; Maayani *et al.*, 1984). The receptors in the HUA and rabbit aorta appear similar both in terms of estimated antagonist affinities (ketanserin, methysergide and phentolamine – Table 3) and of the relative potency of 5-HT and 5-CT in these preparations. In the HUA, at physiological Po_2, 5-CT was approximately 5 fold less potent than 5-HT (Table 2) while in the rabbit aorta

Table 3 Comparison of the affinities of the antagonists ketanserin, methysergide and phentolamine for the 5-HT_2 binding site in brain tissue (rat cortex) and the receptor for 5-HT in the human umbilical artery (HUA) at physiological Po_2 and rabbit aorta

Antagonist	*Binding site*[1]	*Receptor in HUA*[2]	*Receptor in rabbit aorta*[3]
Ketanserin	8.86	8.92 (8.70–9.14)	8.67 (8.38–8.95)
Methysergide	8.57	8.52 (8.32–8.72)	8.49 (7.85–9.14)
Phentolamine	6.06	6.37 (5.69–7.05)	6.21 (5.52–6.92)

[1] Values are $-\log$ D (mean) from Hoyer (1988).
[2] pA_2 values estimated from a Schild analysis and are the mean (95% confidence limits).
[3] pA_2 values estimated from a Schild analysis and are the mean (95% confidence limits), from Apperley *et al.* (1976) and Feniuk *et al.* (1985).

5-CT was 26 fold less potent than 5-HT (Feniuk *et al.*, 1985).

Increasing the Po_2 from physiological levels ($\sim$15 mmHg) to a higher level ($\sim$120 mmHg) resulted in an increased potency of the agonists 5-HT and 5-CT. This effect was particularly evident with 5-CT whose concentration-response curve became distinctly biphasic at high Po_2. For either agonist this increased potency was due to an involvement of cyclo-oxygenase products since indomethacin was able to reverse it.

At high Po_2, contractions induced by 5-HT and 5-CT were mediated via both 'ketanserin-resistant' and 'ketanserin-sensitive' receptors, i.e. 5-HT receptors in the HUA are heterogeneous. The ketanserin-sensitive receptor is undoubtedly the 5-HT_2 receptor: at the level of the EC_{75} (taken as the mid-point location of the 2nd, ketanserin-sensitive, phase) the log agonist CR for either 5-HT or 5-CT in the presence of ketanserin is consistent and similar to that at physiological Po_2.

A quantitative comparison of the potency of the two tryptamines was made at the level of the EC_{25}, as an estimate of their relative potency at the 'ketanserin-resistant' receptor, and at the EC_{75} as an estimate of their potency at the 5-HT_2 receptor. At the 1st (ketanserin-resistant) phase 5-CT was more potent than 5-HT, while at the 5-HT_2 receptor the converse was found. These two pieces of evidence, i.e. a resistance to blockade by ketanserin and the higher potency of 5-CT relative to 5-HT, suggest that the receptor through which low concentrations of 5-HT and 5-CT exert their effects can be described as '5-HT_1-like' in accordance with the guidelines of Bradley *et al.* (1986a).

This order of potency is similar to that found in other vascular smooth muscle preparations where the receptor has been described as 5-HT_1-like. For example, 5-CT is more potent than 5-HT at receptors mediating contraction of saphenous veins from dogs (Feniuk *et al.*, 1985) and rabbits (Martin *et al.*, 1988), and at other receptors that mediate relaxation of porcine vena cava (Trevethick *et al.*, 1986), cat jugular vein (Feniuk *et al.*, 1983) and at the smooth muscle receptor in rabbit jugular vein (Martin *et al.*, 1987). If the EC_{25} for 5-CT at high Po_2 (0.25 nM) is taken as an approximation of its affinity at the 5-HT_1-like receptor (i.e. the EC_{50} of the 1st phase), then 5-CT has an affinity at this receptor in the HUA greater than that found for each of the receptor types mentioned above.

A further similarity between the 5-HT_1-like receptors in the HUA and in other tissues is the partial agonist action of methysergide at high Po_2, where it was 15 fold less potent than 5-HT. This similarity extends only to the 5-HT_1-like receptors in tissues where the receptor-mediated response is contraction since, in tissues where relaxation is seen (see above), methysergide is a silent, albeit weak, antagonist. Thus, an agonist action of methysergide at 5-HT_1-like receptors has been obtained in dog saphenous vein (Apperley *et al.*, 1980), rabbit basilar artery (Bradley *et al.*, 1986b) and rabbit saphenous vein (MacLennan *et al.*, 1988a). In other tissues which may contain a heterogeneous population of 5-HT_2 and 5-HT_1-like receptors, for example dog coronary (Brazenor & Angus, 1981; 1982) and basilar arteries (Muller-Schweinitzer, 1980), methysergide is a weak agonist, but it has not been clearly shown which receptor mediates this agonism.

Since the response to methysergide was partially sensitive to indomethacin, this suggests an action at the 5-HT_1-like receptor. However, in contrast with the additional responses to 5-HT and 5-CT, although the agonist action of methysergide required a higher than physiological Po_2, this was only partially antagonised by indomethacin. A possible involvement of 5-HT_2 receptors and α_1- adrenoceptors was ruled out, as the agonist response was resistant to the selective antagonists ketanserin and

prazosin. However, some further O_2-induced factor may be involved: the α_1-receptor in this tissue also requires high Po_2 for its functional expression and, like methysergide, is resistant to indomethacin (McLennan, 1986). Thus, it is possible that some further action of high Po_2 can facilitate the responses to methysergide and adrenaline.

5-HT has been shown to cause liberation of prostaglandin-like substances from the perfused rat lung (Alabaster & Bakhle, 1976) and to increase the activity of cyclo-oxygenase in ram seminal vesicles (Takeguchi *et al.*, 1971), but the nature of the receptors was not identified. In bovine cultured aortic smooth muscle cells (Coughlin *et al.*, 1984) and in the dog saphenous vein smooth muscle (Kokkas & Boeynaems, 1988) the receptor mediating prostacyclin synthesis has been characterized and appears to belong to the 5-HT_2 subtype.

In other preparations where a functional response to 5-HT has been shown to be mediated by 5-HT_1-like receptors, a direct or indirect action involving cyclo-oxygenase products has been ruled out. Thus, the contraction of dog saphenous vein (Apperley *et al.*, 1980) and rabbit basilar artery (Bradley *et al.*, 1986b) does not involve cyclo-oxygenase products since the response is resistant to indomethacin. Similarly, cyclo-oxygenase products are not involved in the receptor mediating relaxation of cat saphenous vein and guinea-pig ileum (Feniuk *et al.*, 1983). Therefore, unlike the receptor uncovered in the HUA, whose expression relies on cyclo-oxygenase products, other 5-HT_1-like receptor-mediated responses do not directly or indirectly involve cyclo-oxygenase products.

Clearly, the 5-HT_2 receptor-mediated response is unchanged on moving between oxygen tensions but oxygen somehow unmasks the response to activation of the 5-HT_1-like receptor. The only clue to the basis of this modulation by the prevailing oxygen tension is that it requires functional cyclo-oxygenase. Raising the oxygen tension across the same range causes a contraction of the vessel which is blocked not only by indomethacin but also by aspirin and flurbiprofen (McGrath *et al.*, 1986; MacLennan *et al.*, 1988b). It seems reasonable to postulate that oxygen stimulates the formation of cyclo-oxygenase products, which in turn activate the smooth muscle to produce contraction. On prolonged exposure to high Po_2 this contraction wanes but presumably activation continues. The simplest explanation for the uncovering of the 5-HT_1-like receptor is that it cannot, on its own, activate the contractile process without the additional effect produced by cyclo-oxygenase products. It is likely that this synergism occurs beyond the receptor, possibly at the 2nd messenger. If the effect occurred beyond this stage, then it would not be likely to be specific for the 5-HT_1-like compared with the 5-HT_2-receptor. We cannot exclude the alternative possibility that the 5-HT_1-like receptor itself mediates the formation of a contractile cyclo-oxygenase product, which in turn causes contraction, and that either this synthesis or the resultant action on muscle is oxygen-dependent. Indeed, in the perfused HUA, 5-HT can stimulate the synthesis of thromboxane A_2 (Bjoro, 1986) a potent agonist in this tissue (Svensson *et al.*, 1977). However, since we know that oxygen is itself contractile this latter explanation may be over-complicated.

An interesting observation was that phentolamine and the selective α_2-adrenoceptor antagonist Wy 26703 (Lattimer *et al.*, 1984) antagonised both the 5-HT_2 and 5-HT_1-like receptor-mediated responses. The cross-reactivity of α-adrenoceptor antagonists and 5-HT_2 receptors is well known (Apperley *et al.*, 1976; Kaumann, 1983). Our results support previous studies which have shown that α_2-adrenoceptor antagonists possibly interact with modest affinity for 5-HT_1-like receptors. Two studies have demonstrated that phentolamine is a competitive antagonist (pA_2 6.11 and 6.05) at the 5-HT_1-like receptor in dog saphenous vein (Humphrey, 1978; Curro *et al.*, 1978).

A heterogeneous population of 5-HT receptors mediates contraction of the dog coronary (Frenken & Kaumann, 1985) and basilar arteries (Frenken & Kaumann, 1986) and human saphenous veins (Docherty & Hyland, 1986). In each preparation 5-HT has a high affinity for a ketanserin-resistant receptor (which may be 5-HT_1-like but has not been fully characterized) and a somewhat lower affinity for 5-HT_2 receptors. In these two arteries from dogs phentolamine has micromolar affinity for the ketanserin-resistant (5-HT_1-like) receptor (Muller-Schweinitzer, 1980) while in the human saphenous vein yohimbine weakly antagonised the 5-HT_1-like (and 5-HT_2) mediated effects of 5-HT (Docherty & Hyland, 1986). Clearly, α_2-adrenoceptor antagonists have significant affinity for 5-HT_1-like receptors.

Since α_2-adrenoceptor antagonists generally have greatest affinity for the 5-HT_{1a} subtype of the 5-HT_1 recognition site (see Hoyer, 1988), this may suggest a similarity between the 5-HT_{1a} binding site and the 5-HT_1-like receptor in the HUA. However, at concentrations which saturate more than half of these binding sites buspirone and (±)-pindolol had no detectable affinity for the receptor in HUA.

In conclusion, raising the Po_2 above the physiological level found *in vivo* in this vessel uncovers a second 5-HT-receptor, besides the 5-HT_2 receptor. An analysis of this receptor suggests that it can be described as 5-HT_1-like, in accordance with the guidelines of Bradley *et al.* (1986a). It will be interesting to see whether this type of unmasking occurs more generally, possibly highlighting physiological

mechanisms for engaging or disengaging different forms of activation.

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Use of geographutoxin II (μ-conotoxin) for the study of neuromuscular transmission in mouse

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1 Endplate potentials (e.p.ps) were investigated in the presence of geographutoxin II (GTXII) in the mouse phrenic nerve diaphragm preparation. This toxin preferentially blocks muscle Na^+ channels which allows the study of e.p.ps in the absence of nicotinic receptor antagonists or substances to depress acetylcholine release.

2 GTXII abolished muscle action potentials and antagonized the depolarization of the muscle membrane produced by the crotamine-induced opening of Na^+ channels.

3 E.p.ps as large as 19–25 mV were observed after 2–4 $\mu g\,ml^{-1}$ GTXII. These concentrations of GTXII did not cause discernible changes of resting membrane potential and frequency and amplitude of miniature e.p.ps.

4 Lower concentrations (1–2 $\mu g\,ml^{-1}$) of GTXII caused incomplete blockade of the muscle Na^+ channel resulting in exaggerated 'e.p.ps', while higher concentrations of GTXII (8 $\mu g\,ml^{-1}$) abolished e.p.ps by a prejunctional effect.

5 Trains of e.p.ps on repetitive stimulation after GTXII neither ran down, as in tubocurarine-treated preparations, nor facilitated, as in low Ca^{2+} and/or high Mg^{2+}-treated preparations, and were indistinguishable from those of untreated cut muscle preparation.

6 In cut muscle preparations, GTXII did not affect the rise and decay times, amplitude or run-down of e.p.ps.

7 It is concluded that GTXII is a useful agent for studying neuromuscular transmission. This method provides e.p.ps which are neither attenuated nor modified because manipulations that alter transmitter release and postjunctional receptor responses are avoided.

Introduction

Studies of skeletal neuromuscular transmission have relied very much on recordings of endplate potentials (e.p.ps) or endplate current. The information obtained by electrophysiological methods is unrivalled by biochemical or classical pharmacological methods with respect to time resolution especially when pulse to pulse changes of acetylcholine (ACh) release and receptor response are studied. It has been impossible, however, to record the unattenuated e.p.p. in normal preparations because of the ensuing muscle action potential. In order to abolish the muscle action potential and contraction, it is necessary to reduce the amplitude of the e.p.p. pharmacologically, either by decreasing the evoked ACh release in low Ca^{2+} and/or high Mg^{2+} media or by diminishing the postjunctional endplate response with tubocurarine. Another method is to inactivate muscle Na^+ channels by cutting the muscle (Barstad & Lillehel, 1968). These manipulations impose serious limitations on the interpretation of data from such experiments.

Uncontaminated e.p.ps or endplate currents might be revealed if a selective blockade of Na^+ channels of the muscle (and not nerve) can be achieved. Geographutoxin II (GTXII), a novel 22 amino acid peptide toxin isolated from the venom of *Conus geographus* (Nakamura *et al.*, 1983; Sato *et al.*, 1983; Cruz *et al.*, 1985) inhibits the contraction and abolishes the action potential of skeletal muscles at concentrations that scarcely affect conduction in neurones. Binding experiments indicate that GTXII interacts competitively with saxitoxin at neurotoxin receptor site 1 on the Na^+ channel in a tissue-specific manner (Cruz *et al.*, 1985; Moczydlowski *et al.*, 1986; Ohizumi *et al.*, 1986b). The present experiments studied the effect of GTXII on e.p.ps in cut and intact mouse diaphragm preparations, in order to explore the potential of GTXII for obtaining an

unattenuated e.p.p. in the investigation of neuromuscular transmission. It was found that large e.p.ps free from undesirable pre- or postjunctional interferences could be disclosed by treatment with an appropriate concentration of GTXII.

Methods

Nerve-muscle preparations

Phrenic nerve diaphragm preparations were isolated from 20–25 g mice (ICR strain) and bathed in 12 ml Tyrode solution (composition mM: NaCl 137, KCl 2.8, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.33, $NaHCO_3$ 11.9 and dextrose 11.2) maintained at 37 ± 0.5°C and aerated with 95% O_2 + 5% CO_2.

For preparing cut muscle preparations, muscles of the diaphragm were dissected at the costal end and crushed at the junction to central tendon. The cut preparations were equilibrated with Tyrode solution for 2–2.5 h, unless otherwise indicated, until the resting membrane potential declined to −44 to −36 mV at which values the Na^+ channels were completely inactivated.

Electrophysiological studies

Intracellular membrane potentials and e.p.ps of superficial muscle fibres were recorded with a d.c.-coupled waveform recorder by using glass microelectrodes (3–10 MΩ when filled with 3 M KCl). E.p.ps were evoked by delivering supramaximal single pulses (width 0.05 ms) to the phrenic nerve, either at 0.66 Hz or by trains of stimulation at 100 Hz for 400–700 ms, at intervals not less than 100 s to minimize post-tetanic potentiation. Square pulses of 0.5 ms duration and of suitable voltage were applied for direct stimulation of diaphragm muscles.

A single electrode current clamp/bridge amplifier (Dagan, Minneapolis) was used to inject current (2.5–60 nA, 5 ms) into muscle fibres.

For each endplate, means of amplitudes, rise times (from 10 to 90%), decay times (to 1/e) and rates of rise of e.p.ps or miniature e.p.ps were calculated from 30–50 successive events. The quantal content of e.p.ps was estimated either by the method of variance or by the direct method (del Castillo & Katz, 1954). E.p.ps were corrected for non-linear summation according to Chang *et al.* (1986) for this purpose. Toxins were added cumulatively because of the very limited amount available and 8–15 endplates were tested for each concentration applied in each preparation.

Statistics

The results are given as mean ± s.e.mean; number of experiments is indicated by *n*. The significance of differences was evaluated by Student's *t* test.

Drugs

Geographutoxin II (GTXII) was a generous gift from Dr C.H. Wu (Northwestern University Medical School, Chicago) and was the same batch as used by Kobayashi *et al.* (1986). Crotamine was kindly supplied by Dr C. Bon (Pasteur Institute, Paris). Tetrodotoxin and tubocurarine were purchased from Calbiochem (San Diego, California).

Results

Effects of GTXII on e.p.ps

Intact diaphragm preparation In uncut mouse diaphragm twitch responses elicited by stimulation of the phrenic nerve were suppressed markedly by GTXII at concentrations greater than 0.7 μg ml^{-1}. For intracellular recordings of e.p.ps to be successful, the concentration of GTXII had to be raised to 1 μg ml^{-1} to avoid dislocation of the microelectrode

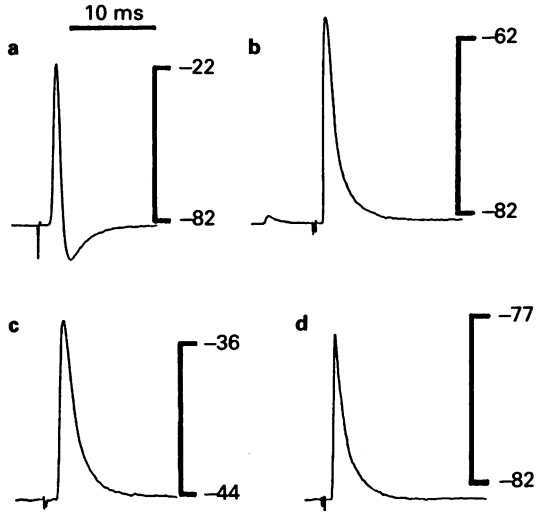

Figure 1 Effects of geographutoxin II (GTXII) or tubocurarine on the e.p.ps of mouse phrenic nerve diaphragm preparations. E.p.ps were recorded in uncut preparations treated with 1 μg ml^{-1} GTXII (a), 2 μg ml^{-1} GTXII (b) or 2 μM tubocurarine (d). The e.p.p. in (c) was obtained from a cut muscle preparation without GTXII or tubocurarine. Note that, in preparations treated with 1 μg ml^{-1} GTXII, about 15% of junctions showed a response similar to that in (a) (the 'e.p.p' was overwhelmed by the active membrane response) and the others showed responses similar to (b). Also note the miniature e.p.p. in (b).

Table 1 Effects of geographutoxin II (GTXII) on the amplitude, decay time and rate of rise of e.p.ps and the amplitude of miniature e.p.ps (M.e.p.ps) in the mouse phrenic nerve diaphragm preparations

		Uncut preparation[a]			*Cut muscle preparation*[a]		
	M.e.p.ps	*E.p.ps*			*E.p.ps*		
GTXII[b] ($\mu g\,ml^{-1}$)	*Amplitude* (mV)	*Amplitude* (mV)	*Decay time* (ms)	*Rate of rise* ($V\,s^{-1}$)	*Amplitude* (mV)	*Decay time* (ms)	*Rate of rise* ($V\,s^{-1}$)
0	1.4 ± 0.3	—	—	—	8.3 ± 0.9	1.26 ± 0.02	27 ± 3
1	1.3 ± 0.2	28.3 ± 2.8	1.14 ± 0.01	97 ± 8	8.9 ± 0.8	1.28 ± 0.07	30 ± 4
2	1.2 ± 0.3	22.3 ± 1.1*	1.15 ± 0.03	75 ± 5*	7.5 ± 0.6	1.20 ± 0.02	22 ± 3
4	1.3 ± 0.3	20.9 ± 1.2*	1.11 ± 0.08	73 ± 5*	7.3 ± 0.6	1.26 ± 0.1	21 ± 3
8	1.3 ± 0.3	7.8 ± 2.1*,**	1.22 ± 0.14	37 ± 11*,**	—	—	—

[a] The number of endplates was at least 24 and 16, respectively, for uncut ($n = 3$) and cut ($n = 2$) phrenic nerve diaphragm preparations except that treated with 8 $\mu g\,ml^{-1}$ GTXII for which values of e.p.ps were obtained from 5 junctions in one preparation before complete block. The resting membrane potentials of uncut and cut muscle preparations were −84.4 ± 2.5 and −42.1 ± 1.2 mV (before GTXII) and −81.9 ± 1.7 and −42.4 ± 1.8 mV (after 4 $\mu g\,ml^{-1}$ GTXII), respectively. The frequencies of miniature e.p.ps of uncut preparations before and after 4 $\mu g\,ml^{-1}$ GTXII were 0.93 ± 0.14 and 1.21 ± 0.27 s^{-1}, respectively.

[b] GTXII was added cumulatively and the incubation time was more than 30 min for each concentration added except that treated with 8 $\mu g\,ml^{-1}$ (7–15 min).

* $P < 0.05$, compared with those after 1 $\mu g\,ml^{-1}$ GTXII.

** $P < 0.05$, compared with those after 2 $\mu g\,ml^{-1}$ GTXII.

by movement of the diaphragm. At this concentration, about 15% of muscle fibres still generated propagating Na^+-spikes in response to nerve stimulations, albeit the amplitudes were reduced (Figure 1a). The amplitude of the spikes measured at the endplate area was 57 ± 11 mV ($n = 3$) with pronounced after-hyperpolarization, while that taken at the ends of the muscle fibre (about 5 mm distal to the endplate) was 25–80% lower. In the rest (85%) of the junctions, stimulation of the nerve produced e.p.ps of 28.3 ± 2.8 mV without triggering propagative potentials. Table 1 shows a comparison of the effects of GTXII on the amplitude, rate of rise and decay time of e.p.ps and on the amplitude of miniature e.p.ps. It should be noted that GTXII affected neither the resting membrane potential of the muscle nor the frequency and amplitude of miniature e.p.ps to any significant extent (cf. Table 1). However, the depolarization of muscle membrane induced by crotamine, a rattlesnake toxin acting preferentially and irreversibly on muscle Na^+ channels at neurotoxin receptor site 3 (Hong & Chang, 1983), was antagonized by GTXII (Figure 2). The membrane potential depolarized again after washout of both toxins, indicating that the binding of GTXII is reversible and the site is distinct from that of crotamine binding.

When the concentration of GTXII was raised to 2 $\mu g\,ml^{-1}$, the diaphragm was paralysed completely and e.p.ps as large as 19–25 mV (Figure 1b) could be recorded only at the endplate area, indicating that the propagative capability of the muscle membrane was lost whereas that of the nerve membrane was still functioning. The amplitude and rate of rise of e.p.ps were 20% smaller than those after 1 $\mu g\,ml^{-1}$ GTXII (Table 1). The rise time was slightly increased from 0.52 ± 0.06 to 0.64 ± 0.05 ms.

Further increase of the concentration of GTXII to 4 $\mu g\,ml^{-1}$, however, had no greater inhibitory effect

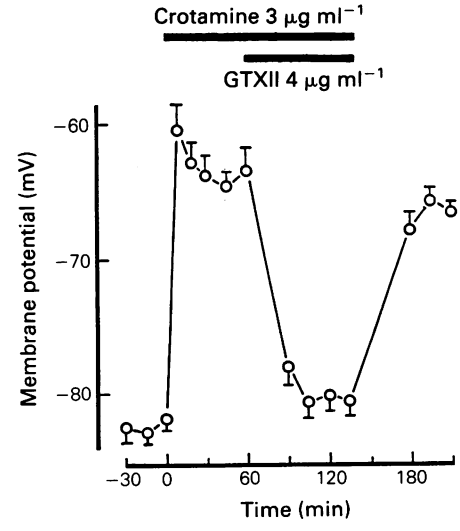

Figure 2 Antagonism of crotamine-induced membrane depolarization by geographutoxin II (GTXII) in uncut mouse diaphragms. Diaphragms ($n = 2$) were treated firstly with 3 $\mu g\,ml^{-1}$ crotamine at 0 min and, 60 min later, co-incubated with 4 $\mu g\,ml^{-1}$ GTXII. Both toxins were removed at 140 min.

so that the amplitudes as well as the rate of rise of e.p.ps were not reduced. Finally, as the concentration of GTXII was increased to $8\,\mu g\,ml^{-1}$, e.p.ps were abolished within 15 min. Before total failure, e.p.p. amplitudes were reduced progressively as the time of toxin incubation increased. The e.p.p. amplitude recorded from 5 junctions before complete failure was 7.8 ± 2.1 mV. The rate of rise of e.p.p. was also depressed. By contrast, the rise and decay times of e.p.ps and the amplitudes of miniature e.p.ps remained unaffected even after treatment with this high concentration of GTXII (Table 1). Washout of GTXII caused e.p.ps to reappear and eventually muscles began to twitch in response to nerve stimulation.

Quantal contents of e.p.ps obtained after GTXII at 2 and $4\,\mu g\,ml^{-1}$ were calculated, by the direct method, to be 21.9 and 18.71 respectively.

Cut muscle preparation GTXII seemed to depress e.p.ps in the uncut diaphragm at two ranges of concentrations, up to $2\,\mu g\,ml^{-1}$ and over $4\,\mu g\,ml^{-1}$. The effect of GTXII was therefore studied in the cut muscle preparation. The e.p.p. amplitude, rise time and rate of rise of the untreated cut muscle at resting membrane potentials of -44 to -36 mV were 8.3 ± 0.9 mV, 0.66 ± 0.07 ms and $27 \pm 3\,V\,s^{-1}$, respectively (cf. Figure 1c). The amplitude of miniature e.p.ps was 0.3–0.5 mV. These values, except the rise time, were about 60–70% less than those obtained in the uncut muscle treated with 2–$4\,\mu g\,ml^{-1}$ GTXII. Unlike in the intact muscle, GTXII up to $4\,\mu g\,ml^{-1}$ caused no statistically significant reduction of these values (Table 1), indicating that GTXII up to $4\,\mu g\,ml^{-1}$ does not affect the quantal release of neurotransmitter. The quantal contents of e.p.ps evoked at 0.66 Hz, calculated by the direct method, were 23.2 and 21.5 respectively before and after GTXII.

In a separate experiment, the effect of GTXII on e.p.p. amplitude was studied in a more depolarized (-32 to -24 mV) cut muscle preparation which would ensure a complete inactivation of muscle Na^+ channels. GTXII $4\,\mu g\,ml^{-1}$ had no apparent depressant effect on the e.p.p. amplitude (control 4.2 ± 0.7 mV, after toxin 3.8 ± 0.6 mV).

Effects of GTXII on the run-down of e.p.ps

The effect of GTXII on e.p.ps evoked at 100 Hz was studied in the uncut diaphragm preparation at higher toxin concentrations which produced a satisfactory immobilization of muscle. In the presence of 2–$4\,\mu g\,ml^{-1}$ GTXII, e.p.ps exhibited an initial facilitation for the first 2–3 e.p.ps, followed by a gradual decline from the 5th onwards and finally levelled off after about 20 pulses; the amplitude being approximately 75% of that of the 1st e.p.p. of the train stimulation (Figures 3c and 4). This profile of run-down of e.p.ps on repetitive stimulation was indistinguishable from that of cut muscle in the absence of drugs. In the cut muscle preparation, GTXII up to $4\,\mu g\,ml^{-1}$ did not alter the pattern of e.p.p. run-down (Figures 3a,b and 4).

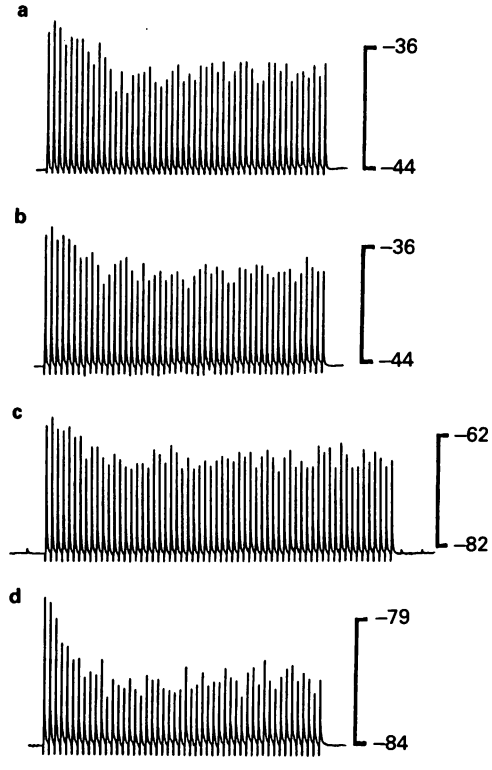

Figure 3 Effects of geographutoxin II (GTXII) or tubocurarine on the run-down of e.p.ps in the mouse phrenic nerve diaphragm preparations. E.p.ps were evoked by trains of stimulation of the phrenic nerve at 100 Hz for 500–630 ms. (a) Taken from an untreated cut muscle preparation. (b) The same endplate as in (a) but after 60 min treatment with $4\,\mu g\,ml^{-1}$ GTXII. (c and d) Different uncut preparations after 60 min incubation with $4\,\mu g\,ml^{-1}$ GTXII and $2\,\mu M$ tubocurarine, respectively.

Figures 3d and 4 compare the run-down of e.p.ps of uncut preparations paralysed with tubocurarine 1.5–$2\,\mu M$, which is the minimum concentration to immobilize the diaphragm. After 60 min incubation, miniature e.p.ps were undetectable and the amplitude of e.p.ps was only 4.9 ± 0.8 mV (cf. Figure 1d) despite the high resting membrane potential (-84.4 ± 1.38 mV, $n = 3$). Noticeably, the run-down of e.p.ps was twice as great as those obtained without tubocurarine. In cut muscle preparations,

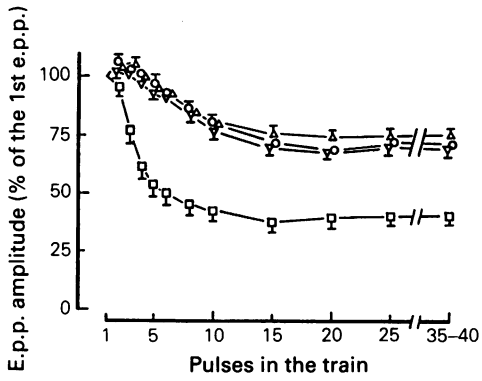

Figure 4 Effects of geographutoxin II (GTXII) on the run-down of e.p.ps in the mouse phrenic nerve diaphragm preparations. E.p.ps were evoked by train stimulations of the phrenic nerve at 100 Hz on uncut (△, □, $n = 3$) or cut (○, ▽, $n = 2$) diaphragms. The uncut preparations were treated with either 4 $\mu g\,ml^{-1}$ GTXII (△) or 1.5–2 μM tubocurarine (□) for 60 min. The cut preparations were either not treated (○) or treated with 4 $\mu g\,ml^{-1}$ GTXII (▽).

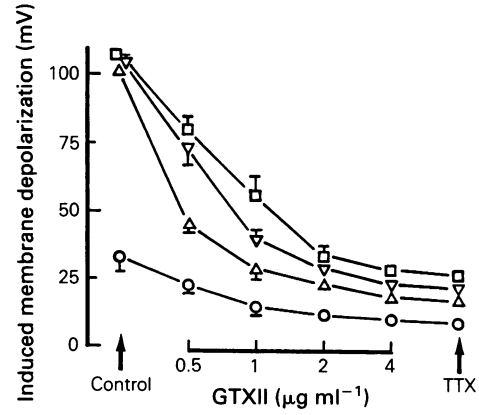

Figure 5 Effects of geographutoxin II (GTXII) and tetrodotoxin (TTX) on the muscle membrane depolarization induced by current injection in the uncut mouse diaphragm. Depolarizing currents of 5 (○), 10 (△), 20 (▽) and 40 (□) nA were injected into the endplate area ($n = 3$). The electrotonic components of the induced membrane depolarization was indicated by the membrane potential shift after treatment with 120–350 $ng\,ml^{-1}$ TTX for 60 min. The resting membrane potentials of control, after 4 $\mu g\,ml^{-1}$ GTXII and after tetrodotoxin were -83.5 ± 1.1, -82.3 ± 1.8 and -85.6 ± 2.2 mV, respectively.

tubocurarine accelerated the run-down of e.p.ps as previously observed (Glavinovic, 1979; Chang *et al.*, 1988).

Effects of GTXII on potential changes induced by current injection

In the uncut muscle, the muscle resting membrane potential is as high as −80 mV and most Na^+ channels should be available for opening on depolarization provided that channels are not plugged. The question then arises as to whether the large e.p.ps observed in the uncut preparation after GTXII treatment resulted from a superimposed activation of residual unblocked Na^+ channels in addition to opening of ACh channels. To assess the contribution of Na^+ channels to the large e.p.ps, the inhibitory effects of GTXII on depolarization induced by current injection were investigated at the endplate area where the density of Na^+ channels is high (Beam *et al.*, 1985). Sufficient current was injected to simulate the depolarizing effects caused by the opening of ACh channels. Depending on the amount of current injected, muscle membranes generated either a simple sustained electrotonic potential, or a brief local depolarization on top of the electrotonic potential due to a local opening of Na^+ channel or, as the threshold was reached, a spike which overshot zero potential by 15–25 mV. Tetrodotoxin 120 $ng\,ml^{-1}$ completely suppressed these responses except the electrotonic potential. The concentration of tetrodotoxin producing half inhibition of the active response was about 20 $ng\,ml^{-1}$. Similar to the effect of tetrodotoxin, GTXII 0.5–4 $\mu g\,ml^{-1}$ inhibited the local depolarization and the spike dose-dependently (Figure 5). The concentration of GTXII producing half inhibition was about 0.5 $\mu g\,ml^{-1}$. With this concentration of GTXII, action potentials, though reduced in amplitude, could be obtained when large amounts of current (20–40 nA) were applied. The action potentials were completely abolished and local active depolarizations were largely suppressed as the concentration of GTXII was raised to 2 $\mu g\,ml^{-1}$. As the concentration of GTXII reached 4 $\mu g\,ml^{-1}$ the local depolarizations were negligible and the total membrane depolarization was suppressed to the same extent as in 120 $ng\,ml^{-1}$ tetrodotoxin (Figure 5). With regard to the inhibitory action on muscle Na^+ channels, the effect of 2 $\mu g\,ml^{-1}$ GTXII was approximately equal to that produced by 60 $ng\,ml^{-1}$ tetrodotoxin.

Discussion

Ligand binding studies have revealed that GTXII binds to the Na^+ channel competitively against tetrodotoxin and saxitoxin, but, in contrast to the latter two toxins, GTXII had higher affinity for skeletal muscle than nerve (Moczydlowski *et al.*, 1986; Ohizumi *et al.*, 1986b; Gonoi *et al.*, 1987; Yanagawa

et al., 1987). Electrophysiological studies confirmed the preferential blocking action of GTXII on Na^+ channel of muscle (Cruz *et al.*, 1985; Kobayashi *et al.*, 1986; Ohizumi *et al.*, 1986a). The present results showed that GTXII, at concentrations that did not appear to impair nerve action potential, abolished the muscle action potential and effectively reversed the membrane depolarization caused by crotamine, a peptide toxin which opens the Na^+ channel of mammalian skeletal muscle (Chang *et al.*, 1983). With low concentrations of GTXII an appreciable fraction of Na^+ channels in the diaphragm seemed unaffected and could be opened by large step depolarizations (Figure 5). It is likely, in this respect, that the end-plate depolarization caused by excitation of the ACh receptor may activate some of the residual unblocked Na^+ channels and result in an overestimate of e.p.p. amplitude. Indeed, in the uncut muscle preparations, the apparent e.p.p. amplitude was reduced by about 20% when GTXII was increased from 1 to 2–4 $\mu g\,ml^{-1}$, while no change of e.p.p. amplitude was observed up to 4 $\mu g\,ml^{-1}$ GTXII in the cut muscle preparations in which the muscle Na^+ channel was already inactivated due to depolarization (Table 1). This contamination from Na^+ channel activation should become greatly reduced on increasing the GTXII concentration to 2–4 $\mu g\,ml^{-1}$, since the membrane depolarization due to Na^+ channel activation was negligible as far as the amplitude of step depolarization was within the range of e.p.ps. In the rat cultured myoball, these concentrations of GTXII were sufficient to block the tetrodotoxin-sensitive binding or Na^+ current (Gonoi *et al.*, 1987).

The e.p.p. amplitude and rise time appeared constant in 2–4 $\mu g\,ml^{-1}$ GTXII, either in the cut or in the uncut preparations, indicating that the release of neurotransmitter was not affected. E.p.ps were finally abolished after incubation with 8 $\mu g\,ml^{-1}$ GTXII. Since amplitudes of miniature e.p.ps were unchanged, the blockade of e.p.ps must derive from a prejunctional failure of transmitter release, probably due to an inhibitory action on the nerve Na^+ channel.

In the present experiments with mouse phrenic nerve diaphragm preparations, GTXII seems not to discriminate in favour of muscle against neuronal Na^+ channels as effectively as previously observed (Nakamura *et al.*, 1983; Cruz *et al.*, 1985; Ohizumi *et al.*, 1986a). Part of the reason for the discrepancy in the relative potency of GTXII with respect to muscle and nerve may be that we looked at the effects on e.p.p. amplitude instead of muscle action potential or axonal conduction. Yet, a careful treatment of vertebrate neuromuscular preparations with a proper concentration (2–4 $\mu g\,ml^{-1}$) of GTXII may be the best known procedure to study uncontaminated e.p.ps and thus to study the prejunctional as well as the postjunctional events with unblocked ACh receptors and unimpaired ACh release. The resting membrane potential is unchanged, acetylcholinesterase activity is not inhibited, the e.p.ps are large and can be recorded together with the miniature e.p.ps. Tetrodotoxin and saxitoxin cannot substitute for GTXII for these purposes because muscle Na^+ channels are not preferentially blocked.

To date, with normal Ca^{2+} and Mg^{2+} concentrations, the quantal content of e.p.ps in uncut preparations can be measured in the presence of tubocurarine either by the method of variance, which deviates from poisson statistics when the release is high (Ginsborg & Jenkinson, 1976), or by the 'semi-direct' method, which relies on the determination of tubocurarine's dissociation constant (Ceccarelli & Hurlbut, 1975). It is now possible to estimate the normal quantal release by directly comparing the amplitudes of e.p.ps with those of miniature e.p.ps. After correcting for non-linear summation of e.p.ps, the quantal content of the intact and cut mouse diaphragms is estimated to be only 20–25 in contrast to 127 ± 19 estimated by the method of variance from the same set of data. The lesser extent of e.p.p. run-down on high frequency stimulation in GTXII-treated or cut muscle preparations than in tubocurarine-treated preparations may further support the view that the marked e.p.p. run-down after tubocurarine is due to a prejunctional effect (Glavinovic, 1979; Bowman *et al.*, 1986; Chang *et al.*, 1988).

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Phentolamine and structurally related compounds selectively antagonize the vascular actions of the K^+ channel opener, cromakalim

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1 The effects of cromakalim, a novel vasodilator agent believed to open K^+ channels, were studied in a range of large and small arteries *in vitro*. In dog isolated coronary artery, precontracted with U46619 (a thromboxane A_2-mimetic), cromakalim caused concentration-dependent relaxation which could be inhibited by phentolamine (10–100 μM).

2 The ability of phentolamine to antagonize cromakalim was selective since it did not affect responses to a number of other vasodilators including isoprenaline, nitroprusside or nicorandil.

3 The effect of phentolamine was not related to its α-adrenoceptor blocking actions since other α-adrenoceptor antagonists (prazosin 10 μM, rauwolscine 10 μM and phenoxybenzamine 1 μM) failed to influence the action of cromakalim.

4 A number of compounds structurally related to phentolamine were also able to block the vasorelaxant response to cromakalim in the dog isolated coronary artery. The rank order of potency was alinidine = phentolamine = ST91 > tramazoline = naphazoline. Clonidine and tolazoline were inactive. The most potent compounds (alinidine and phentolamine) were effective only at concentrations above 1 μM.

5 Electrophysiological studies, in which resting membrane potential and tension were measured simultaneously, were carried out on rat isolated femoral artery. Phentolamine (30 μM) antagonized both the vasorelaxation and hyperpolarization caused by cromakalim.

6 These results suggest that phentolamine and some structurally related compounds, may inhibit K^+ channel opening, an action which would account for their ability to antagonize the actions of cromakalim. Such compounds may prove useful in determining the role of K^+ channels in regulating vascular smooth muscle tone *in vivo* and *in vitro*.

Introduction

Cromakalim (BRL 34915) is a novel relaxant of both vascular (Hamilton *et al.*, 1986; Quast, 1987) and non-vascular (Weir & Weston, 1986a; Hollingsworth *et al.*, 1987) smooth muscle. This compound appears to reduce tone by opening specific K^+ channels and thereby causing hyperpolarization of the cell membrane. Recent studies have shown that K^+ channel opening may be important in the mechanism of action of endogenous vasodilator substances (see Taylor & Weston, 1988). However, assessment of the role of K^+ channels in the actions of these substances has been hindered by the lack of specific inhibitors of the various types of K^+ channels known to exist in smooth muscle. In this paper we show that phentolamine, and some structurally related compounds, selectively antagonize the actions (mechanical and electrophysiological) of cromakalim in a concentration-dependent manner. These compounds may therefore prove useful in the study of vascular K^+ channels in general.

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Methods

Mechanical studies

Dog isolated coronary artery Circumflex coronary artery was obtained from greyhound dogs (20–30 kg) killed by pentobarbitone (60 mg kg^{-1}, i.v.). A ring segment (4 mm long) was mounted on wire hooks in a 25 ml organ bath containing a modified Krebs buffer (composition in mM: NaCl 119, KCl 4.7,

$MgSO_4.7H_2O$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, $CaCl_2$ 2.5 and glucose 11) maintained at 37°C and gassed with 5% CO_2 in O_2. Changes in isometric force development were monitored with Grass (FT03) force displacement transducers coupled to a Grass polygraph (model 7). Each tissue segment was placed under an initial passive force of 4 g and allowed to equilibrate for 30 min before the addition of any drugs. Active tone was induced by the addition of U46619 (a chemically stable thromboxane A_2-mimetic) at a concentration (10–30 nM) that caused approximately 80% of the maximum response to this agent. Vasorelaxant responses to increasing concentrations of a number of agents (cromakalim, isoprenaline, nitroprusside and nicorandil) were assessed in the absence and in the presence of phentolamine (1, 10 and 100 μM). Phentolamine was added to the bath once the response to the U46619 had reached a plateau. Ten minutes later one of the vasorelaxant drugs was tested. Preliminary studies indicated that this contact time was sufficient to cause maximal antagonism. The effects of increasing concentrations of phentolamine were obtained in consecutive dose-response curves to the vasorelaxants with a 30 min equilibration period between each curve. Similar experiments were also performed assessing the actions of a number of α-adrenoceptor antagonists (phenoxybenzamine 1 μM without washout, prazosin 10 μM and rauwolscine 10 μM) on vasorelaxant responses caused by cromakalim.

The ability of a number of compounds, structurally related to phentolamine, to antagonize vasorelaxant responses to cromakalim was also assessed. In these experiments the vessel segments were precontracted with U46619 (10 nM) and cromakalim (0.5 μM) added to relax the segment by approximately 50%. The ability of a number of compounds (alinidine, phentolamine, ST91, tramazoline, naphazoline, clonidine and tolazoline, see structures Figure 1) to reverse the cromakalim-induced dilator response was assessed by adding increasing concentrations of each compound to the bath. The concentration of the drug required to cause 50% of its maximal effect in reversing cromakalim-induced relaxation (EC_{50}) was calculated graphically and used as an index of the potency of the drug as an antagonist. To eliminate any α-adrenoceptor-mediated actions of these compounds, the tissues were pretreated with phenoxybenzamine (1 μM) for 10 min followed by a 30 min washout period. Preliminary studies with the α-adrenoceptor agonist, methoxamine (1–100 μM), indicated this treatment regime was sufficient to eliminate completely the response to this compound.

Rat isolated femoral artery and guinea-pig basilar artery Wistar-Kyoto rats (200–250 g) or guinea-pigs (400–600 g) were killed by a blow to the head. Two mm segments of rat femoral or guinea-pig basilar artery were mounted in a small vessel myograph as previously described (Angus *et al.*, 1988). Briefly, two 40 μm wires were threaded through the lumen of the vessel segment. One wire was attached to a stationary support driven by a micrometer while the other wire was attached to an isometric transducer which measured force development. An initial force of 1 g and 0.5 g was applied to the rat femoral and guinea-pig basilar artery respectively. The vessel was allowed to equilibrate for 30 min before the addition of drugs. In the rat femoral artery, tone was induced by the addition of a submaximal concentration of U46619 (10–30 nM). Vasorelaxant responses to cromakalim were assessed in the absence and in the presence of phentolamine (10 and 100 μM). The guinea-pig basilar artery was precontracted with

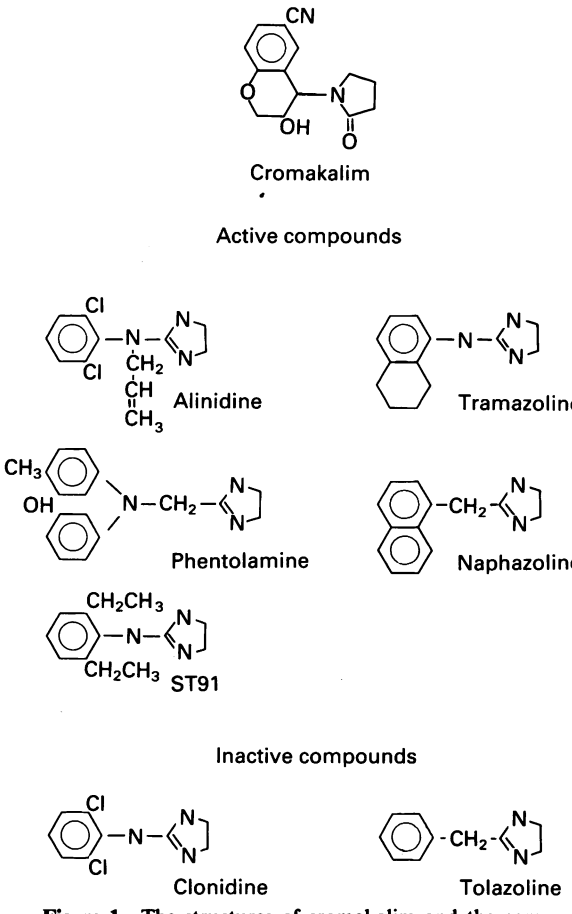

Figure 1 The structures of cromakalim and the compounds tested for their ability to reverse the vasorelaxant response of cromakalim.

ouabain (10 μM). Ouabain was chosen as a tone-inducing agent in this tissue to check whether the interaction between cromakalim and phentolamine was dependent upon the spasmogen used to induce tone. Responses to cromakalim (10 μM) were assessed in the absence and in the presence of phentolamine (10 μM).

Electrophysiological studies

In a separate series of experiments the electrophysiolgical and mechanical effects of cromakalim and phentolamine were simultaneously assessed on the rat femoral artery. A segment of rat femoral artery, 2 mm in length, was mounted in the small vessel myograph as described above (see also Angus *et al.*, 1988). The membrane potential of a single cell was monitored by use of conventional glass microelectrodes (1 mm blanks, World Precision Instruments Inc. New Haven, U.S.A.) filled with 0.5 M KCl. The resistance of the microelectrode was approximately 100 MOhms. The microelectrode was advanced (vertically to the plane of the vessel) into the vessel by use of a pneumatic micromanipulator (L.S. Starrett, Great Britain).

The bath solution was gassed with a stream of 5% CO_2 in O_2 located behind a wave guard mounted in the bath. The bath contents were mixed with a recirculating pump. This allowed drugs to be added directly to the bath and the response followed until a plateau had been reached. At the end of a procedure the bath contents could be exchanged with fresh buffer by use of a separate pumping system. The effect of cromakalim (1 and 3 μM) was assessed in the absence and presence of phentolamine (30 μM). Experiments were performed in the absence or in the presence of tone induced by U46619 (10–30 nM).

Drugs

The following drugs were used: cromakalim (Beecham); U46619 (1,5,5-hydroxy-11α,9α(epoxymethano)prosta-52,13E-dienoic acid (Upjohn); nicorandil (Chugai); (−)-isoprenaline bitartrate (Sigma); phenoxybenzamine hydrochloride (Smith, Kline and French); prazosin hydrochloride (Pfizer); rauwolscine hydrochloride (Carl Roth); phentolamine hydrochloride or mesylate, naphazoline nitrate, tolazoline hydrochloride (Ciba-Geigy); alinidine (ST 567) bromide, ST 91 hydrochloride, tramazoline hydrochloride, clonidine hydrochloride, (Boehringer-Ingelheim); sodium nitroprusside (Nipride-Roche).

Stock solutions (10 mM) of most drugs were made in distilled water and diluted each day with the same vehicle. Cromakalim (10 mM) was dissolved in 70% (v/v) ethanol. Isoprenaline (10 mM) stock was made with 0.01 M HCl. Phenoxybenzamine, prazosin and sodium nitroprusside solutions were made fresh daily.

Data analysis and statistics

pD_2 ($-\log EC_{50}$ value) and concentration-ratios were calculated by standard graphical procedures. Statistical comparisons between two groups were made by Student's t test. Multiple comparisons of dependent samples were made with Bonferroni's t test. Multiple comparisons between independent samples were made with Scheffe's test (see Wallenstein *et al.*, 1980). Results in the text are the mean ± s.e.mean for the specified number of experiments.

Results

Mechanical activity

Cromakalim (0.1–100 μM) caused marked vasorelaxant responses in the dog isolated coronary artery precontracted with U46619 (30 nM; Figure 2). Phentolamine, at concentrations less than 100 μM had no direct effect on tone. In 21 experiments with phentolamine (100 μM), 3 preparations responded to phentolamine addition with a small (<15%) increase in tone. Despite its negligible effects on U46619-

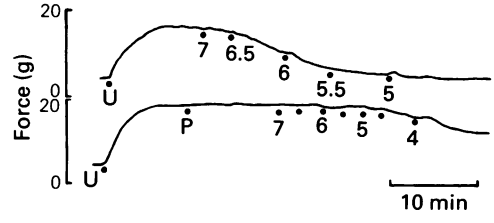

Figure 2 Representative trace of the cromakalim-mediated vasorelaxant response in the absence (upper record) and presence (lower record) of phentolamine (100 μM) in a single segment of dog isolated coronary artery precontracted with U46619 (U, 30 nM). Phentolamine (P, 100 μM) was added once a plateau response was obtained. After 10 min the cromakalim curve was reconstructed. Numbers represent −log molar concentration of cromakalim.

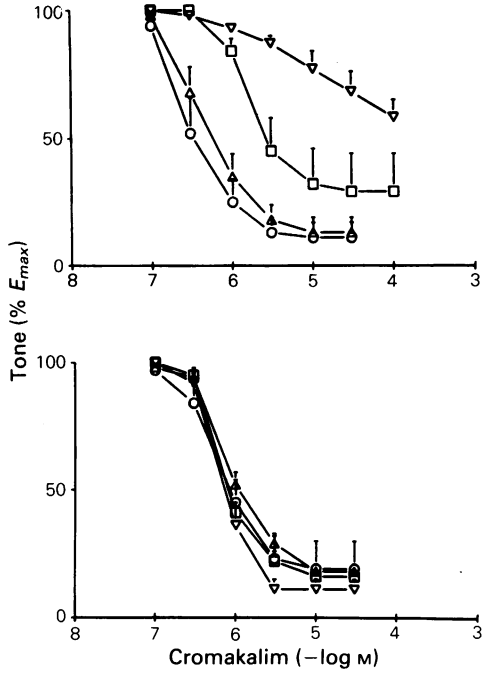

Figure 3 Mean concentration-relaxation curves for cromakalim in the dog isolated coronary artery precontracted with U46619 (30 nM; maximum induced tone, E_{max} = 100%) in the absence (○) and in the presence of increasing concentrations of phentolamine (a; 1 (△), 10 (□) and 100 (▽) μM). Panel (b) shows data from corresponding time-matched control experiments performed in vessels not exposed to phentolamine. Results are the mean ± s.e.mean for 6 separate experiments with s.e.mean shown by vertical bars

induced tone, phentolamine (1–100 μM) antagonized cromakalim in a concentration-dependent manner (Figure 3). Thus the pD_2 value (−log molar concentration required to cause 50% of the maximum inhibitory response) for cromakalim was 6.62 ± 0.09 (n = 6) in the absence of phentolamine and 6.18 ± 0.14, 5.63 ± 0.08 and 5.08 ± 0.13 (n = 6) in the presence of 1, 10 and 100 μM phentolamine respectively. The pD_2 values in the presence of 10 and 100 μM phentolamine were significantly different from the control value obtained in the absence of phentolamine ($P < 0.05$, Bonferroni's t test). In addition, the maximum relaxant response was also attenuated (tone induced by U46619 = 100%) from 89% ± 6% in the absence of phentolamine to 87% ± 6%, 71% ± 15% and 42% ± 7% (n = 6) in the presence of phentolamine 1, 10 and 100 μM respectively. In the presence of phentolamine 10 and 100 μM the maximum relaxant response was significantly less than the control ($P < 0.05$, Bonferroni's t test). Corresponding time-matched control experiments (Figure 3b) indicated that the location of the cromakalim concentration-effect curve and the maximum relaxant effect of cromakalim were not significantly altered by repeated administration. The ability of phentolamine to antagonize the responses of a number of other vasorelaxants was also determined. Phentolamine, at concentrations (10–100 μM) that significantly antagonized the response to cromakalim, failed to alter the vasorelaxant responses to isoprenaline, nitroprusside or nicorandil (Figure 4 a-c).

Studies were also performed to determine whether the effect of phentolamine was related to its α-adrenoceptor blocking actions. Prazosin (10 μM, α_1-selective antagonist), rauwolscine (10 μM, α_2-selective antagonist) and phenoxybenzamine (1 μM, irreversible α-adrenoceptor antagonist), at concentrations that would cause blockade of α-adrenoceptors, failed to affect significantly the vaso-

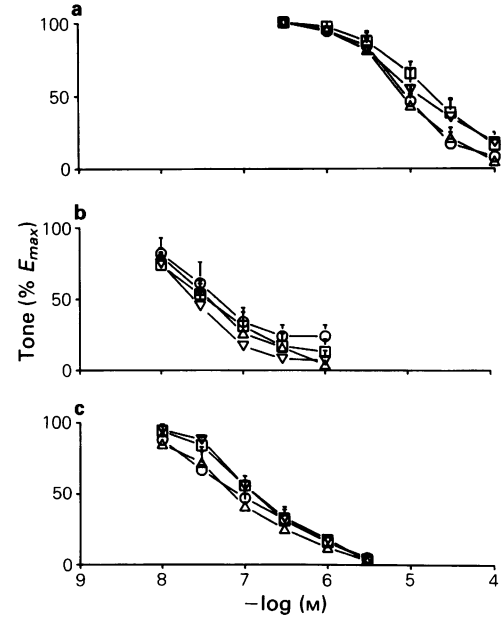

Figure 4 Mean concentration-relaxation curves obtained for nicorandil (a), isoprenaline (b) and nitroprusside (c) obtained in dog isolated coronary artery precontracted with U46619 (30 nM) in the absence (○) and in the presence of increasing concentrations of phentolamine (1 (△), 10 (□), and 100 (▽) μM). Results are the mean for 4 to 5 separate experiments with s.e.mean shown by vertical bars.

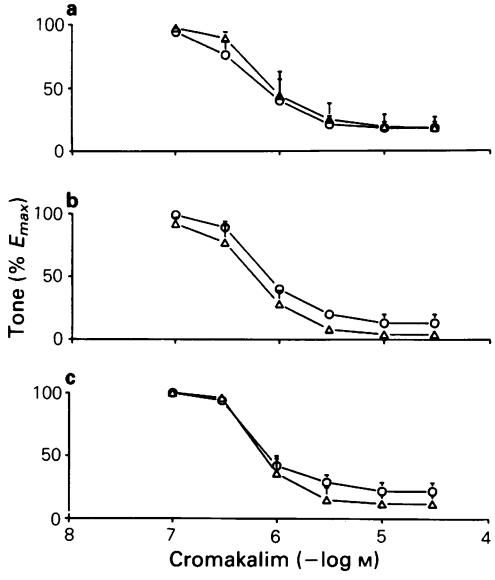

Figure 5 Mean concentration-relaxation curve for cromakalim in the dog isolated coronary artery, precontracted with U46619 (30 nM), obtained in the absence (○) and presence (△) of phenoxybenzamine (1 μM, a), prazosin (10 μM, b) and rauwolscine (10 μM, c). Results are the mean for 3 separate experiments with s.e.mean shown by vertical bars.

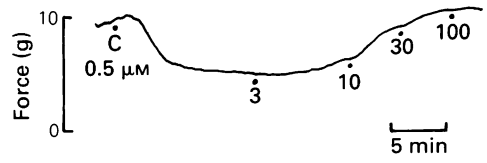

Figure 6 Representative trace obtained in dog isolated coronary artery where the ability of alinidine to reverse the relaxation to cromakalim was determined. Artery segments were precontracted with U46619 (10 nM). A submaximal concentration of cromakalim (C, 0.5 μM) was added to cause a steady partial relaxation. Increasing concentrations of alinidine (3, 10, 30, 100 μM) were then added. The concentration of antagonist required to produce 50% reversal of the cromakalim relaxation was calculated and used as an index of the potency of the drugs.

relaxant responses to cromakalim (Figure 5 a-c) under conditions where phentolamine caused significant antagonism.

A number of compounds structurally related to phentolamine were tested for their ability to inhibit the cromakalim vasorelaxant response in the dog isolated coronary artery. The results from these studies are summarized in Table 1. An index of the potency of these compounds was determined by calculating the concentration of the compound required to cause 50% reversal of the vasorelaxant response to cromakalim (0.5 μM). The result of an experiment with alinidine is shown in Figure 6. The rank order of potency of the ability of the compounds to reverse the cromakalim vasorelaxant response was: alinidine = phentolamine = ST91 > tramazoline = naphazoline ≫ clonidine = tolazoline. All compounds except clonidine and naphazoline were able to reverse the vasorelaxant response to cromakalim to a level of force that was not significantly different from that before the addition of cromakalim ($P > 0.05$, Scheffe's test). Clonidine and naphazoline were generally ineffective in reversing the response to cromakalim (see Table 1).

Table 1 Vasorelaxant effects of cromakalim in dog coronary artery precontracted with U46619 (10 nM): antagonist actions of a number of compounds structurally related to phentolamine

Compound	n	pD_2	*Maximal reversal* (% reversal of relaxation by 0.5 μM cromakalim)
Alinidine	5	5.32 ± 0.08^{1}*	109 ± 3
Phentolamine	6	$5.24 \pm 0.04^{1,2}$	98 ± 4
ST 91	6	$5.13 \pm 0.10^{1,2,3}$	91 ± 8
Tramazoline	3	$4.77 \pm 0.3^{2,3}$	94 ± 10
Naphazoline	5	$4.76 \pm 0.02^{2,3}$	103 ± 4
Clonidine	3	<4	
Tolazoline	6	<4	

* Values marked with the same number were not significantly different ($P > 0.05$, Scheffe's test).

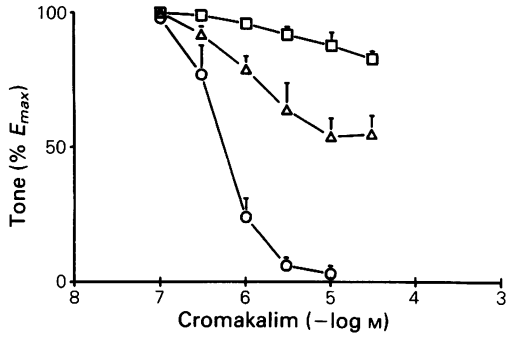

Figure 7 Mean concentration-responses curves to cromakalim in the absence (○) and in the presence of phentolamine (10 (△) and 100 (□) μM) in rat isolated femoral artery precontracted with U46619 (30 nM). Phentolamine antagonized the response to cromakalim in a manner similar to that observed in dog isolated coronary artery. Results are the mean from 4 separate experiments with s.e.mean shown by vertical bars.

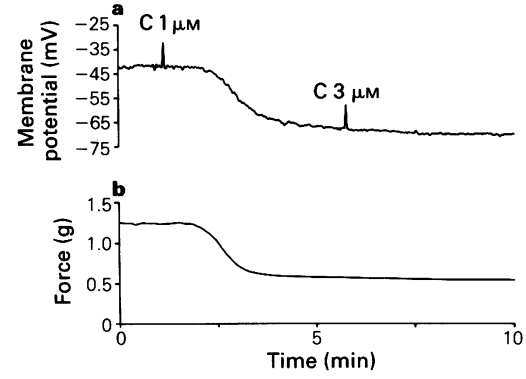

Figure 8 Representative trace from an electrophysiological experiment with rat isolated femoral artery. The artery was precontracted with U46619 (30 nM). Changes in membrane potential (a) and isometric force (b) were measured simultaneously. U46619 caused a marked depolarization in association with the contraction of the vessel (not shown). The two spikes are 10 mV event markers superimposed on the membrane potential output. Cromakalim (C) 1 μM and 3 μM was added as indicated by the event markers. Cromakalim caused relaxation and hyperpolarization thereby reversing the effects of U46619.

Mechanical experiments were also performed to characterize the interaction between cromakalim and phentolamine in the rat isolated femoral artery. Figure 7 shows mean concentration-response curves for cromakalim in the absence and in the presence of phentolamine (10 and 100 μM). As in the dog isolated coronary artery, phentolamine caused a marked attenuation of the cromakalim vasorelaxant response. pD_2 values for cromakalim were 6.27 ± 0.09 in the absence and 5.92 ± 0.05 ($n = 4$) in the presence of phentolamine (10 μM). The marked inhibition of the response to cromakalim in the presence of phentolamine (100 μM) precluded estimation of a pD_2 value for cromakalim. The maximum vasorelaxant response (tone caused by U46619 = 100%) was 97 ± 3, 45 ± 7 and 17 ± 3 ($n = 4$) in the absence and in the presence of phentolamine 10 and 100 μM respectively. Phentolamine (10 μM) also antagonized the relaxation caused by cromakalim in the guinea-pig basilar artery contracted with ouabain (10 μM, $n = 3$).

Electrophysiological effects

Electrophysiological studies were performed on the rat isolated femoral artery by the small vessel myograph technique. From the mechanical studies just described, a concentration of cromakalim (1 and 3 μM) was selected that caused approximately 75–80% of the maximal vasorelaxant response. The ability of phentolamine (30 μM) to affect the response to these two concentrations of cromakalim was monitored in the absence of tone and in the presence of tone produced by U46619 (30 nM). Cromakalim produced marked hyperpolarization of the rat isolated femoral artery in association with the vasorelaxant response (Figure 8). Phentolamine (30 μM) had no significant effect ($P > 0.05$; membrane potential or mechanical response). However it inhibited both the mechanical and electrophysiological responses to cromakalim. The mean data are shown in Figure 9. Under basal conditions the rat isolated femoral artery had a resting membrane potential of -59 ± 2 mV ($n = 6$). Cromakalim (1 and 3 μM) hyperpolarized the preparation to -68 ± 1 and -72 ± 1 mV ($n = 6$) respectively. U46619 (30 nM) caused a marked depolarization of the femoral artery from -62 ± 2 to -38 ± 4 mV ($n = 6$). In the presence of U46619, cromakalim (1 and 3 μM) again hyperpolarized the preparation to -59 ± 4 and -67 ± 2 mV ($n = 6$) respectively. Phentolamine (30 μM) significantly ($P < 0.05$, Bonferroni's t test) attenuated the hyperpolarization and mechanical responses to cromakalim (1 and 3 μM) (Figure 9).

Discussion

The major finding in this work is that phentolamine has a property additional to α-adrenoceptor antago-

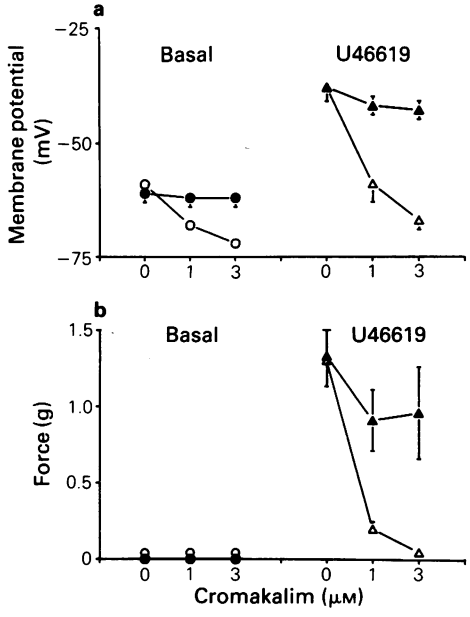

Figure 9 The interaction between cromakalim and phentolamine on membrane potential (a) and mechanical response (b) in the rat isolated femoral artery. Experiments were performed in the absence of tone (left side, Basal) or in the presence of active force (right side, U46619) induced by U44619 (30 nM). Measurements of membrane potential and force were made in the absence and in the presence of cromakalim (1 and 3 μM). The experiments were performed in the absence (open symbols) and in the presence (closed symbols) of phentolamine (30 μM). Results are the mean for 4 separate determinations with s.e.mean shown by vertical bars.

nism, that of antagonizing the novel vasorelaxant, cromakalim. If the current view is correct that cromakalim causes relaxation by opening K^+ channels and hyperpolarizing the cell membrane, then it is possible that phentolamine acts as a K^+ channel inhibitor. This mechanism of action of phentolamine is unrelated to α-adrenoceptor antagonism for the following reasons. First, phentolamine antagonized cromakalim responses at concentrations (10 to 100 μM) well in excess of those required to block α-adrenoceptors. For example, pA_2 values for phentolamine in rat mesenteric (7.1, McPherson *et al.*, 1984) and thoracic aorta (7.9, Digges & Summers, 1983) and guinea-pig spleen (8.3, Digges *et al.*, 1981) indicate a potency of phentolamine against α-adrenoceptors 100–1000 times greater than that found in the present study. Second, a number of α-adrenoceptor antagonists, prazosin, rauwolscine and phenoxybenzamine, were inactive in antagonizing the vasorelaxant action of cromakalim even when they were tested at concentrations 100–1000 fold in excess of that required to inhibit α-adrenoceptor activation. Phentolamine displayed similar activity on both the dog isolated coronary and rat isolated femoral artery. However, there were some quantitative differences. For example, in the dog isolated coronary artery phentolamine (10 μM) produced an approximately 10 fold rightward shift in the concentration-effect curve to cromakalim. In the rat however, the shift was only approximately 2 fold but the maximum response was substantially reduced. The greater apparent effectiveness of phentolamine in blocking cromakalim responses in the rat isolated femoral artery as opposed to the dog coronary artery may indicate some quantitative differences in the K^+ channels of these two vessels.

The interaction between cromakalim and phentolamine was also characterized in the guinea-pig basilar artery. We wished to determine whether the mechanism of action of the tone-inducing agent influenced the nature of the interaction. The finding that phentolamine blocked the response to cromakalim in the guinea-pig basilar artery precontracted with ouabain would indicate that this is not the case. On the basis of these experiments and those with the dog coronary and rat femoral artery it would appear that the ability of phentolamine to block the response to cromakalim is not species-dependent, not restricted to a particular vascular bed and not dependent on the agent used to induce vascular tone.

Phentolamine (10–100 μM), a concentration that significantly attenuated the response to cromakalim, did not alter the vasorelaxant responses to a number of other vasodilators i.e. isoprenaline, nitroprusside or nicorandil. The result with nicorandil was unexpected since this compound is thought to act as a K^+ channel opener (Inoue *et al.*, 1984) in a manner similar to cromakalim. However, apart from opening K^+ channels, it has been shown that nicorandil can also raise cyclic GMP levels as do other directly acting nitrovasodilators such as sodium nitroprusside (Weir & Weston, 1986b; Coldwell & Howlett, 1987). This dual mechanism of action displayed by nicorandil may account for the lack of antagonism displayed by phentolamine in the present study.

In the absence of constrictor tone, cromakalim caused a hyperpolarization in rat isolated femoral artery, a finding similar to that found in other studies with vascular and non-vascular tissue (Hamilton *et al.*, 1986; Weir & Weston, 1986a,b; Allen *et al.*, 1986; Hollingsworth *et al.*, 1987). When this vessel was exposed to U46619 there was a marked depolarization (approximately 30 mV) in association with contraction. Both effects were rapidly reversed by cromakalim. More importantly,

phentolamine effectively blocked both the mechanical and electrophysiological response. This finding suggests that cell hyperpolarization plays a pivotal role in the vasorelaxant effects of cromakalim in this particular blood vessel.

The mechanism of the interaction between cromakalim and phentolamine is unknown. If the results are interpreted assuming simple competitive kinetics it would appear that phentolamine behaves as a non-competitive antagonist since it depressed the maximum response to cromakalim but produced little or no shift in the concentration-effect curve. This interpretation assumes that the effects of cromakalim are mediated through a receptor mechanism and that phentolamine occupies this receptor but does not produce a response. However, it is currently unclear how the effects of cromakalim are mediated (i.e. receptor-mediated or a direct effect on the K^+ channel). Consequently the precise nature of the interaction between phentolamine and cromakalim should be viewed cautiously.

A number of compounds structurally related to phentolamine were tested for their ability to inhibit the vasorelaxant response caused by cromakalim. The structures of these compounds are shown in Figure 1. Three imidazolines (phentolamine, tolazoline and naphzaoline) and four imidazolidines (alinidine, clonidine, tolazoline and tramazoline) were tested. Although too few compounds were studied to examine structure-activity relationships in detail, some observations can be made. First, the ability to block the response to cromakalim was not related to the compound being an imidazoline or imidazolidine since both phentolamine and alinidine displayed equal potency. Second, the most potent compounds (alinidine and phentolamine) are tertiary amines with a benzene ring as a substituent. The idea that this configuration is important in determining activity is supported by the finding that alinidine (N-allyl clonidine) was most potent whereas clonidine was least potent. Clearly more work is required in this area.

The compounds used in this study display varying activity at α-adrenoceptors. Phentolamine and tolazoline behave as antagonists (Malta *et al.*, 1981). Naphazoline, clonidine, ST91, tramazoline are partial agonists (Malta *et al.*, 1981) while alinidine is effectively devoid of activity at α-adrenoceptors (Heinzow *et al.*, 1982). The pretreatment of the dog isolated coronary artery with phenoxybenzamine precluded the action of these compounds on the α-adrenoceptor. The result obtained with alinidine may be particularly significant. This compound causes bradycardia (see Heinzow *et al.*, 1982) which again appears to be unrelated to specific α- or β-adrenoceptor-mediated actions. In the heart, cromakalim causes a decrease in action potential duration (Scholtysik, 1987; Osterrieder, 1988) by increasing K^+ conductance. The bradycardia observed with alinidine could involve a similar mechanism (K^+ channel) to that activated by cromakalim. This possibility is currently being examined in our laboratory.

It has recently been proposed that the endothelium of vascular smooth muscle releases a hyperpolarizing factor (EDHF) which may be involved in the actions of some vasodilators such as acetylcholine (see Taylor & Weston, 1988). While the hyperpolarization is associated with a K^+ efflux similar to that observed with cromakalim, it is uncertain whether EDHF is the endogenous ligand at the cromakalim receptor. Given the present results, it is possible that compounds such as phentolamine or alinidine may be valuable experimental tools in this area of vascular research. For example, using these compounds we have shown that rat isolated mesenteric resistance vessels have a basal secretion of a hyperpolarizing factor which contributes to the resting membrane potential (McPherson & Angus, unpublished observations). The exact role of this hyperpolarizing compound in regulating blood pressure *in vivo* is now being investigated in our laboratory.

In conclusion the results of this study have shown that the action of cromakalim can be markedly attenuated by phentolamine and a number of other structurally related compounds. These compounds, or compounds more potent and devoid of other actions, may be valuable research tools to study the K^+ channel(s) regulating vascular smooth muscle tone.

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The action of dopamine and vascular dopamine (DA_1) receptor agonists on human isolated subcutaneous and omental small arteries

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1 Human small arteries were obtained from surgical specimens and studied *in vitro* by use of a myograph technique. Following induction of tone with a potassium depolarizing solution, dopamine in the presence of β-adrenoceptor and catecholamine uptake blockade relaxed isolated omental and subcutaneous arteries. Preincubation of tissues with phentolamine increased the maximum relaxation in response to dopamine.

2 The selective vascular dopamine receptor agonists, fenoldopam and SKF 38393 also relaxed isolated subcutaneous and omental arteries in a concentration-dependent manner. The order of potency for agonists was dopamine > fenoldopam > SKF 38393.

3 Dopamine-induced relaxation was competitively antagonized by SCH 23390, (**R**)- and (**S**)-sulpiride, and fenoldopam induced relaxation by SCH 23390 and (+)- but not (−)-butaclamol.

4 These results indicate the presence of vascular dopamine receptors (DA_1 subtype) on human isolated resistance arteries from omental and subcutaneous sites.

Introduction

Considerable evidence exists in many species to suggest the existence of vascular dopamine (DA) receptors in mediating vasodilatation (Goldberg, 1972; 1984). These receptors have been classified by Goldberg & Kohli (1979) as DA_1 subtype to differentiate them from the peripheral dopamine receptors present presynaptically on sympathetic nerves (DA_2 receptors).

DA_1 receptors have been demonstrated *in vitro* in a variety of preparations in several species (Brodde, 1982), including man (Ueda *et al.*, 1982; Toda, 1983; Forster *et al.*, 1983). However, these *in vitro* studies have generally been conducted using arteries of a size unlikely to contribute substantially to peripheral resistance (Mulvany, 1987). Mulvany & Halpern (1977) recently developed a technique which allows small arteries with an internal diameter less than 200 μm to be studied *in vitro*. Arteries of this diameter make an important contribution to peripheral resistance (Mulvany, 1987; Bohlen *et al.*, 1987). Consequently, we have used this technique to study the effect of dopamine and some selective DA_1 receptor agonists on human small isolated arteries. Some of this work has previously been presented to the British Pharmacological Society (Hughes & Sever, 1988).

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Methods

Arteries were obtained from tissue removed at surgery from a total of 31 patients (14 male, age range 25–80 years). Tissue was collected in cold physiological saline (PSS) of composition (mM): NaCl 118, KCl 4.7, $MgCl_2$ 1.2, $NaHCO_3$ 21, glucose 20, NaH_2PO_4 1, $CaCl_2$ 2.5, Na_2EDTA 0.002. Arteries (approximate external diameter 100–200 μm) were dissected free from surrounding tissue and mounted on two 40 μm wires in a myograph to allow measurement of isometric tension (Mulvany & Halpern, 1977). The myograph chamber contained 10 ml PSS aerated with 95% O_2:5% CO_2 maintained at 37°C. Following equilibration for 30 min the vessels were set to a resting internal circumference Lo corresponding to 0.9 L100, where L100 equals the internal circumference producing a wall tension equivalent to that produced by a distending

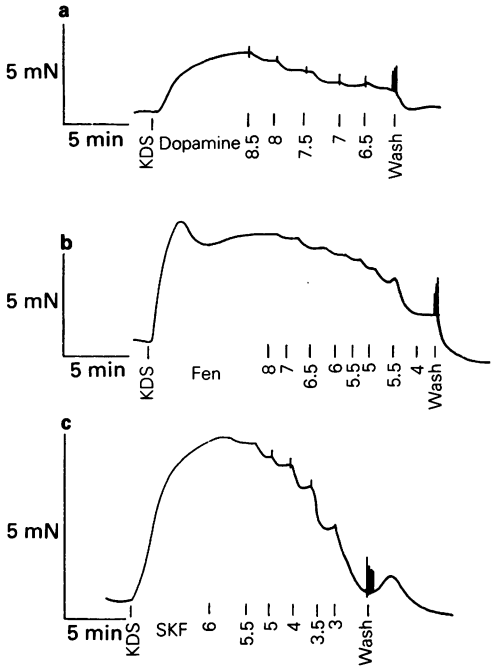

Figure 1 Representative traces showing the effect of dopamine and dopamine receptor agonists on isolated human arterioles: (a) omental arteriole internal diameter = 233 μm; (b) omental arteriole internal diameter = 485 μm; (c) omental arteriole internal diameter = 298 μm. Potassium depolarizing solution (KDS), dopamine, fenoldopam (Fen) or SKF 38393 (SKF) were added at the points shown, concentrations of drug are shown as $-\log$ [M].

pressure of 100 mmHg calculated from the Laplace relationship as described by Mulvany & Halpern (1977). Under these conditions vessels generated contractile responses to potassium-induced depolarization that were near to maximal (unpublished data).

Following a further 30 min equilibration, vessels were exposed to potassium depolarizing solution (KDS) composed of PSS in which the NaCl was replaced by an equimolar quantity of KCl (118 mM). Arteries not generating a tension equivalent to 90 mmHg (calculated by Laplace's relationship) were not used for further studies. Only 2 out of 60 vessels studied failed to fulfil this criterion. Following washout and recovery, the vessels were exposed to PSS containing noradrenaline (10^{-5} M). The functional integrity of the endothelium was assessed by the addition of acetylcholine (10^{-6} M) once stable tone had been induced by noradrenaline; relaxation was taken as indicative of release of endothelium-derived relaxing factor and the presence of a functional endothelium (Furchgott, 1983). In some vessels the endothelium was disrupted by passing a third 40 μm wire through the lumen of the vessel; the effectiveness of this procedure was confirmed by the abolition of relaxation in response to acetylcholine.

Concentration-response curves to dopamine, fenoldopam and SKF 38393 were generated by cumulative addition of agonist. Stable tone was induced by KDS and responses were calculated as % relaxation of KDS-induced tone. Propranolol (4×10^{-6} M), cocaine (10^{-5} M) and 17β-oestradiol (10^{-5} M) were present in studies where dopamine was the agonist to block β-adrenoceptors and catecholamine uptake processes respectively. Phentolamine (10^{-5} M) was also included in some studies with dopamine to block any possible α-adrenoceptor agonist action of this agent.

Concentration-response data obtained from individual arteries were fitted to a logistic function by use of a computer programme (Barlow, 1983) and values for EC_{50}, namely the concentration of agonist producing 50% of the maximum response to the same agonist, and maximum responses derived. pA_2 values were similarly calculated from data derived from individual vessels; after contraction by KDS a concentration-response curve to an agonist was generated in the absence of an antagonist. Following washout, the tissue was equilibrated with the appropriate concentration of antagonist for 20 min, contracted by KDS containing the antagonist at the appropriate concentration and the agonist concentration-response curve was repeated. The tissue was then washed and the same sequence of contraction and agonist concentration was used for a higher concentration of antagonist. Concentration-ratios of EC_{50} values in the presence and absence of antagonist were calculated and pA_2 values obtained by the method of Arunlakshana & Schild (1959). Values for EC_{50} are geometric means (95% confidence limits) and maximum responses are expressed as mean $\pm$ s.e.mean. pA_2 values and slope of the regression line are presented as means $\pm$ s.e.mean; deviation of the slope of the regression line from unity was tested by analysis of variance; $P < 0.05$ was taken as indicating statistical significance. Concentration-response curves have been drawn by the horizontal averaging method advocated by Carpenter (1986).

Drugs

Acetylcholine HCl (Sigma), cocaine HCl (McCarthy's), (+)- and (−)-butaclamol HCl (Research Biochemicals Inc.), dopamine HCl (Sigma), fenoldopam methanesulphonate (a gift from SK&F Ltd.), noradrenaline bitartrate (Sigma), 17β-oestradiol (Sigma), phentolamine mesylate (Ciba Geigy),

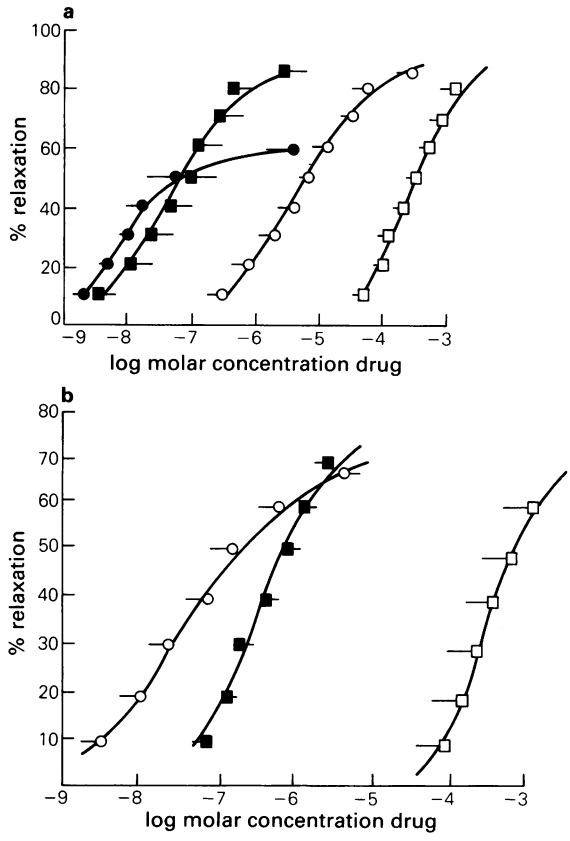

Figure 2 Concentration-response curves for dopamine and DA_1 receptor agonists: (a) in isolated omental arterioles, (●) dopamine ($n = 5$); (■) dopamine + phentolamine 10^{-5} M ($n = 15$); (○) fenoldopam ($n = 17$) and (□) SKF 38393 ($n = 5$). (b) In isolated subcutaneous arterioles, (○) dopamine + phentolamine 10^{-5} M ($n = 7$); (■) fenoldopam ($n = 4$); (□) SKF 38393 ($n = 3$). Points represent mean percentage relaxation of KDS-induced tone; s.e.means are indicated by horizontal bars.

(±)-propranolol HCl (Sigma), SCH 23390 ((R)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5,-tetrahydro-1H-3-benzazepine maleate, a gift from Schering Corp.), (R)- and (S)-sulpiride (gifts from Ravizza SpA), SKF 38393 HCl (1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol HCl, Research Biochemicals Inc.). (+)- and (−)-butaclamol, SCH 23390, and (R)- and (S)-sulpiride were made up freshly as 10^{-3} M stock solutions in methanol and 17β-oestradiol was made up as a 10^{-2} M stock solution in polyethylene glycol; these were then diluted appropriately in distilled water. Other drugs were made up in distilled water.

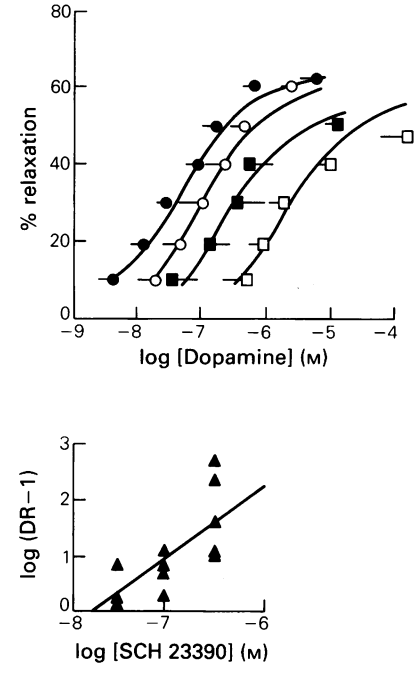

Figure 3 The effect of SCH 23390 on dopamine-induced relaxation of isolated subcutaneous arterioles: (●) dopamine alone ($n = 6$); (○) SCH 23390 3×10^{-8} M ($n = 3$); (■) SCH 23390 10^{-7} M ($n = 4$); (□) SCH 23390 3×10^{-7} M ($n = 5$); Points represent mean percentage relaxation of KDS-induced tone, s.e.means are indicated by horizontal bars. Schild analysis of the data is shown as an inset.

Results

Addition of dopamine usually had no effect on the tone of resting arteries, although occasionally a small contraction was observed in some vessels at concentrations greater than 10^{-6} M. Following contraction with KDS, addition of dopamine (10^{-9}–10^{-5} M) caused concentration-dependent relaxation of both omental and subcutaneous arteries (Figure 1, Figure 2). Preincubation with phentolamine (10^{-5} M) increased the maximum response to dopamine in omental arteries from 58 ± 16% ($n = 4$) to 86 ± 5% ($n = 15$) without causing much alteration in the EC_{50} for dopamine-induced relaxation, 2.19 (0.56–8.5) × 10^{-8} M in the absence of phentolamine and 3.8 (1.1–13.7) × 10^{-8} M in the presence of phentolamine (Figure 2a). No artery failed to relax in response to dopamine. Relaxation in response to dopamine was not dependent on a functional endothelium: EC_{50} and maximum response to dopamine was 3.8 (0.1–15) × 10^{-8} M and 74 ± 7% ($n = 5$) in arteries relaxing in response to acetylcholine compared with 3.2

(0.9–10.8) $\times 10^{-8}$ M and 67 ± 12% ($n = 5$) in those relaxing less than 10% to acetylcholine.

The selective DA_1 receptor agonists, fenoldopam and SKF 38393, both relaxed isolated omental and subcutaneous arteries in a concentration-dependent manner as can be seen in Figure 2a and b.

Responses to dopamine in the presence of phentolamine (10^{-5} M) were competitively antagonized by SCH 23390 (3×10^{-8}–3×10^{-7} M) in subcutaneous arteries. Schild analysis of these data gave a pA_2 value of 7.8 ± 0.2 with a slope of 1.3 ± 0.5 which was not significantly different from unity (Figure 3). (R) and (S)-sulpiride also antagonized dopamine-induced relaxation in omental arteries in the presence of phentolamine (10^{-5} M) ($pA_2 = 4.9 \pm 0.1$, slope = 1.3 ± 0.3 (NS), and $pA_2 = 4.7 \pm 0.1$, slope = 1.5 ± 0.4 (NS) respectively).

Fenoldopam-induced relaxation of isolated omental arteries was also competitively antagonized by SCH 23390 (3×10^{-8}–3×10^{-7} M) ($pA_2 = 7.6 \pm 0.1$, slope = 1.1 ± 0.2 (NS) (Figure 4) and by (+)-butaclamol (10^{-7}–10^{-6} M) ($pA_2 = 7.7 \pm 0.1$, slope = 1.2 ± 0.4 (NS)) (Figure 5) but not by (−)-butaclamol (10^{-6} M); the concentration-ratio for fenoldopam in the absence and the presence of (−)-butaclamol was 1.0 ± 0.1 ($n = 4$).

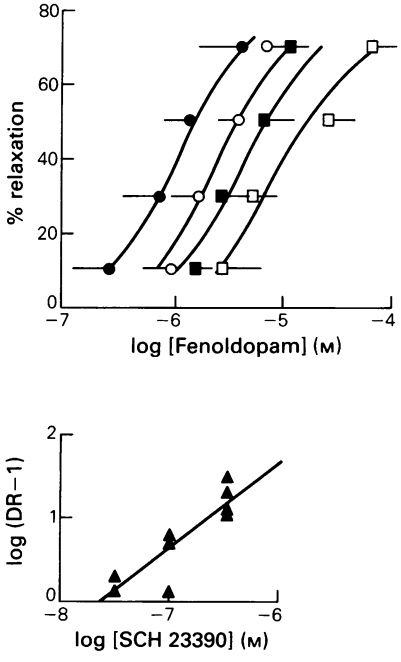

Figure 4 The effect of SCH 23390 on fenoldopam-induced relaxation in omental arterioles: (●) fenoldopam alone ($n = 5$); (○) SCH 23390 3×10^{-8} M ($n = 3$); (■) SCH 23390 10^{-7} M ($n = 3$); (□) SCH 23390 (3×10^{-7} M ($n = 4$). Points represent mean percentage relaxation of KDS-induced tone, s.e.means are indicated by horizontal lines. Schild analysis of the data is shown as an inset.

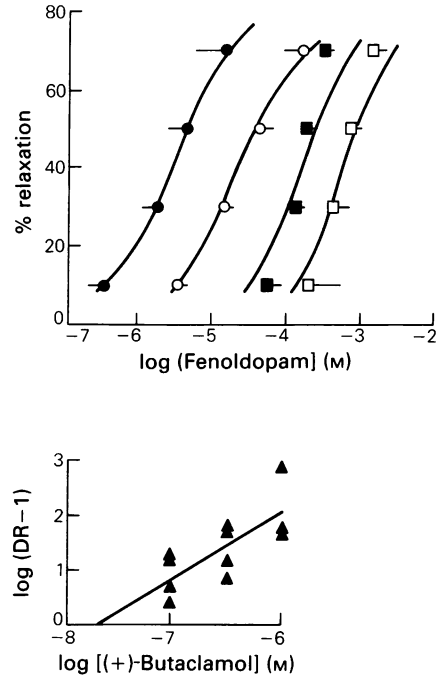

Figure 5 The effect of (+)-butaclamol on fenoldopam-induced relaxation in omental arterioles: (●) fenoldopam alone ($n = 6$); (○) (+)-butaclamol 10^{-7} M ($n = 4$); (■) (+)-butaclamol 3×10^{-7} M ($n = 4$); (□) (+)-butaclamol 10^{-6} M ($n = 4$). Points represent mean percentage relaxation of KDS-induced tone, s.e.means are indicated by horizontal lines. Schild analysis of the data is shown as an inset.

Analysis of the relationship between normalised internal diameter and both maximum response and EC_{50} for dopamine (in the presence of phentolamine) did not indicate any significant correlation between these variables, in contrast to results obtained with other agonists (Aalkjaer *et al.*, 1987; Nielsen *et al.*, 1987). Neither was there any correlation between the age or sex of the patient from whom tissue was obtained and the maximum response to or potency of dopamine or fenoldopam in these studies.

Discussion

These results indicate that dopamine and the selective dopamine receptor agonists, fenoldopam and

SKF 38393, relax human isolated omental and subcutaneous arteries. The relaxant effect of dopamine and fenoldopam observed in this study was competitively antagonized by the selective DA_1 receptor antagonists SCH 23390, (+)- but not (−)-butaclamol and weakly by (R)- and (S)-sulpiride. These findings suggest that the relaxant effect of dopamine and the selective dopamine agonists in this preparation are mediated by DA_1 receptors.

Comparison of these data with other studies of DA_1 receptors in man and other species show some differences. The finding that dopamine is a more potent agonist than SKF 38393 is similar to the report of Forster and colleagues (1983) in their study of human basilar arteries. Fenoldopam was not studied by these workers but we have found it to be a full agonist in human isolated cerebral arteries (unpublished data). In contrast, (Hilditch & Drew, 1981; 1985b) found both fenoldopam and SKF 38393 to be inactive or weak partial agonists in isolated splenic artery of the rabbit, whereas Ohlstein and others (1984) found fenoldopam to be a full agonist in this preparation. Brodde (1982) found SKF 38393 to be a partial agonist but more potent than dopamine in rabbit isolated mesenteric artery and Edwards (1986) found fenoldopam and dopamine to have similar potencies in rabbit renal arterioles *in vitro*. Using the perfused rat kidney *in situ*, Schmidt's group (Schmidt, *et al.*, 1982; 1985) have reported an agonist potency series of fenoldopam > SKF 38393 ≫ dopamine and in the cat Edvinsson and coworkers (1985) also reported that SKF 38393 was more potent than dopamine as a relaxant of cerebral arterioles *in situ*.

In the case of the antagonist studies, there are also some anomalous results with reference to the literature. In our study sulpiride was a weak antagonist of the DA_1 receptor and showed little enantiomeric selectivity. This finding contrasts with the enantiomeric selectivity of sulpiride ((S)- > (R)-) at DA_2 receptors and is similar to a previous report on this drug as a DA_1 antagonist (Schmidt *et al.*, 1983). Similarly, most studies but not all (see Hilditch & Drew 1985b; Brodde, 1982) find racemic sulpiride to be a weak DA_1 receptor antagonist. The pA_2 value for (+)-butaclamol obtained in these studies (7.7) is around ten fold higher than reported by Brodde (1982) in rabbit isolated mesenteric artery but around ten fold lower than that obtained by Schmidt & Imbs (1980) in the *in situ* perfused rat kidney and by Drew in isolated rabbit splenic artery (cited in Brodde, 1982). The pA_2 value calculated for SCH 23390 in these studies is also lower than that reported in most other studies; $pA_2 = 10.65$ in rabbit splenic artery (Hilditch & Drew 1985a), $pA_2 = 9.7$ in rat perfused kidney (Schmidt *et al.*, 1987), $K_i = 10^{-9}$ M in cultured smooth muscle cells obtained from rat mesenteric artery (Balmforth *et al.*, 1988), IC_{50} between 10^{-9} M and 10^{-8} M in cat cerebral arteries *in situ* (Edvinsson *et al.*, 1985). The reasons for these discrepancies are unclear. Inter-species variation cannot be excluded. It is also possible that some differences relate to the contractile agonist used or the use of phenoxybenzamine, an irreversible α-adrenoceptor antagonist with weak dopamine receptor antagonist properties (Walton *et al.*, 1978) in many of these studies. In this context, Berkowitz & Ohlstein (1984) have reported that the K_B value for SKF 83566, a DA_1 receptor antagonist, structurally related to SCH 23390 differs by almost 1000 fold when calculated using dopamine as an agonist in splenic arterial rings treated with phenoxybenzamine and compared with the value obtained with fenoldopam in untreated rings. Alternatively, the possibility of the existence of different subtypes or affinity states of the DA_1 receptor as previously suggested by Hilditch & Drew (1987), and by other workers on the basis of ligand binding studies of the central D_1 receptor (Andersen & Braestrup 1986; Bzowej *et al.*, 1988) cannot be ruled out. Further work is necessary to clarify these issues.

No evidence was found in these studies to indicate an involvement of the endothelium in the response to DA_1 agonists. However, since endothelium-dependent relaxation is usually difficult to demonstrate using potassium-induced tone (Furchgott 1983) this does not necessarily exclude any role for the endothelium in mediating responses to dopamine. Nevertheless, it seems likely that the effects observed in this study represent action of these agonists solely on DA_1 receptors located on vascular smooth muscle.

These studies were conducted on isolated arteries small enough to contribute significantly to peripheral resistance (Mulvany, 1987). In contrast to previous studies in larger arteries (Ueda *et al.*, 1982; Forster *et al.*, 1983), dopamine-induced relaxation was seen in the absence of α-adrenoceptor blockade and relaxant responses to dopamine and dopamine agonists were consistently found in all the vessels studied. Furthermore, dopamine was found to be more potent in these studies of resistance arteries than has been found in previous work on larger arteries (Ueda *et al.*, 1982; Forster *et al.*, 1983). It is interesting that Edwards (1985) also found that dopamine was a considerably more potent relaxant of renal afferent and efferent arterioles than interlobular arteries in the rabbit. These findings may therefore suggest that the concentration of dopamine necessary *in vivo* to produce vasodilatation of resistance arteries may be less than has been inferred from previous studies of large arteries and while the concentrations of dopamine found to relax isolated resistance arteries in this study considerably exceed

plasma levels of free dopamine, the levels of dopamine occurring in the vicinity of dopaminergic nerves such as those identified by Bell and colleagues in the dog kidney (Bell *et al.*, 1978) and paw pad (Bell & Lang, 1979) could well be sufficient to activate DA_1 receptors in the vasculature.

At present the physiological role of DA_1 receptors in the vasculature is not understood. However, in view of the marked vasodilator and hypotensive effects of DA_1 agonists *in vivo* (Stote *et al.*, 1983; Hughes *et al.*, 1987) an important role for DA_1 receptors in cardiovascular physiology cannot be excluded.

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The dihydropyridine niguldipine modulates calcium and potassium currents in vascular smooth muscle cells

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1 Vascular smooth muscle cells were isolated from the portal vein and from pial vessels of the cow. They were voltage-clamped with a single patch electrode technique (whole cell recording) in order to analyse the effects of niguldipine on ionic membrane currents. Due to adsorption of niguldipine to plastic and glass, the effective concentrations are lower than the nominal concentrations by a factor of about 3.

2 Niguldipine reduced Ca-currents (I_{Ca} of the L-type, voltage operated) at nominal concentrations $>0.1\,\mu$M up to a complete block at $1\,\mu$M (50% block at $0.4\,\mu$M). Nominal concentrations between 50 and 200 nM facilitated I_{Ca} ('Ca-agonistic effect'). The Ca-agonistic effects of niguldipine showed modest use- but strong voltage-dependence.

3 Niguldipine increased the outward currents at nominal concentrations >10 nM. The extra outward currents reversed at -85 mV, the result suggesting that niguldipine had increased potassium currents, I_K. Maximal facilitation of I_K by niguldipine was about 400% and was obtained at $1\,\mu$M, half-maximal facilitation was obtained with a nominal concentration of 20 nM.

4 Both reduction of I_{Ca} and facilitation of I_K may contribute to vasodilatation by niguldipine. Due to its greater sensitivity, the effects on I_K may dominate.

Introduction

The compound niguldipine HCl, (±)-3-methyl-5-[3-(4,4-diphenyl-1-piperidinyl)-propyl]-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-pyridine-3,5-dicarboxylatehydrochloride, is a novel antihypertensive drug, structurally related to the dihydropyridine calcium entry blockers (Sanders & Kolassa, 1986). It has been demonstrated that its antihypertensive potency in animals exceeds that of dihydropyridine-congeners, that the onset of action is retarded and that its duration of action is prolonged. Thus, a once daily administration in man may be possible (Sanders & Kolassa, 1986; Sanders *et al.*, 1987).

At the final, postsynaptic level of the vascular smooth muscle cell, an anti-hypertensive drug can relax the vessel via different mechanisms: Ca antagonists hinder the entry of Ca ions from the extracellular space into the cytosol. Furthermore, treatment with Ca antagonists reduces the intracellular Ca load. Both processes reduce the degree of contractile activation via the amount of activator calcium. The principle of Ca antagonists is well established in the treatment of hypertension.

Recently, activation or facilitation of K channels ('K-agonism') has been introduced as another principle of vasodilatation. Drugs that facilitate outward potassium currents can hyperpolarize the membrane, thereby preventing the Ca entry through voltage operated Ca-channels. This may explain the action of BRL 34915, which can relax vascular and tracheal smooth muscle tissue (Hamilton *et al.*, 1986; Weir & Weston, 1986a,b; Allen *et al.*, 1986).

To determine which of the above principles might be responsible for the anti-hypertensive effects of niguldipine, the present study was undertaken. Ca-antagonism or K-agonism induce electrophysiological changes measurable with the voltage clamp technique. This powerful method analyses the transmembrane movement of Ca and K ions by measuring the corresponding ionic currents. It has been successfully applied to isolated visceral and vascular smooth muscle cells (Brown *et al.*, 1986; Benham *et al.*, 1987; Klöckner & Isenberg, 1985a,b,c; 1986). Therefore, in the present study the voltage-clamp technique was used to analyse the effects of niguldipine on the ionic membrane currents and to define its mode of action at the level of the smooth muscle cell.

Methods

The experiments were performed on vascular myocytes isolated from the portal vein or from the pial vessels of the cow. The principles for cell isolation have been described recently for the urinary

bladder (Klöckner & Isenberg, 1985a). The protocol for the bovine vessels was similar, and here only a brief outline is given. The processes of cell isolation as well as the electrophysiological experiments were performed at 35°C.

Portal vein The muscularis was separated from the adventitia and the intima. Chunks of muscular tissue (diameter about 2 mm) were stirred in 25 ml of a Ca-free solution composition, mM: NaCl 90, KH_2PO_4 1.2, $MgCl_2$ 5, glucose 20, taurine 50 and HEPES 5, adjusted to pH 7.1 with NaOH, for 6 periods of 5 min each. Thereafter, the chunks were incubated in the same calcium-free solution but complemented by 1.5 mg per ml collagenase (Sigma, St. Louis, C0130) and 1 mg per ml pronase (Serva, Heidelberg, Germany, Type E). The enzyme treatment delivered only broken material within the first incubation period (30 min) but numerous elongated cells after 60 and 90 min. The cells were harvested in the supernatant and suspended in KB-medium (composition, mM: KCl 85, K_2HPO_4 30, $MgSO_4$ 5, Na_2 ATP 2, Na-pyruvate 5, creatine 5, taurine 20, 5-OH-β-butyrate 5, fatty acid free albumin 1 gl^{-1} adjusted with KOH to pH 7.4) for storage at 5°C. For the experiments, cells were superfused with physiological salt solution (PSS) (composition, mM: NaCl 150, KCl 5.4, $CaCl_2$ 3.6, $MgCl_2$ 1.2, glucose 10, HEPES 5, adjusted with NaOH to pH 7.4). At 35°C they had resting potentials between −55 and −68 mV.

Pial vessels Vessels having diameters between 0.1 and 0.5 mm were selected and cut from the surface of the brain. They were opened from the inside, which resulted in thin sheets 2 mm long and 1 mm wide. The sheets were stirred in the calcium-free solution for 6 periods of 5 min each. For enzymatic cell dissociation, the calcium-free solution was complemented with 0.5 mg per ml pronase (Serva, Heidelberg, Germany, Type E), 0.5 mg per ml collagenase (Sigma, St. Louis, C0130), 10 nM isoprenaline (Sigma, St. Louis) and 2 mg per ml fatty acid-free albumin (Sigma, St. Louis). The specimens were stirred for 3 × 15 min each, and the supernatant with the isolated cells was harvested. The cells were suspended and stored in KB-medium for up to 4 days (at 5°C). In PSS at 35°C the cells had resting potentials between −50 and −65 mV.

For the experiment, a drop of the KB-medium containing the cells was pipetted into the experimental chamber (volume 100 μl). When the cells had settled down to the glass bottom, they were continuously superfused with a physiological salt solution (PSS). For the electrical measurements, we used patch electrodes in the 'whole cell clamp mode' (Hamill *et al.*, 1981). The tips were fire polished to an inner diameter of about 1 μm. When filled, they had resistances of about 3 MΩ. The Cs-electrode solution (composition, mM: CsCl 130, Na-pyruvate 5, Cs-oxalacetate 5, Cs-succinate 5, EGTA 1, HEPES 10, adjusted with CsOH to pH 7.4) was used for the experiments in part A, where the Ca antagonistic effects of niguldipine were analysed because intracellular Cs ions depress the potassium currents (Klöckner & Isenberg, 1985a,b). The amplitude of I_{Ca} was defined as the most negative net negative current surge. The K-electrode solution (composition, mM: KCl 140, Na_2ATP 2, HEPES 10, EGTA 0.1, pH adjusted with KOH to 7.4) was used in part B, where the K agonistic effects of niguldipine were investigated. The electrodes were connected via Ag/AgCl wires to an input amplifier with facilities for constant current injection (see Klöckner & Isenberg, 1985a). A PDP 11-23 minicomputer generated the pulse protocol, digitized the recorded membrane currents (1024 points of 12 bit resolution) and stored them. The data were not corrected for leakage and capacitive currents. The illustrations are playbacks from the computer to a graphics terminal with line printer.

The compound niguldipine was obtained from Byk Gulden Pharmaceuticals, Konstanz (batch number UL 19/114). A 2 mM stock solution of niguldipine was prepared by dissolving 13 mg of the drug in 10 ml of DMSO at 40–50°C. Adequate aliquots of this stock solution were dissolved in the PSS to a final concentration between 1 nM and 1 μM. The aqueous solutions were freshly prepared every day.

Dihydropyridine type calcium antagonists and niguldipine adsorb with high affinity to plastic and glass (deJong & Huizer, 1984). Chemical analysis of the PSS containing nominally 1 μM niguldipine from the superfusate in the experimental chamber revealed that the effective free concentration was only 0.3 μM. After washing the chamber with drug-free solution for 5 min, the concentration of niguldipine was below the detection limit (< 5 nM). Chemical analysis of these samples was performed in the laboratories of Byk Gulden, Konstanz, using h.p.l.c. and ultraviolet detection. All concentrations refer to nominal applied concentrations of niguldipine; the 'free' concentrations are approximately one third of these values.

Results

(A) *Calcium currents under the influence of niguldipine*

Calcium currents flow mostly through L-type Ca-channels In vascular myocytes, two types of Ca-channel currents have been described. According to the terminology of Benham *et al.* (1987) a long

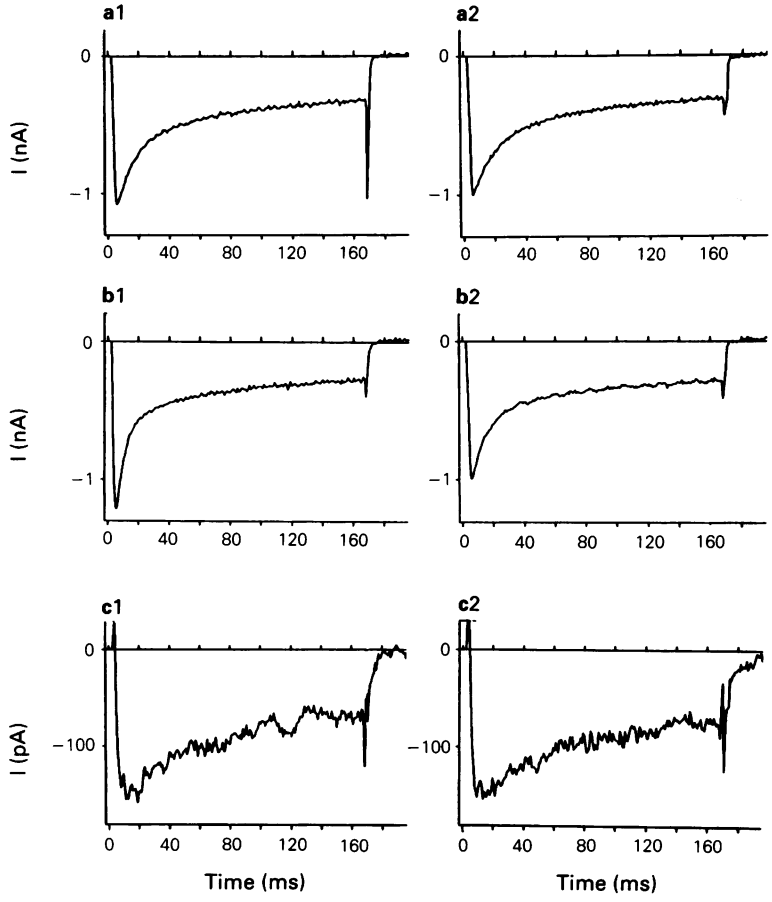

Figure 1 Separation of the Ca-channel current into L- and T-components. (a and b) Myocyte from portal vein, PSS containing 3.6 mM $CaCl_2$ without (a1, b1) or with 50 μM $NiCl_2$ (a2, b2). Pulses, duration 170 ms, to −5 mV were applied from a holding potential of −65 mV (a) or −95 mV (b). (c) Myocyte from pial vessels, PSS containing 20 mM $BaCl_2$. Clamp steps to +5 mV started from a holding potential of −55 mV (c1) or −95 mV (c2).

lasting or L-type current is distinguished from a transient or T-type current. In the myocytes analysed here, the L-type current seems to dominate I_{Ca}. We demonstrate this by blocking the T-channel with 50 μM nickel (Cerbai *et al.*, 1988) or by changing the degree of T-channel inactivation with the holding potential.

Figure 1a shows I_{Ca} of a myocyte of the bovine portal vein. The membrane was held at −65 mV and the depolarizations stepped to −5 mV (0.1 Hz). Under control conditions (panel a1), I_{Ca} peaked with 3 ms to −1.10 nA. Nickel reduced this peak to 1.02 nA (panel a2). If the T-type current is defined as the difference current, it amounts to −0.08 nA or to 7.3% of total I_{Ca}. The record of panel (a) represents an example where the T-component was large. For a total of 10 cells investigated with a holding potential of −65 mV, the contribution of channel current was 4.5 ± 2% of total I_{Ca} on average (mean ± s.e.mean).

Hyperpolarization is known to increase the contribution of the T-channel current (cf. Benham *et al.*, 1987). Therefore, we repeated the analysis with a holding potential of −95 mV (panel b). Peak I_{Ca} was now −1.24 nA before and −1.01 nA after addition of nickel, respectively. Thus the contribution of the nickel-sensitive component to total I_{Ca} was 19% for (b) or 13 ± 4% ($n = 5$) on average. When we used a holding potential of −45 mV, peak I_{Ca} was −0.903 nA and −0.912 nA in the absence and presence of 0.05 mM nickel, respectively. Thus, the more positive holding potential had inactivated the T-channel component.

I_{Ca}, as it is studied in Ca-containing PSS, inactivates along a two exponential time course (time

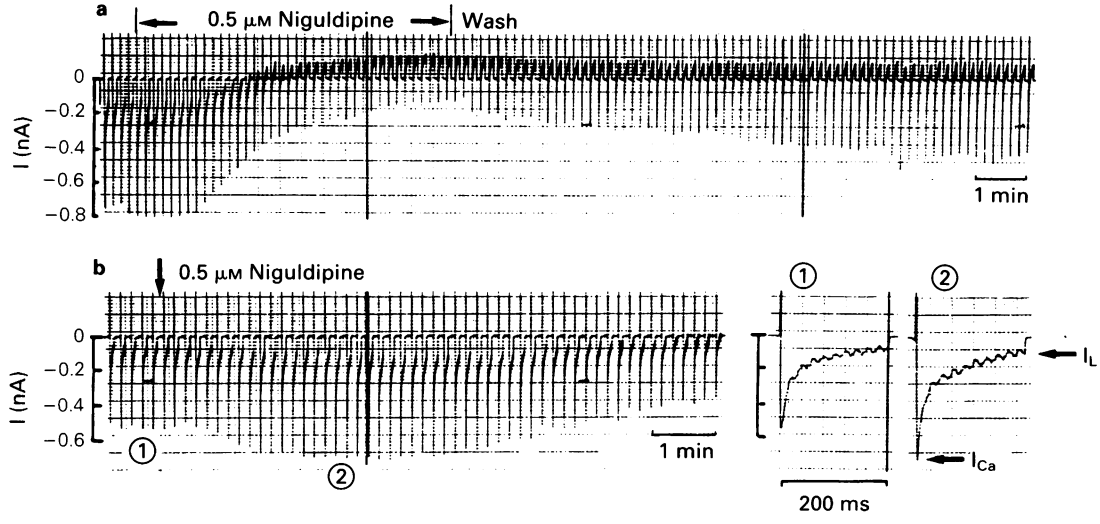

Figure 2 Effect of 0.5 μM niguldipine on the calcium current (I_{Ca}) (myocyte from portal vein). Membrane currents in response to 200 ms clamp steps from −65 mV (holding potential) to −5 mV (pulse potential). Peak I_{Ca} is indicated by the downward deflections. At the end of the pulse, the 'late current' I_L is measured. Pulsing rate 0.2 Hz. The tracings are computer printouts of stored data and not an on line record. For reasons of space, the pulse interval is shortened. (a) Niguldipine reduces within 5 min I_{Ca} from −0.78 nA to −0.15 nA (by 81%) and induces a positive I_L of 0.2 nA. The effect is partially reversed upon washout of the drug. (b) Ca-agonistic effect of niguldipine; 90 s after drug application, I_{Ca} was increased from −0.53 nA (inset 1) to −0.7 nA (inset 2).

constants about 6 and 60 ms) to a non-inactivating component of about 25%. Application of 50 μM nickel reduced the fast exponential but did not block it, i.e. in the presence of nickel the fit of inactivation required two exponentials and the non-inactivating component. The same complex description was necessary when the T-channel component was inactivated with a holding potential of −45 mV. These results suggest that the inactivation time course alone is not an adequate criterion for separation of I_{Ca} into T- and L-channel components.

In myocytes from pial vessels the amplitude of I_{Ca} was small. In order to improve the signal-to-noise ratio and to facilitate the separation of L- and T-type currents (Benham *et al.*, 1987), we substituted in the PSS the 3.6 mM $CaCl_2$ by 20 mM $BaCl_2$ (panel c1 of Figure 1). The intervention is known to shift the gating parameters by more extensive screening of surface charges (see Wilson *et al.*, 1983). We corrected for this shift by setting the holding and step potential to −55 and +5 mV, respectively. In the Ca-free Ba solution, one expects the Ca-mediated inactivation component to be absent. Figure 1 (c1) shows the Ba-current to inactivate slowly and along a single exponential (time constant about 60 ms) to the non-inactivating component. The peak value of this current was not modified when the holding potential was changed from −55 mV (c1: −161 pA) to −95 mV (c2: −159 pA). When we added 0.05 mM nickel to the bath, the current did not change either. A similar ineffectiveness of strong hyperpolarizations or of 50 μM nickel on calcium channel current was observed in 7 other myocytes (either 3.6 mM $CaCl_2$ or 20 mM $BaCl_2$) suggesting that the contribution of T-channel current to the I_{Ca} of pial cells is insignificant.

The results suggest that Ca currents, when elicited from a holding potential close to the resting potential (−65 mV), are mostly L-type currents (Benham *et al.*, 1987). We therefore used a holding potential of −65 mV because it is close to the resting potential and because it effectively removes inactivation of I_{Ca} both in the presence and the absence of the drug.

Ca-antagonistic effects of niguldipine Most experiments were performed on smooth muscle cells isolated from the portal vein of the cow. Examples of the effects of 0.5 μM niguldipine on I_{Ca} are illustrated in Figure 2. Niguldipine (0.5 μM) reduced I_{Ca} within 8 min to 20 ± 8% of control (mean ± s.e.mean; $n = 8$). The reduction of I_{Ca} by 0.5 μM niguldipine did not start immediately after drug administration, but after an initial delay of 1–2 min. During this period, I_{Ca} exceeded the control level by 20–40% ('Ca-agonistic effect', see panel at the bottom of Figure 2). When niguldipine was applied for less than 8 min,

removal of the drug led to a partial recovery of I_{Ca}. The effects of exposure to niguldipine for longer than 10 min could not be reversed by washing for 20 min.

'Use dependence' of the Ca-antagonistic niguldipine effect The reduction of I_{Ca} by Ca antagonists of the verapamil type has been shown to depend on the number of depolarizations applied. This suggests that the channel must open or inactivate before the drug can interact with its binding site (Ehara & Kaufmann, 1978; McDonald *et al.*, 1980; Klöckner & Isenberg, 1986). For such a block, the expressions use-dependence or frequency-dependence are taken to be synonymous.

To evaluate the use-dependence, niguldipine (0.5 μM) was applied under resting conditions for 3 min. The first pulse after the rest evoked an I_{Ca} that was already strongly attenuated; on average the 'initial block' amounted to $62 \pm 7\%$ ($n = 4$, 3–4 min exposure time). The following repetitive pulsing further reduced I_{Ca}. At steady state, niguldipine reduced I_{Ca} by $80 \pm 8\%$ ($n = 8$). Thus, the conditioned block was $18 \pm 8\%$. This number is small for a Ca-antagonist of the dihydropyridine type (compare Sanguinetti & Kass, 1984).

Suppression of I_{Ca} depends on the potential of the clamp step The influence of the clamp step potential on the voltage operated calcium channel is shown in Figure 3. Beyond a threshold potential of −35 mV, the amplitude of I_{Ca} increased at more positive potentials up to −5 mV, but it fell again when the potential became positive. The corresponding I-V curve (Figure 3a) shows a descending branch that reaches maximal inward currents at −5 mV (at 0 mV on average, $n = 15$). At stronger depolarizations, the I-V curve ascended again and intersected the voltage axis of about +50 mV. Niguldipine (0.5 μM) reduced I_{Ca} over the whole range of test potentials and changed the shape of the I-V curve moderately (see below).

Niguldipine shifted the intersection of the ascending branch with the voltage axis by about −5 mV, which could indicate an increase in superimposed outward currents (Figure 3). Thus niguldipine modified not only I_{Ca} but also the late current, I_L, as well as the currents at pulses negative to the threshold potential of I_{Ca}. Under control conditions, the currents negative to −40 mV are almost zero. Five min after application of niguldipine, the I-V curve was shifted in an outward direction (Figure 3b). This increase in outward current also holds true for the potentials positive to −40 mV: the curve of I_L lies above the voltage axis, whereas the control curve was below. Neither the induction of outward currents at potentials negative to the I_{Ca} threshold nor the positivity of net currents can be attributed to Ca-antagonistic effects. Therefore, we postulate that the increase in I_L results from additional effects on a potassium channel. this 'K-agonist effect' will be described in section B.

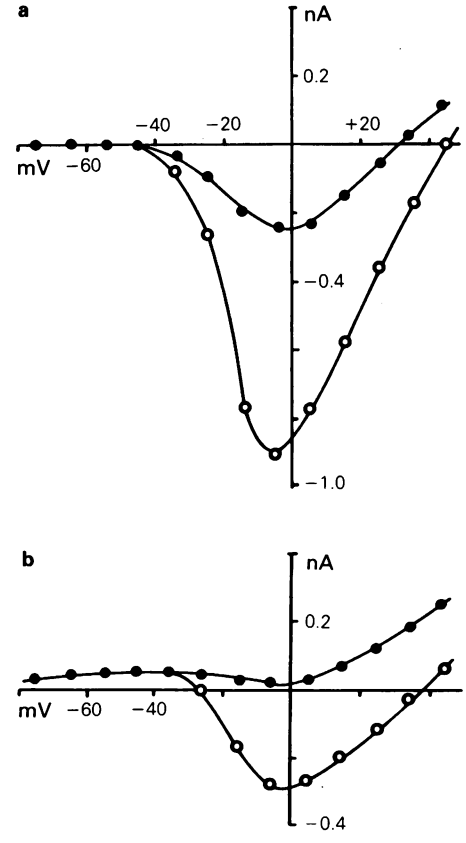

Figure 3 Current-voltage relation of the calcium current (I_{Ca}, ○) and 'late current' (I_L, ●) in control (a) and in the presence of 0.5 μM niguldipine (b). Data obtained with a double pulse protocol. Starting from a holding potential of −65 mV, a conditioning 170 ms long pre-pulse went to voltages between −100 and +50 mV. After a 20 ms repolarization to −65 mV, the 170 ms long test-step to +5 mV started. In this figure, the I_{Ca} and I_L were evaluated as a function of the pre-pulse.

Figure 4 shows the steady-state activation and inactivation curve of I_{Ca} in control solution and during niguldipine. The activation curve was plotted from the data of Figure 3 by dividing the current by the driving force (clamp step potential minus reversal potential) and normalizing the result to give 1 for maximal activation at +35 mV. The activation curve was fitted by the Boltzmann-equation (see legend to Figure 4) adjusting the parameters k (slope of the curve) and V_h (potential of half-maximal activation).

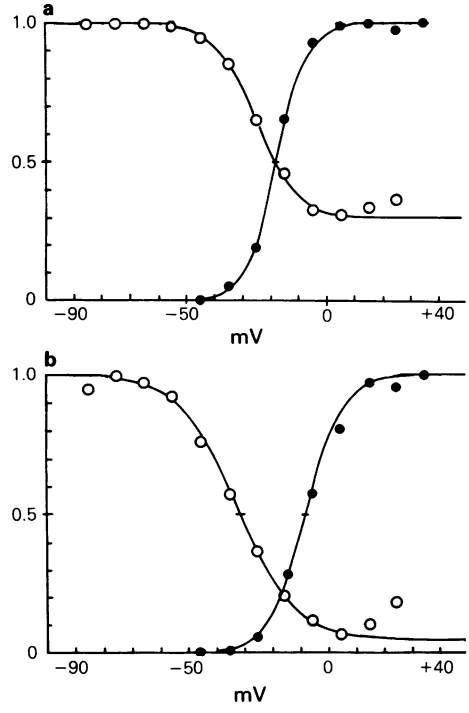

Figure 4 Steady-state activation (●) and inactivation curve (○) of the calcium current (I_{Ca}). (a) Before and (b) after exposure to 0.5 μM niguldipine. The activation curves were fitted with $d_\infty = (1 + \exp[(V - V_h)/k])^{-1}$, where the slope factor k was −7 mV and −8 mV for control and 0.5 μM niguldipine, respectively, and the potential of half-maximal activation (V_h) was −9 mV and −8 mV. The inactivation curve was fitted with $f_\infty = R + (1 - R)(1 + \exp[(V - V_h)/k])^{-1}$. Niguldipine changed k from +7 to +10 mV, V_h from −25 mV to −32 mV, and the residual or non-inactivating fraction R from 32% to 5%.

Niguldipine did not significantly modify these 2 parameters which suggests that the voltage-dependence of activation is not modified.

The inactivation curve was analysed with a paired-pulse protocol. I_{Ca} evoked by a test step to +5 mV (second of the paired pulses) was plotted against the potential of the pre-step. (The curves only approximate the steady state inactivation, since the 170 ms prepulse did not completely inactivate I_{Ca}). Under control conditions, the curve is bell-shaped with a potential of half-maximal availability of −18 mV. Niguldipine 0.5 μM depressed the availability over all of its range, shifted the curve to the left and changed its shape. In terms of the inactivation parameters, the potential of half-maximal inactivation was shifted from -19 ± 7 mV to -37 ± 8 mV ($n = 6$) and the slope factor (of the descending branch) was increased from $+7 \pm 1.2$ to $+11 \pm 2$ mV. Finally, the residual fraction of I_{Ca}, i.e. the fraction that did not inactivate during the 170 ms prepulse to 0 mV, was reduced from $31 \pm 4\%$ to $6 \pm 4\%$ ($n = 6$). These results suggest that niguldipine modifies the voltage-dependence of the steady-state inactivation curve in such a way that membrane depolarizations, evoked either artificially by pre-steps or naturally by transmitters, potentiate the Ca-antagonistic effect of niguldipine. On the other hand, more negative potentials (i.e. membrane hyperpolarizations) attenuate the effects. Thus, niguldipine reduced I_{Ca} by 60% when the pulse started from −85 mV, by 80% after the pre-pulse to −25 mV, and by 94% after the pre-pulse to 0 mV.

The Ca-agonistic effects of niguldipine Figure 2 has already shown that 0.5 μM niguldipine transiently facilitated I_{Ca} by a factor of 1.4. Transient enlargement of I_{Ca} by 0.5 μM niguldipine was seen in 5 of the 8 experiments. Usually, the effect peaked within 1–3 min and disappeared within the following 5–8 min. We attribute the transient 'Ca-agonistic effect' to a slow increase in niguldipine concentration near to the channel, whereby low niguldipine concentrations increase I_{Ca}. Such an argument is supported by the steady-state effect of 50 or 100 nM niguldipine, which consistently increased I_{Ca}. The maximal increase amounts to 30% (Figure 5). Ca-agonistic effects of low concentrations of Ca-antagonists have been found repeatedly for dihydropyridine derivatives (Hess *et al.*, 1984; Brown *et al.*, 1986).

Concentration-response curve for the effects of niguldipine on I_{Ca} Figure 5 summarizes the pooled responses for a total of 17 vascular myocytes (from both portal vein and pial vessels). No material differences were observed in the two cell types. The curve shows no effects of niguldipine at concentrations below 30 nM, a Ca-agonistic effect between 30 and 180 nM (the curve exceeds 1), and a Ca-antagonistic effect at higher concentrations. Due to adsorption of niguldipine to plastic and glass, the effective concentrations are probably lower than the nominal concentrations by a factor of 3 (see Methods).

(B) *Niguldipine facilitates potassium currents*

Niguldipine induces extra outward currents Figure 2 shows that niguldipine, besides reducing the calcium inward current I_{Ca}, induces extra outward currents. The late current (I_L), which was close to zero or inward before application of the drug, became outward. In these experiments, the patch electrodes were filled with the Cs-electrode solution to block the outward potassium currents (Klöckner & Isenberg, 1985b). Therefore, under control conditions,

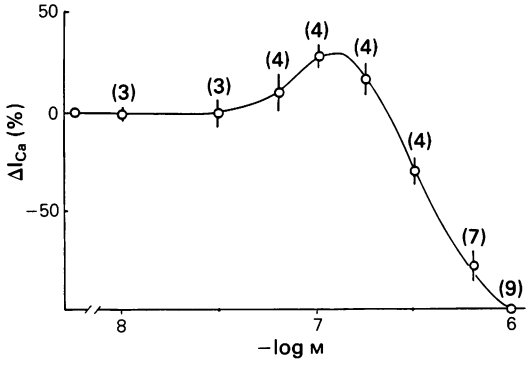

Figure 5 Concentration-response curve for the effect of niguldipine on the calcium current (I_{Ca}). Ordinate scale: current in the presence of niguldipine ($*I_{Ca}$) normalized to $\Delta I_{Ca} = (*I_{Ca} - I_{Ca})/I_{Ca}$. The −log of the drug concentration is plotted on the abscissa scale. The symbols represent the mean of the number of experiments in parentheses; vertical lines show s.e.mean.

the current I_L may result from a fraction of I_{Ca} that had not inactivated within the period of 170 ms. As expected for a 'residual I_{Ca}', I_L was almost zero at all potentials negative to −35 mV (the threshold of I_{Ca}), and at more positive potentials its voltage-dependence followed by voltage-dependence of peak I_{Ca} (Figure 3a).

Under the influence of niguldipine, I_L became outward. A positive or outwardly directed current cannot be explained as non-inactivating, inwardly directed I_{Ca}. Furthermore, these outward currents were also recorded at potentials negative to the threshold of I_{Ca} (−35 mV). Finally, the voltage-dependence of I_L was changed such that it no longer paralleled the voltage-dependence of peak I_{Ca}. Therefore, it is unlikely that the niguldipine-induced outward currents flow through calcium channels. We suggest that niguldipine modified K-channels, and that the current is carried by either K or Cs ions. In order to investigate the effect of niguldipine on the outward potassium currents, additional experiments were performed using electrodes filled with K-electrode solution (see Methods). The effect of 0.1 μM niguldipine on the potassium outward currents is illustrated in Figures 6 and 7. Figure 6 shows the results recorded from a smooth muscle cell of the portal vein. Under control conditions, an outwardly directed current flowed at −65 mV, indicating that the resting potential of the unclamped cell would be negative to −65 mV. The depolarizing clamp step evoked a small time-dependent outward current; the current increased (activated) within about 100 ms to a maximum, from which it slowly decayed (inactivated). A 5 min exposure to 0.1 μM niguldipine

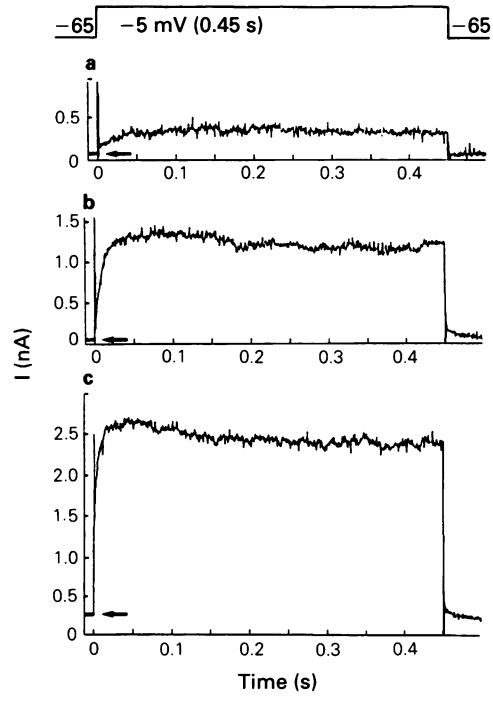

Figure 6 Effect of 0.1 μM niguldipine on the outward currents of a vascular myocyte from the portal vein. The 450 ms clamp steps ranged from −65 mV to −5 mV. (a) Before, (b) 5 min and (c) 10 min after application of the drug.

increased the outward current during the pulse from 0.3 to 1.3 nA and induced a decaying outward directed tail current upon repolarization to −65 mV. The 10 min exposure to niguldipine (Figure 6) increased the holding current (current before the clamp step, marked by arrow), the current during the pulse (maximal 2.5 nA, 50 ms after the start of the depolarization, 1.9 nA for I_L), as well as the tail current upon repolarization. After an exposure time of 15 min, the current was similar to the one recorded at 10 min.

Figure 7 shows the effects of 0.1 μM niguldipine on the outward currents of a pial vascular myocyte. Many of these pial cells are very small in size: the cell of Figure 7 was only 40 μm long and less than 5 μm thick. As expected for such a small cell, the membrane currents were in the range of pA. Thus, the contribution of currents through individual channels can be visualized in the record. A 5 min exposure to 0.1 μM niguldipine induced the following changes: the holding current at −65 mV increased from 1 to 20 pA (marked by the arrow). The current during the pulse was on average larger than before

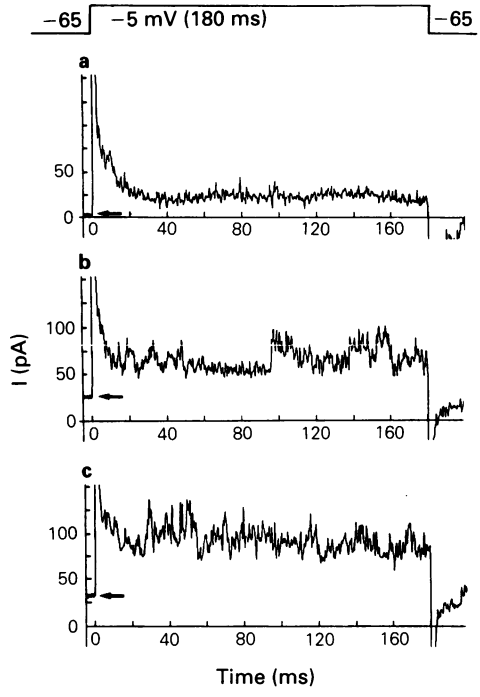

Figure 7 Effect of 0.1 μM niguldipine on the outward currents of a pial vascular myocyte. The 180 ms clamp steps were from −65 mV to −5 mV. (a) Before, (b) 5 min and (c) 7 min after application of the drug.

the application of the drug (50 pA in comparison to 20 pA). In addition, step-like current-jumps with an amplitude of 20–30 pA were evident in the current trace, which can be attributed to the opening and closing of channels with a high conductance. Seven min exposure to 0.1 μM niguldipine further increased the outward currents both at the holding potential (25 pA) and during the pulse (70 pA).

Time course and reversibility of the increase in outward current At all concentrations, niguldipine induced the increase in outward currents with a delay of several min. For a test depolarization to +20 mV and a drug concentration of 0.1 μM, the first significant increments in outward current were recorded 2–4 min after application of niguldipine. This latency is significantly longer than the time required to change the bath fluid completely (20 s). Usually, niguldipine increased the outward current with a 'biphasic' time course: after a first increment, a second, larger increment was frequently recorded about 10 min after drug application. The time course of the increase in outward current did not depend on the frequency of depolarizing clamp-pulses, and in cells that remained 'un-pulsed' at a holding potential of −65 mV, niguldipine increased the outward currents as well.

The niguldipine-induced outward current was largely reversed by drug removal, provided the concentrations remained below 0.1 μM and the exposure time was shorter than 10 min. When niguldipine was applied at 1 μM and/or for 20 min, the effects were only partially reversible after washing in drug-free solution for up to 1 h. Frequently, the cells became 'leaky' during the wash-out, i.e. for the holding potential −65 mV, a rather large negative holding current was required.

The drug-induced current is a potassium current The outward current induced by niguldipine might be carried by K or by Cl ions (an inflow of negative charge would generate an outward current). These 2 possibilities can be distinguished on the basis of the voltage-dependence of the drug-induced current, especially by its reversal potential. The I-V curves from a representative experiment are illustrated in Figure 8. Figure 8a shows an I-V curve from a smooth muscle cell isolated from the portal vein. Under control conditions, the curve is very flat and nearly matches the zero current line between −100 and −20 mV. Positive to −20 mV, the curve bends up showing 'outward rectification'. Five min administration of 0.3 μM niguldipine more than doubled the outward currents at potentials more positive than −20 mV. For more negative clamp potentials, however, the I-V curve was almost unchanged i.e. there was no intersection of the 2 curves and therefore no reversal potential. A 15 min exposure to 0.3 μM niguldipine increased the current further over the potential range +50 mV to −85 mV. At the reversal potential of −85 mV, the 2 curves intersected due to more negative currents at −100 mV. The reversal potential of −85 mV is close to the calculated potassium equilibrium potential of −84 mV. This finding supports the hypothesis that the drug-induced current is a potassium current. Using the nomenclature of Weir & Weston (1986a), we would like to conclude that niguldipine exerts K-agonistic effects on the vascular smooth muscle cells, but, the conclusion has to be proven by single-channel analysis.

Figure 8b supports the above conclusion with an experiment from a pial cell. Seven min after application of 0.3 μM niguldipine, an I-V curve was measured that lies above the control curve within the potential range between +50 mV to −75 mV. For more negative potentials, the I-V curve measured in the presence of niguldipine lies below the control curve. Thus, in this experiment the reversal potential was −75 mV. Again, this value is close to the calculated potassium equilibrium potential, supporting

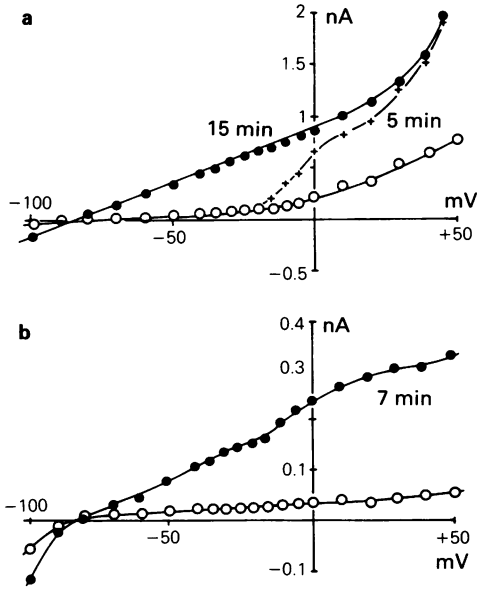

Figure 8 Effect of 0.3 μM niguldipine on the current-voltage curves of the late outward currents. Abscissa scale: pulse step potential. Ordinate scale: current measured at the end of the 180 ms clamp step, the holding potential was −65 mV. (a) Smooth muscle cell from the portal vein; (○) before, (+) 5 min, (●) 15 min after application of the drug. (b) Smooth muscle cell from the pial vessels; (○) before, (●) 7 min after application of the drug.

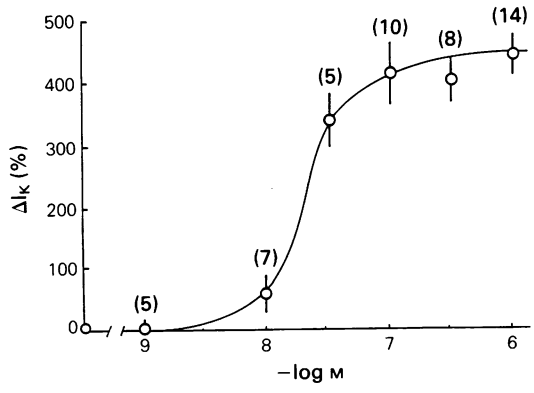

Figure 9 Concentration-response curve for the effect of niguldipine on potassium current. The current was measured with clamp steps to between −5 and +20 mV. Ordinate scale: effect of niguldipine expressed as $(*I_K - I_K)/I_K$ where I_K and $*I_K$ are the currents before and after application of niguldipine. The −log of the drug concentration is plotted on the abscissa scale. Each point represents the mean of the number of experiments indicated in parentheses and vertical lines show s.e.mean.

the view that the drug-induced current is a potassium current.

The concentration-effect curve for the facilitation of I_K by niguldipine The dependence of the increase in potassium outward current on the concentration of niguldipine was evaluated from a total of 49 vascular myocytes. As described above, the effect started with a long latency and needed about 10 min to reach steady-state. Since the small myocyte survives on the patch electrode only 15–30 min, most of the data were taken from a single application of the drug to one cell; only in 8 cells were 2 concentrations tested in a cumulative manner.

The first significant increase in outward current was measured at a nominal concentration of 10 nM. This concentration is considered to be the threshold concentration, it increased I_K of pial myocytes by 55 ± 30% ($n = 3$), I_K from the myocytes of the portal vein by 50 ± 25% ($n = 4$). At a concentration of 100 nM, the increase in I_K was 420% ($n = 3$) and 410% ($n = 7$), respectively. Maximal effects were observed with nominal 1 μM niguldipine, the increase was 460% (pial myocytes, $n = 4$) and 440% (myocytes from the portal vein, $n = 10$), respectively. From the concentration-effect curve of Figure 9 we extrapolated a half-maximal nominal concentration of 20 nM. The curve shown in Figure 9 is based on pooled data. Data were calculated from the current I_L recorded during the clamp step to potentials of −5, +10 or +20 mV. In addition, data from myocytes of the portal vein and of the pial vessels were pooled together since the niguldipine-sensitivity of these two cell types was almost identical (see above).

Discussion

Niguldipine lowers the blood pressure in animals, most probably via a reduction in peripheral vascular resistance (Sanders & Kolassa, 1986; Fischer *et al.*, 1987; Sanders *et al.*, 1987). Our studies on the effect of niguldipine have shown that calcium and potassium currents are modified in such a way that vasorelaxation can be explained at the level of the single myocyte.

Niguldipine influenced I_{Ca} in a similar manner to other dihydropyridines. That is, the Ca-antagonistic

effect showed a steep concentration-dependence with an EC_{50} of nominally 0.4 μM (effective concentrations 0.1–0.2 μM). As shown for nitrendipine and Bay K 8644 (Thomas *et al.*, 1984; Brown *et al.*, 1986), we found a 'Ca-agonistic effect' on niguldipine in concentrations between 50 nM and 200 nM. Thus, the concentration-effect curve of Figure 5 is typical for dihydropyridines.

The Ca-antagonistic effect of niguldipine was characterized by a large initial block (about 62%) on which only a small use-dependent component was superimposed. Thus, block of I_{Ca} by niguldipine (or other dihydropyridines) does not require the Ca-channel to open, whereas block by Ca antagonists of the verapamil-type does (e.g. Klöckner & Isenberg, 1986). Also in line with the known influence of dihydropyridines is the effect on the steady-state inactivation-curve. A similar shift of the activation curve was expected (ct. ref. Lee & Tsien, 1983) but was not found in the present experiments.

In vascular smooth muscle cells, a major part of potassium conductance is activated by an increase in intracellular calcium concentration $[Ca^{2+}]_c$ (Benham *et al.*, 1986). Thus, the facilitation of potassium current could 'secondarily' arise from an increase in cytosolic $[Ca^{2+}]$ as it ought to result from the increase in I_{Ca} (measured between 50 and 200 nM niguldipine). We consider the Ca-agonism unlikely to be the only causal mechanism by which niguldipine stimulates potassium currents, since the concentration-dependence of the two effects is quite different. Stimulation of I_K started at 10 nM niguldipine, which is 5 fold lower than the threshold concentration for the modulation of I_{Ca}. Maximal facilitation of I_K was found at 1 μM niguldipine, a concentration that blocked I_{Ca}. Finally, the increase in I_K by niguldipine was recorded at a constant holding potential of -65 mV, i.e. it was observed under conditions that did not activate I_{Ca}.

The increase in I_K is expected to move the membrane potential towards the potassium equilibrium potential which is thought to hyperpolarize most smooth muscle cells. In addition, facilitation of I_K should stabilize this negative membrane potential, thereby reducing excitability (e.g. in smooth muscle cells of small vessels, such as the pial cells investigated here). In the non-excitable cells of large arteries, the depolarizing effects of neurotransmitters will be reduced. In this paper, we did not demonstrate the expected hyperpolarization, as the isolated myocyte does not suit those measurements. The input resistance of the isolated cell is 3 GΩ (myocytes from portal vein) or 5 GΩ (pial myocytes), thus a small fluctuation in current (e.g 2 pA) changes the membrane potential appreciably (6 or 10 mV, respectively). The fluctuations in current could originate as an artifact of the amplifier system, or physiologically, by the opening of one or two single potassium channels (see Figure 7). Studies with conventional microelectrodes, applied to multicellular tissue where many thousands of cells are coupled to each other, may bypass this problem. The preparations have a much smaller input resistance and the effects originating from the channel fluctuations are bypassed by spatial superimposition. Thus, the postulated hyperpolarization should be tested by future complementary studies.

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Endothelin- and neuropeptide Y-induced vasoconstriction of human epicardial coronary arteries *in vitro*

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1 The effects of the recently discovered peptide endothelin and neuropeptide Y (NPY) on human epicardial coronary arteries were studied *in vitro*.

2 Endothelin induced a concentration-dependent, endothelium-independent, long-lasting vasoconstriction regardless of vessel size. NPY evoked contractions of small coronary arteries with a similar potency to that of endothelin, although to a significantly lower degree. Large coronary arteries did not respond to NPY. Endothelin did not relax coronary arteries precontracted with potassium.

3 The effect of endothelin was dependent on extracellular Ca^{2+} and, like NPY, significantly reduced by the Ca^{2+}-antagonist nifiedipine.

4 In conclusion, endothelin is a potent human vasoconstrictor *in vitro*. It is suggested that endothelin may be involved in the regulation of coronary blood flow.

Introduction

The recently discovered 21 amino acid-residue peptide endothelin has been shown to exert potent vasoconstrictor effects (Yanagisawa *et al.*, 1988). *In vitro* studies on pig coronary arteries revealed that endothelin induced vasoconstriction at significantly lower concentrations than all other peptides known to date. Incubation in a Ca^{2+}-free solution or with Ca^{2+}-antagonists abolished the effects of endothelin and it has been suggested that the peptide is an endogenous agonist of the dihydropyridine-sensitive Ca^{2+}-channel (Yanagisawa *et al.*, 1988). Furthermore, endothelin may be involved in vasospastic disorders as well as hypertension (Yanagisawa *et al.*, 1988; Tomobe *et al.*, 1988). However, the effects of endothelin seem to be species-dependent and *in vivo* vasodilator actions occur in certain vascular beds (Lippton *et al.*, 1988; Wright & Fozard, 1988).

Neuropeptide Y (NPY) (Tatemoto, 1982) is a 36 amino acid-residue peptide co-localized with noradrenaline in peripheral sympathetic nerves (Lundberg *et al.*, 1982). NPY is a potent human coronary vasoconstrictor *in vitro* (Franco-Cereceda & Lundberg, 1987) as well as *in vivo* (Clark *et al.*, 1987). NPY also inhibits cardiac sympathetic (Franco-Cereceda *et al.*, 1985) and parasympathetic neurotransmission (Lundberg *et al.*, 1984; Potter, 1985) and thus could be involved in the regulation of both coronary vascular tone and cardiac contractility.

In the present study the actions of endothelin in relation to the vasoconstrictor effects of NPY on human coronary arteries were investigated.

Methods

This study was approved by the Ethics Committee of the Karolinska Hospital. A total of 53 epicardial coronary arterial segments were obtained from five organ donor patients (1 male, 4 females, age 33 ± 6 years) with total cerebral infarction due to intracranial bleeding and/or traumata. The arteries were dissected out from the left ventricle with the aid of a microscope and divided into two groups; segments with an estimated inner diameter of <0.6 mm representing the second or third branch of the left anterior descending coronary artery (LAD), and segments with an inner diameter of >1.0 mm representing the first branch of the LAD. All vessels were epicardial and careful microscopic examination was made to exclude vessels with atherosclerosis. After

mounting the vessels on two L-shaped metal holders (0.3 mm in diameter) in 2 ml organ baths, a resting tension of 5 mN (small segments) or 10 mN (large segments) was applied by adjusting one of the metal holders (see Högestätt *et al.*, 1983). The other holder was connected to a Grass FT03C transducer connected to a Grass Polygraph for recordings of isometric tension. The mounted arterial segments were kept in Tyrode solution of the following composition (mM): NaCl 137, $NaHCO_3$ 11.9, KCl 2.7, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $CaCl_2$ 1.8 and glucose 5.6. The solution was aerated with 95% O_2 and 5% CO_2 and maintained at 37°C. After 60 min of equilibration, circular contractions were induced by use of Tyrode solution in which the NaCl had been replaced by KCl, giving a final potassium concentration of 127 mM or 40 mM. Only arteries showing two consecutive reproducible contractions were used in the experiments and each drug was tested on arterial segments from at least three patients.

In separate sets of experiments, the inner surfaces of the arteries were rubbed with a silk thread to remove the endothelium. The absence of substance P-induced relaxation of these arteries was taken as an indicator of successful removal of the endothelium. In addition, the effects of 60 min of preincubation with Ca^{2+}-free Tyrode solution containing 0.1 mM EGTA as well as 30 min of preincubation with 5×10^{-7} M nifedipine or nifedipine added after precontraction were studied in some experiments. This concentration of nifedipine has been shown to reduce effectively the NPY-induced contraction of peripheral human vessels (Pernow *et al.*, 1987).

Endothelin and NPY were added in cumulative concentrations directly into the baths since preliminary experiments had revealed that the results obtained by this procedure did not differ from those produced by single concentrations.

Values are given as means ± s.e.mean and expressed as percentages of contractions induced by potassium in controls, due to some variance in absolute values of the contractions. The Mann-Whitney U-test was used for statistical analysis; $P < 0.05$ was considered significant.

Porcine endothelin (which does not differ from the human form, Itoh *et al.*, 1988) was purchased from Peptide Institute Inc. (Osaka, Japan), human NPY was obtained from Cambridge Research Biochemicals Ltd (Cambridge, England) and nifedipine from Bayer (F.R.G.). Endothelin and NPY were dissolved in stock solutions of NaCl (100 $\mu g\,ml^{-1}$) while nifedipine was initially dissolved in 15 ml of 70% ethanol and 15 ml polyethylenglycol-200. This solution was then adjusted to 100 ml with distilled H_2O. To avoid photo-decomposition, the nifedipine solution was freshly prepared and kept in dark containers.

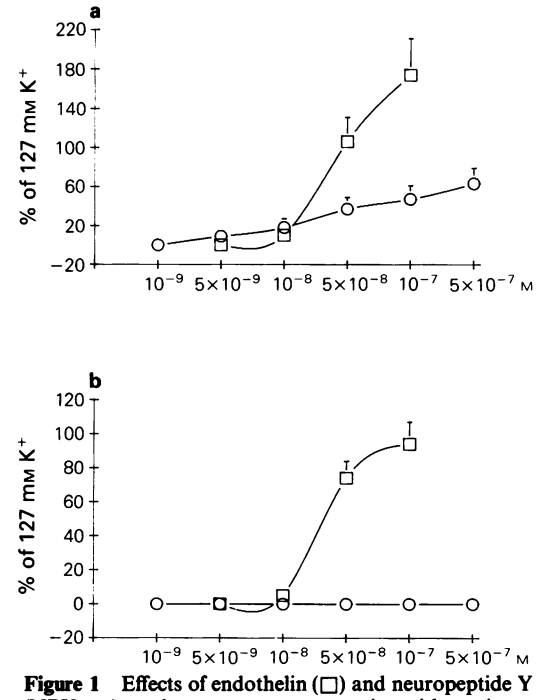

Figure 1 Effects of endothelin (□) and neuropeptide Y (NPY, ○) on human coronary arteries with an inner diameter of <0.6 mm (a) and >1.0 mm (b). Endothelin induced vasoconstriction regardless of vessel size while NPY induced constriction of primarily small arteries. Values are given as means (with s.e.mean shown by vertical bars) after comparison with contractions induced by 127 mM potassium and represent 7–13 arteries in each group.

Results

Potassium (127 mM) caused contractions of the small and large arteries of 4.6 ± 1.3 and 7.1 ± 0.9 mN, respectively. Contractions induced by 40 mM potassium on larger coronary arteries were found to be 3.7 ± 0.5 mN.

Endothelin induced concentration-dependent sustained contractions of human coronary arteries regardless of vessel size (Figures 1, 2). Thus, the threshold concentration of endothelin for inducing contraction was 10^{-8} M, while 10^{-7} M appeared to induce maximal contraction. Repeated rinsing over a period of several hours was usually needed to reverse the contractions induced by endothelin. Compared to potassium- (127 mM) induced contractions, endothelin, at the highest concentration used, evoked contractions of at least the same magnitude (Figures 1, 2). Endothelin (10^{-11} M to 10^{-8} M) did not relax vessels pre-contracted with 40 mM potassium but an increase in the potassium contraction was observed

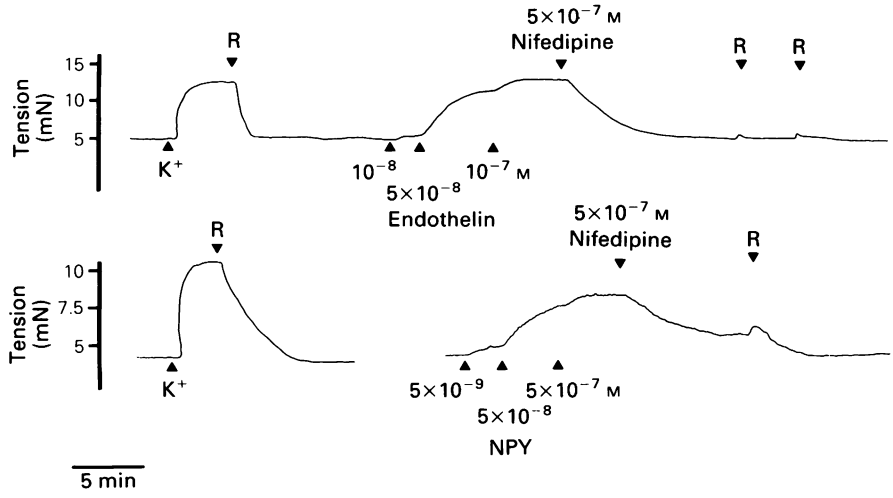

Figure 2 Original tracing showing the effects of 127 mM potassium (K^+), endothelin and neuropeptide Y (NPY) on human epicardial coronary arteries with an inner diameter of <0.6 mm. The two recordings represent experiments performed on two consecutive segments obtained from the same artery. Addition of nifedipine on the maximal contraction induced rapid and stable relaxations. R indicates rinsing.

at 10^{-8} M endothelin (amounting to 121 ± 25% of the 40 mM potassium contraction).

In contrast to the effects induced by endothelin, NPY contracted only small coronary arteries (Figures 1, 2) and did not influence the tone of the larger vessels. The threshold concentration of NPY was similar to that of endothelin. However, compared to the potassium- (127 mM) induced contraction, NPY (in concentrations up to 5×10^{-7} M) was considerably weaker than endothelin as a vasoconstrictor agent (Figure 1).

After preincubation with Ca^{2+}-free Tyrode solution, the contractions induced by potassium (127 mM) in large arteries were reduced by more than 80% (Figure 3). The effects of endothelin were also greatly reduced in Ca^{2+}-free medium. Thus, at both 5×10^{-8} M and 10^{-7} M endothelin, a reduction of about 70% of the contraction induced in the corresponding controls was observed. Preincubation with the Ca^{2+}-antagonist nifedipine (5×10^{-7} M) reduced the potassium- (127 mM) induced contractions by almost 90% (Figure 3) and the endothelin-induced contractions by 75 ± 5% and 65 ± 8% at 5×10^{-8} M and 10^{-7} M endothelin, respectively. Furthermore, after precontracting small arteries with endothelin or NPY, the addition of nifedipine induced a rapid and stable relaxation by 68 ± 8% and 62 ± 6%, respectively (Figure 2).

After mechanical removal of the endothelium in large arteries, the ability of potassium (127 mM) to cause contraction was reduced by 48 ± 8%. However, the contractile effects of endothelin remained unchanged as compared to controls. The contractions induced by endothelin in these vessels were 12 ± 1%, 39 ± 17% and 136 ± 26% for 10^{-8} M, 5×10^{-8} M and 10^{-7} M endothelin, respectively, when calculated as percentages of 127 mM potassium-induced contractions. The relaxant effect of substance P (10^{-8} M; 37 ± 6%; $n = 11$) was completely abolished after the endothelium was removed.

Discussion

The present findings demonstrate that endothelin is a human coronary vasoconstrictor with a potency similar to that of NPY *in vitro*. Several major differences seem to exist regarding the effects of these two peptides on coronary vascular tone. Thus, endothelin induced contractions regardless of vessel size and therefore may influence conductance as well as resistance vessels. NPY, on the other hand, contracted only small arteries, which is consistent with the observed effects of NPY on human epicardial coronary arteries *in vitro* (Franco-Cereceda & Lundberg, 1987) and of local intracoronary infusion of NPY in man (Clarke *et al.*, 1987). Furthermore, the maximal response to endothelin, but not to NPY, was similar to or greater than that seen in potassium-induced contractions, indicating that

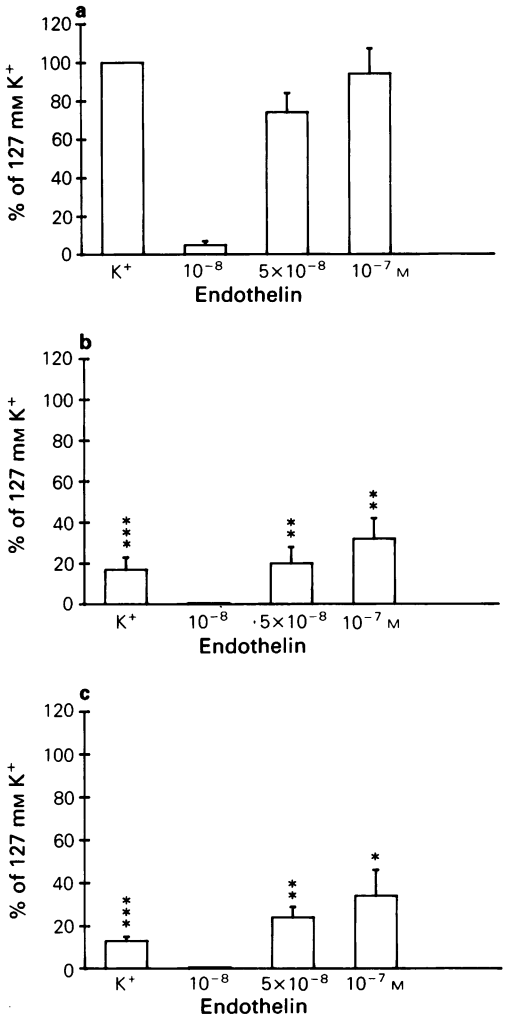

Figure 3 Influence of 60 min of preincubation with Ca^{2+}-free medium (b) as well as 30 min of preincubation with nifedipine (c) on the vasoconstrictor effects of endothelin and potassium (127 mM, K^+) as compared to control potassium-induced contractions of arteries with an inner diameter of > 1.0 mm (a). Values are given as means (with s.e.mean shown by vertical bars) and represent 5–14 arterial segments in each group. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$: Mann-Whitney U-test comparing values in (b) and (c) with the corresponding contractions in (a).

endothelin is an extremely strong constrictor of the human coronary vascular bed. The vasoconstriction induced by endothelin was long-lasting and, in contrast to both potassium- and NPY-induced contractions, repeated rinsing was needed to reverse it, thus confirming the finding in porcine coronary artery strips of an 'almost irreversible' effect of endothelin (Yanagisawa *et al.*, 1988; see also Tomobe *et al.*, 1988).

Endothelin administered i.v. in the cat was shown to induce initial decreases in systemic arterial pressure and renal perfusion pressure (Lippton *et al.*, 1988). In the rat, vasodilatation was the major effect observed in certain vascular beds on administration of endothelin (Wright & Fozard, 1988). In the present studies, potassium at 40 mM induced submaximal contraction of the human coronary arteries, but subsequent administration of endothelin failed to relax these precontracted vessels and only an additional contraction was observed. Furthermore, since i.v. *in vivo* administration of endothelin in the pig only decreased the coronary blood flow (Pernow *et al.*, 1988) the physiological effect of endothelin in the coronary circulation is likely to be vasoconstriction. The present findings further suggest that there might be differences in the response to endothelin depending on the species as well as vascular beds investigated.

Contraction of vascular smooth muscle is considered to be the result of an elevation of the free intracellular Ca^{2+} concentration which may stem from extracellular or intracellular stores (see Tayo & Bevan, 1987). After incubation with Ca^{2+}-free medium containing EGTA, the potassium-induced contractions were almost completely abolished (Pernow *et al.*, 1987). The response to endothelin was also highly sensitive to the removal of Ca^{2+} from the perfusion medium. In addition, the Ca^{2+}-antagonist nifedipine, which is often used in coronary vasospasm, also reduced the effects of endothelin as well as those of potassium and NPY. Thus, these results support the suggestion that an influx of extracellular Ca^{2+} is required for the vasoconstrictor actions of endothelin (see Yanagisawa *et al.*, 1988). However, the suggestion that endothelin may be an endogenous agonist for the dihydropyridine-sensitive Ca^{2+}-channel (Yanagisawa *et al.*, 1988) has been questioned in recent reports (Auget *et al.*, 1988; Miasiro *et al.*, 1988).

The vascular relaxant effects of substance P *in vitro* are not elicited after removal of the endothelium (see Furchgott, 1984). The lack of substance P-induced relaxation of the potassium-precontracted rubbed arteries indicated successful removal of the endothelium (see also Franco-Cereceda *et al.*, 1987). The contractions of these vessels induced by potassium were significantly reduced, probably due to mechanical damage to the vascular smooth muscle. However, endothelin induced contractions of a relative magnitude similar to that in controls. Furthermore, it was recently reported that NPY induced

vasoconstriction of human skeletal muscle arteries in an endothelium-independent manner (Pernow & Lundberg, 1988).

In conclusion, endothelin is a potent coronary vasoconstrictor *in vitro* in man. The effect of endothelin is highly dependent upon an influx of extracellular Ca^{2+} and remains unchanged after removal of the endothelium. In view of the long-lasting effects of endothelin, it is suggested that this peptide may be involved in such pathological conditions as coronary vasospasms and myocardial infarction.

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Actions of guanine nucleotides and cyclic nucleotides on calcium stores in single patch-clamped smooth muscle cells from rabbit portal vein

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1 Single smooth muscle cells were obtained from the rabbit portal vein by enzymic digestion and membrane currents under voltage clamp measured by whole-cell patch clamp technique.

2 When held at depolarized potentials, spontaneous outward currents (STOCs) were discharged; it is likely that these represent the cyclical storage and release within the cell of calcium in relation to Ca-activated K-channels.

3 Application of lower concentrations of carbachol (10^{-5}M) or caffeine (10^{-3}M) accelerated STOC discharge. Higher concentrations of caffeine (10^{-2}M) or carbachol (10^{-4}M), or noradrenaline (10^{-5}M), produced an outward current of 1–5 nA which disappeared within 5–15 s and which was considered to result from the discharge of calcium stores; STOC discharge was abolished for a period.

4 Ryanodine (10^{-5}–10^{-4}M) or a non-hydrolysable GTP analogue, GTPγS (10^{-5}–10^{-3}M) introduced into the cell abolished STOC discharge within 2–5 min. STOCs were large in cells filled with GDPβS (10^{-3}M) and the action of GTPγS introduced at various concentrations was antagonized.

5 GTPγS (10^{-4}–10^{-3}M) in the cell reduced or abolished outward current to caffeine (10^{-2}M) noradrenaline (10^{-5}M) or carbachol (10^{-4}M); the effect on caffeine outward current was antagonized by GDPβS (10^{-3}M) introduced into the cell. GDPβS reduced noradrenaline outward current but not caffeine outward current implying the existence of a G-protein step in noradrenaline-evoked Ca-store release, possibly regulating phospholipase C enzyme activity and D-myo inositol 1,4,5 trisphosphate formation.

6 If cyclic AMP (10^{-3}M) or cyclic GMP (10^{-3}M) was introduced into the cell, or 8-bromo cyclic AMP (0.5×10^{-3}M) or 8-bromo cyclic GMP (0.5×10^{-3}M) applied to the cell in the bathing solution, STOC discharge was only slightly affected. However, the outward current to caffeine applied after noradrenaline was much enhanced.

7 The results could be explained if cyclic GMP and cyclic AMP enhance calcium storage whereas GTPγS depletes calcium stores, an action antagonized by GDPβS.

Introduction

The application of patch clamp technique to single dispersed smooth muscle cells has revealed that in cells voltage-clamped and held at depolarized potential, a sporadic discharge of transient outward potassium currents occurs (Benham & Bolton, 1986; Ohya *et al.*, 1987). The discharge of these spontaneous transient outward currents (STOCs) may reflect calcium overloading of stores during depolarization of the membrane and periodic discharge of calcium from these in relation to calcium-activated potassium channels which are very sensitive to the internal free ionised calcium concentration (Benham *et al.*, 1986). Thus the discharge of STOCs or the appearance of calcium-activated K-current can be used to monitor the effects of various agents on the calcium store. In this paper we examine the effects on the calcium store of single vascular smooth muscle cells, of cyclic nucleotides and guanine nucleotides when these are introduced into the cell held under voltage clamp. The actions of these compounds are compared with those of agents whose actions on the calcium store are better understood.

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Methods

Preparation of cells

Rabbits of either sex, weighing 2–2.5 kg, were killed by injection of an overdose of sodium pentobarbitone into the ear vein. The portal vein was removed and rinsed in physiological salt solution (composition given below). The tissue was dissected free of connective tissue, cut longitudinally, and then the resulting sheet was cut into small pieces (approximately 2 × 2 mm).

These pieces of portal vein were incubated in salt solution containing 10 μM added calcium (low Ca^{2+} solution) at 37°C. After 10 min of incubation, the solution was replaced with fresh low Ca^{2+} solution but with the addition of papain 5 mg ml^{-1}, bovine serum albumin 3 mg ml^{-1} and dithiothreitol 5 mM, and the tissue fragments left to incubate for 25 min. After the completion of enzyme digestion, tissues were washed with enzyme-free, low Ca^{2+} solution. They were then placed in 1 ml low Ca^{2+} solution and agitated by drawing them in and out of a blunt glass pipette 50–80 times. The solution was removed, examined by microscope, and retained. The procedure was repeated six times at room temperature (20–25°C); from microscopical examination it was found that the number of cells in the solution increased gradually with advance of the agitation step and that the solution after the third and fourth, and sometimes fifth, agitation steps was very rich in cells. The cell-rich solutions were combined and centrifuged at about 1000 r.p.m. for 2 min. The cells were resuspended in 0.8 mM Ca^{2+}-containing salt solution. Aliquots were placed on coverslips and kept in a moist atmosphere at 4°C until use on the same day.

Whole-cell recording

Dispersed single smooth muscle cells were transferred to a small chamber (0.6 ml in volume) on a microscope stage and immersed in physiological salt solution. Whole-cell membrane-current recordings were made at room temperature by standard patch-clamp techniques (Hamill *et al.*, 1981). Patch pipettes had a resistance of 2–6 MΩ when filled with a patch pipette solution. The current amplifier used was a List EPC-7 and current records were stored on FM tape and replayed onto a Gould Brush recorder for illustration and analysis.

Amplitudes of STOCs were measured from paper records by hand. The amount of transferred charge in response to a drug was estimated by measuring the area of the drug-induced current.

Solutions

The physiological salt solution used in the experiments had the following composition (mM): NaCl 126, KCl 6, $CaCl_2$ 1.7, $MgCl_2$ 1.2, glucose 14 and HEPES 10.5 (titrated to pH 7.2 with NaOH). For the dispersal procedure, $CaCl_2$ was omitted except for the 10 μM added as described. The patch pipette solution had the following composition (mM): KCl 134, $MgCl_2$ 1.2, ATP 1, EGTA 0.05, glucose 14 and HEPES 10.5 (titrated to pH 7.2 with NaOH). In some cases, when drugs were added to the pipette solution, its pH was adjusted to 7.2 after their addition. Its osmolarity, however, was not adjusted except when caffeine was added, because of the relatively low concentrations of each drug (up to 1 mM). In the case of caffeine (10–40 mM) added to the pipette solution, the KCl concentration was reduced accordingly.

Drugs and chemicals

The following were used; papain type 4, dithiothreitol, bovine serum albumin, ethylene glycol *bis*-(2-aminoethyl) tetraacetic acid (EGTA), adenosine 5′-triphosphate (ATP), caffeine, carbachol chloride, noradrenaline bitartrate, 8-bromoadenosine 3′,5′-cyclic monophosphate (8-bromo cyclic AMP), 8-bromoguanosine 3′,5′-cyclic monophosphate (8-bromo cyclic GMP), adenosine 3′,5′-cyclic monophosphate (cyclic AMP), guanosine 3′,5′-cyclic monophosphate (cyclic GMP), guanosine 5-O-(γ-thio) triphosphate (GTPγS), guanosine 5-O-(β-thio) diphosphate (GDPβS) (all from Sigma) and ryanodine (Agrisystems International, 125, W.7th St. Wind Gap, Pennsylvania, U.S.A.).

The values in the text are the mean ± s.e.mean. Statistical significance was tested by an unpaired *t* test and differences were considered significant when $P < 0.05$.

Results

Recordings of membrane current were made from single cells of rabbit portal vein in normal physiological salt solution held at either −20 or 0 mV under voltage clamp. Spontaneous transient outward currents (STOCs) were seen in many but not all cells. STOCs are believed to represent the opening of up to 500 or so calcium-activated potassium-channels due to the release of calcium from stores in relation to the internal surface of the cell membrane (Benham & Bolton, 1986; Ohya *et al.*, 1987). The concentration of EGTA in the pipette was 0.05 mM which is probably insufficient to clamp the internal free

ionised calcium concentration (cf. Byerly & Moody, 1984).

Calcium release

The application in the bathing solution of caffeine (10^{-2}M), of noradrenaline (10^{-6}–10^{-5}M) or of carbachol (10^{-4}M) often elicited an outward current of up to 5 nA which disappeared within 5–15 s (Figure 1). STOC discharge was abolished for a period. Noradrenaline (10^{-5}M) was applied to cells showing STOC discharge 3–5 min after establishing the whole-cell recording mode; after about 40 s, caffeine (10^{-2}M) was applied. Cells could be classified into 3 types (I, II and III) on the basis of their responses (Figure 1a–c). Type I cells (which constituted 62%) responded with a large (1–5 nA) outward current to noradrenaline (10^{-5}M) but in the continuing presence of noradrenaline no response to caffeine (10^{-2}M) could be evoked. During the outward current to noradrenaline there was an extra outward movement of charge (current × time) of 3.73 ± 0.55 nC (mean ± s.e.mean; $n = 24$). In type II cells (which constituted 23%) outward currents to both noradrenaline and caffeine application were elicited (Figure 1b) although the caffeine response was generally somewhat reduced in size. Noradrenaline caused an extra 2.85 ± 0.79 nC ($n = 10$) and caffeine an extra 1.05 ± 0.31 nC ($n = 10$) outward movement of charge. Type III cells (15% of total) did not response to noradrenaline but responded normally to caffeine (10^{-2}M) which caused the outward movement of 6.53 ± 2.57 nC ($n = 6$) (Figure 1c).

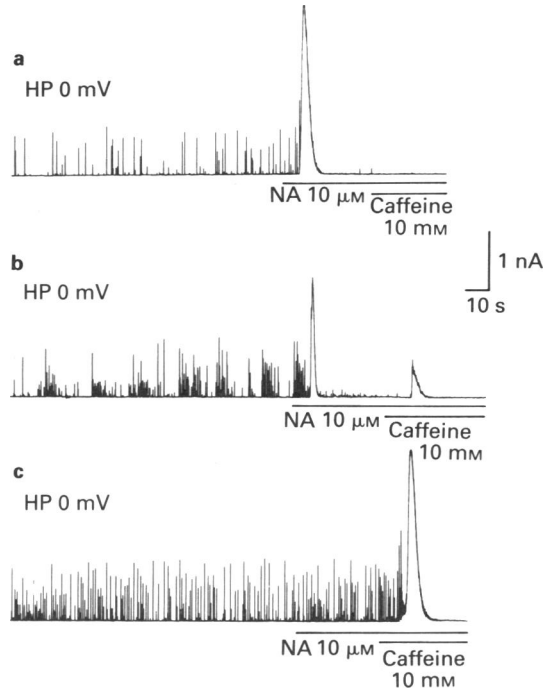

Figure 1 Responses to bath-applied noradrenaline (NA, 10 μM) and caffeine (10 mM) of single vascular smooth muscle cells held under voltage clamp at 0 mV (HP 0 mV). (a) Type I cell: NA produced a transient outward current followed by abolition of STOCs and in the continuing presence of NA, bath-applied caffeine (10 mM) was without any effect. (b) Type II cell: both NA and caffeine elicited outward current, although the caffeine response was small in size. (c) Type III cell: no response to NA was evoked, but caffeine produced a transient outward current followed by abolition of STOCs. Type I, II and III cells constituted 62%, 23% and 15% of the total (39 cells), respectively.

Lower concentrations of caffeine (10^{-3}M) often accelerated the rate of discharge of STOCs and increased their size; the acceleration of STOC discharge by caffeine declined slowly with time (Figure 2a). Following the outward current to caffeine (10^{-2}M) an inward current was sometimes seen during which STOCs were abolished (Figure 2b,c). Transient acceleration of STOC discharge often preceded the outward current. Return to drug-free solution caused STOCs to reappear after a period of a minute or more.

Lower concentrations of carbachol (10^{-5}M) could transiently accelerate STOC discharge and then reduce STOC size; increasing the carbachol concentration to 10^{-4}M then abolished STOC discharge without stimulation (Figure 3). However outward current to caffeine (10^{-2}M) could still be evoked (cf. Type II response to noradrenaline) and the outward movement of charge was 4.30 ± 2.02 nC ($n = 3$) (Figure 3b).

Although accurate quantitative experiments were not performed, it seemed that caffeine could release most of the calcium stores such that noradrenaline or carbachol applied in the presence of caffeine were without calcium-store-releasing action. Noradrenaline or carbachol could also release calcium stores but after their application caffeine could often release further calcium (Figure 1b); this was especially true after carbachol (Figure 3b). The calcium-store releasing potency was thus caffeine (10^{-2}M) > noradrenaline (10^{-5}M) > carbachol (10^{-4}M). However, up to 40 mM caffeine included in the pipette solution was without effect on STOCs or on the response to 10 mM caffeine applied in the bathing solution: outward current in response to noradrenaline could also be elicited in caffeine-filled cells.

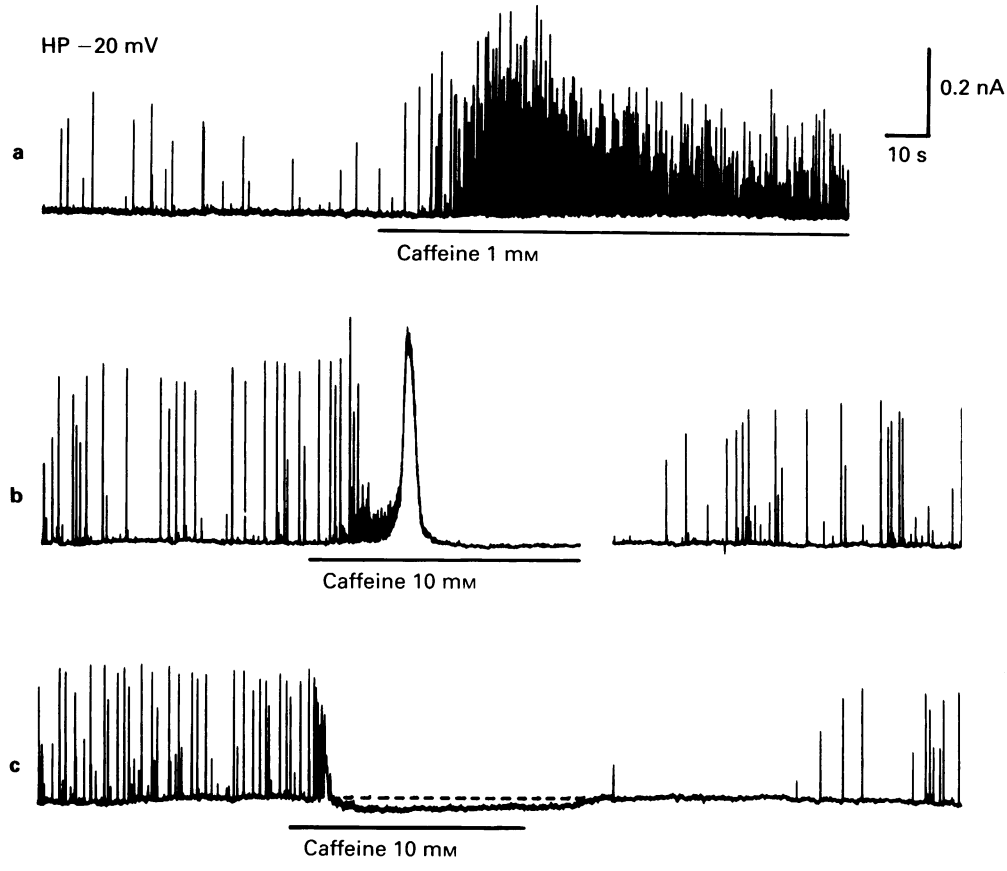

Figure 2 Membrane current responses to bath-applied caffeine of vascular cells held under voltage clamp at −20 mV (HP − 20 mV). (a) Caffeine 1 mM caused an increase in frequency and size of STOCs. (b) Caffeine 10 mM elicited an initial burst of STOCs with a large outward current followed by abolition of STOCs, which recovered 5 min after wash out. (c) A similar response to that observed in (b) but followed by a small inward current which disappeared rapidly upon washing away the caffeine.

Cyclic nucleotides

If 8-bromo cyclic AMP (0.5 mM) was applied to the cells in the bathing solution only a slight increase in frequency and a small increase in size of STOCs was observed (Figure 4a). 8-Bromo cyclic GMP had a similar action (Figure 4b). If cyclic AMP or cyclic GMP (1 mM) were included in the pipette solution, then a small increase in frequency and size of STOCs occurred within 2–5 min of achieving the whole-cell recording mode. Outward current to caffeine or noradrenaline application was not noticeably changed. However, with 1 mM cyclic nucleotide in the pipette solution, the application of caffeine (10^{-2} M) about 40 s after noradrenaline (10^{-5} M) and in its presence, caused a large outward current (Figure 5b,c) which was much larger than the outward current to caffeine seen in similar experiments with cyclic-nucleotide-free pipette solution (cf. Figures 5a and 1b): thus charge transfer during the outward caffeine current was 4.46 ± 1.12 nC ($n = 5$) in cyclic AMP-filled cells and 4.73 ± 2.29 nC ($n = 3$) in cyclic GMP-filled cells compared to 1.05 ± 0.31 nC ($n = 10$) obtained in controls (Type II cells). Such a result could be explained if both cyclic nucleotides promote calcium storage but are without influence on the release processes.

Ryanodine

The inclusion of ryanodine in the pipette solution generally abolished STOC discharge within a few

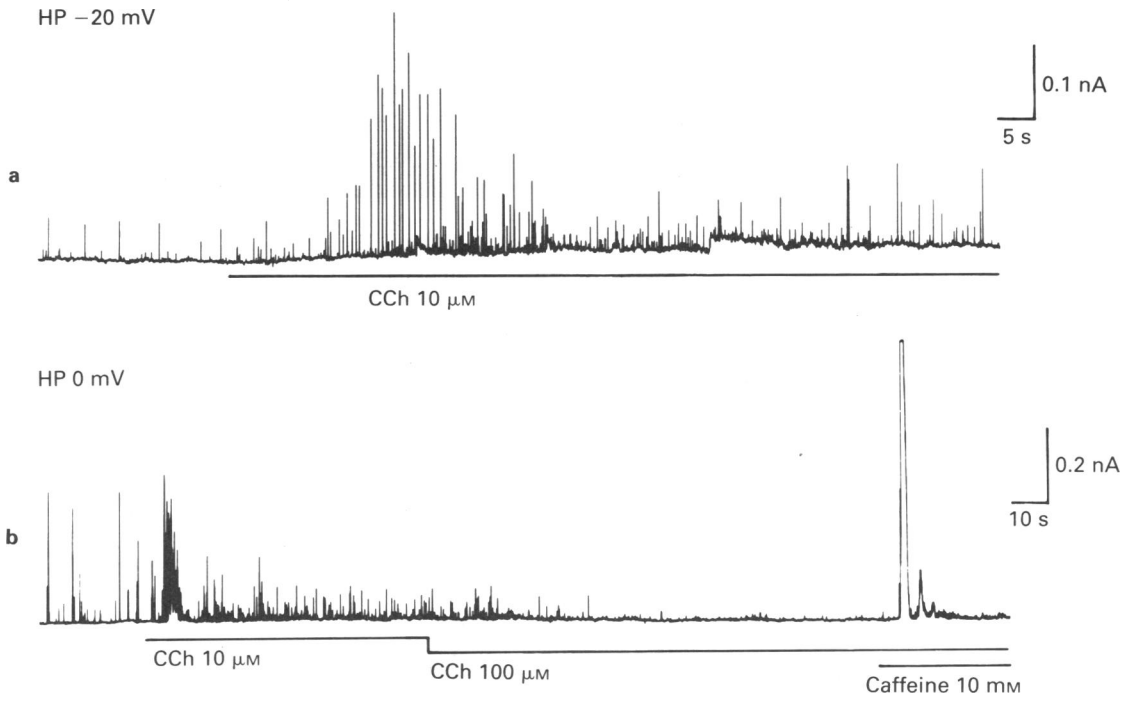

Figure 3 Membrane current responses to bath-applied carbachol (CCh) and caffeine. (a) CCh 10 μM caused a transient increase in size and frequency of STOCs. (b) STOCs in the presence of CCh (10 μM) were abolished after increasing the concentration to 100 μM: in the continuing presence of CCh a large outward current to caffeine (10 mM) was still elicited. Holding potentials (HP) as marked.

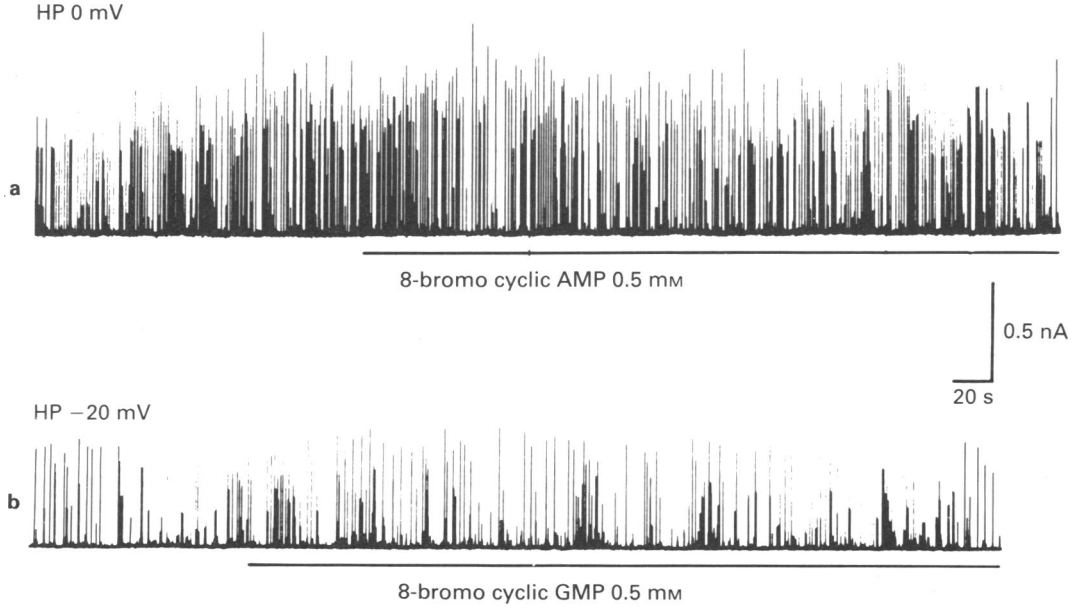

Figure 4 Effects of membrane permeable cyclic nucleotides on STOC discharges of vascular cells held at depolarized potentials: (a) 8-bromo cyclic AMP (0.5 mM); (b) 8-bromo cyclic GMP (0.5 mM). Bath application of the drugs caused only a slight increase in frequency and size of STOCs. Holding potentials (HP) as indicated.

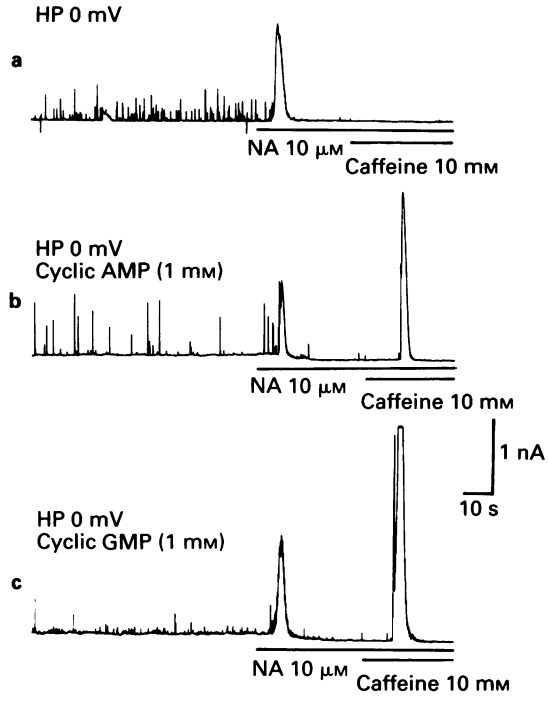

Figure 5 Effects of cyclic nucleotides included in the pipette on membrane current responses to noradrenaline (NA, 10 μM) and caffeine (10 mM). (a) Control experiment, in which a single vascular cell was held under voltage clamp at 0 mV (HP 0 mV) using the pipette filled with cyclic nucleotide-free solution. Caffeine applied to the cell in the bathing solution was without any effects after prior application of NA which elicited an outward current (also see Figure 1a,b). (b) The pipette was filled with cyclic AMP (1 mM)-containing solution. (c) The pipette was filled with cyclic GMP (1 mM)-containing solution. Note that inclusion of either cyclic AMP or cyclic GMP in the pipette allowed caffeine to elicit a large outward current in the presence of NA without noticeable changes of NA-induced outward current.

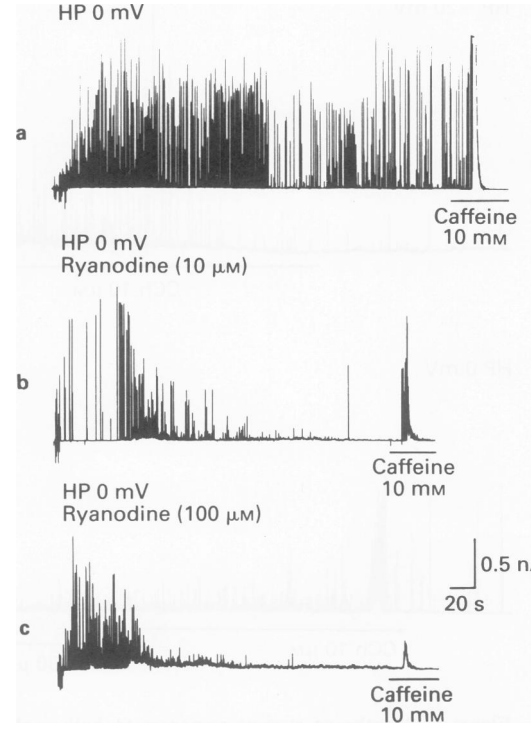

Figure 6 Effects of ryanodine included in the pipette on STOCs and caffeine (10 mM)-induced outward currents of vascular cells held under voltage clamp at 0 mV. (a) Control experiment with no ryanodine in the pipette solution. STOC discharge was sustained before bath application of caffeine (10 mM) which produced a large outward current followed by abolition of STOCs. (b) Inclusion of ryanodine (10 μM) in the pipette abolished STOCs with slight acceleration of STOC discharge shortly after achieving whole-cell recording mode and reduced caffeine-induced outward current. (c) Inclusion of a higher concentration (100 μM) of ryanodine in the pipette abolished STOCs more rapidly and markedly inhibited the caffeine response.

minutes of achieving the whole-cell recording mode. STOCs were abolished at a rate which increased with increasing the concentration of ryanodine (10^{-5}–10^{-4} M). When ryanodine was present in the cell, outward current to caffeine (10^{-2} M) was reduced or absent (Figure 6b,c; cf. Figure 6a).

Guanosine triphosphate and diphosphate analogues

If GTPγS (0.1 mM) was included in the pipette solution, STOCs were rapidly abolished in whole-cell recording mode. The effects were similar to the actions of ryanodine (cf. Figure 7a and Figure 6b,c). STOCs were often large if GDPβS (0.5–1 mM) was included in the pipette solution (Figure 8b,c). GTPγS (0.1 mM) generally abolished the outward current to caffeine (10^{-2} M), noradrenaline (10^{-5} M) or carbachol (10^{-4} M) whereas inclusion of GDPβS in the pipette solution did not (Figure 8b,c). However, quantitative comparison of responses to noradrenaline (10^{-5} M) in normal and in GDPβS-filled cells showed a reduction in the outward current response: in 6 controls the average outward charge transferred upon noradrenaline application was 3.85 ± 0.94 nC whereas in 6 cells which responded to noradrenaline and which were patched with a pipette solution containing GDPβS (0.5–1 mM) it averaged

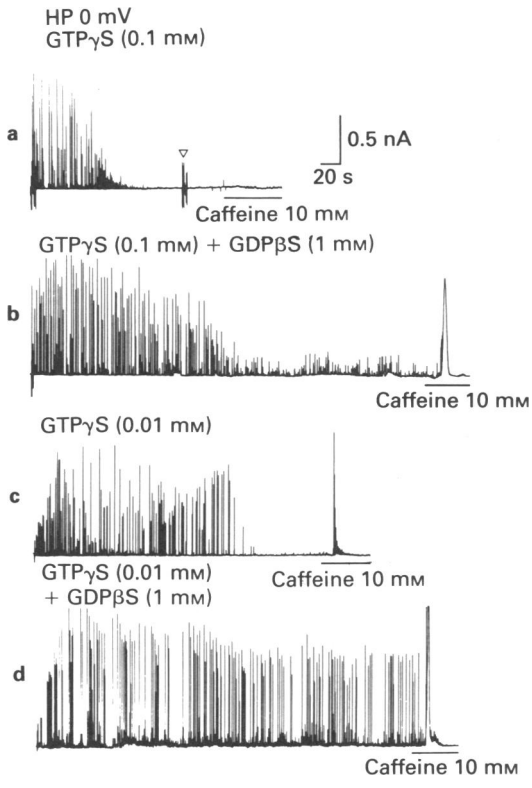

Figure 7 Effects of guanine nucleotides included in the pipette on STOCs and on caffeine-induced outward current of vascular smooth muscle cells held under voltage clamp at 0 mV. (a) Use of a pipette filled with guanosine 5-O-(γ-thio) triphosphate (GTPγS) (0.1 mM)-containing solution, abolished STOCs shortly after achieving whole-cell recording mode and bath-applied caffeine (10 mM) produced almost no effect. At ▽, access resistance was checked with a train of hyperpolarizing voltage pulses (10 mV in size, 200 ms in duration). (b) Inclusion of both guanosine 5-O-(β-thio) diphosphate (GDPβS, 1 mM) and GTPγS (0.1 mM) in the pipette delayed the abolition of STOCs by GTPγS, and a transient outward current to caffeine was elicited. (c) With 0.01 mM GTPγS in the pipette solution, STOCs were reduced in size more slowly, compared with (a) and caffeine could still produce an outward current. (d) With both GDPβS (1 mM) and GTPγS (0.01 mM) in the pipette solution STOC discharge was barely suppressed, and caffeine elicited a large outward current followed by abolition of STOCs.

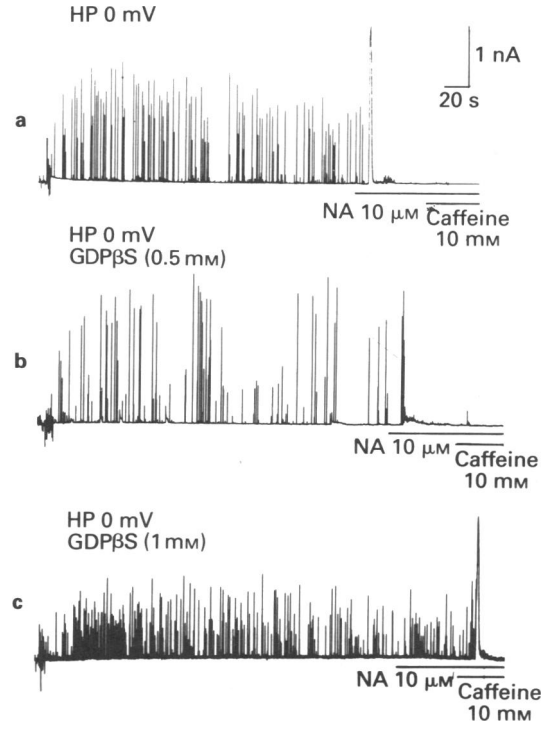

Figure 8 Effects on the outward current response to noradrenaline (NA, 10 μM) of guanosine 5-O-(β-thio) diphosphate (GDPβS) included in the pipette. (a) Control experiment with no GDPβS in the pipette solution. Bath-applied NA produced a large outward current and caffeine produced no response in the presence of NA. (b) With 0.5 mM GDPβS in the pipette solution, NA-induced outward current was relatively small, compared with that in (a) and caffeine also elicited a very small response. (c) With 1 mM GDPβS in the pipette solution, the cell was insensitive to NA but responded to caffeine normally with an outward current followed by abolition of STOC discharge. Only 6 out of 21 cells filled with GDPβS (0.5–1 mM) responded to NA with an outward current.

0.88 ± 0.26 nC. The difference is significant ($P < 0.05$). Furthermore, noradrenaline-insensitive type III cells constituted only 15% of a control series ($n = 39$) whereas they constituted 71% of 21 cells patched with GDPβS-containing pipettes. In the 15 of 21 cells not responding to noradrenaline, the outward charge transferred in response to caffeine was normal averaging 3.90 ± 1.30 nC. GDPβS (1 mM) had no noticeable effect on the outward current to caffeine in cells not previously treated with noradrenaline as measured by transferred charge, this being 3.67 ± 0.62 nC ($n = 12$), very similar to the control value of 4.05 ± 1.02 nC ($n = 7$) (Figure 9).

GDPβS (1 mM) also antagonized the inhibitory effect of GTPγS on STOC discharge and on caffeine (10^{-2} M) outward current. In a series of experiments, pipettes were filled with solution containing 0.01, 0.1

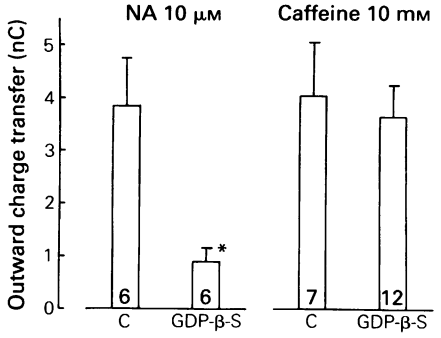

Figure 9 Summary of the effects of guanosine 5-O-(β-thio) diphosphate (GDPβS) on membrane current responses of vascular cells to bath-applied noradrenaline (NA, 10 μM) and caffeine (10 mM). Single cells were held under voltage clamp at 0 mV using pipettes filled with normal solution (control; C) or with GDPβS (0.5–1 mM)-containing solution, and 4–6 min after achieving whole-cell recording mode either NA or caffeine was externally applied. Ordinate scale: outward charge transfer (nano coulomb; nC) which was estimated by measuring the area of NA- or caffeine-induced outward current response. Each column represents the mean obtained on the indicated number of cells; vertical line indicates one s.e.mean. Asterisk indicates significant difference from the control value ($P < 0.05$).

or 1 mM GTPγS and the time for STOCs to decrease to half their maximum size was measured from beginning whole-cell recording mode (Figure 7). This was 1–2 min at 0.1 or 1 mM GTPγS and averaged over 4 min at 0.01 M GTPγS although in some cases (2 out of 13) STOCs did not decline in size by more than 50% and these results were omitted (Figure 10). If 1 mM GDPβS was added to the above solutions and the experiment repeated, then the STOCs did not decline in size to less than 50% in 7 of 10 cells if the pipette contained 0.01 mM GTPγS (Figure 7c,d); i.e. the inclusion of GDPβS in the pipette generally prevented any reduction in STOC size caused by 0.01 mM GTPγS. No point is shown in Figure 10a for the remaining 3 cells. With the two higher concentrations of GTPγS, the presence of 1 mM GDPβS increased the time taken for 50% reduction in STOC

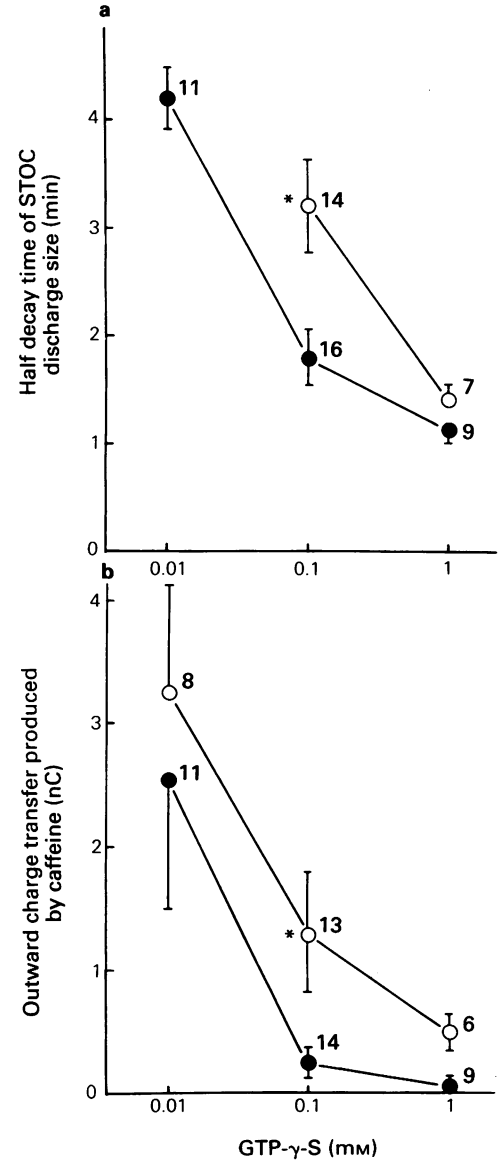

Figure 10 Effects of guanosine 5-O-(β-thio) diphosphate (GDPβS) on the dose-response curves of guanosine 5-O-(γ-thio) triphosphate (GTPγS) for the inhibition of STOC discharge and for inhibition of caffeine-induced outward current. Various concentrations of GTPγS were included in the pipettes without (●) and with (○) GDPβS (1 mM) used to hold vascular cells under voltage clamp at 0 mV. (a) The time for STOCs to decrease to half their maximum size was measured from beginning whole-cell recording mode. (b) After abolition of STOCs or reaching a steady state in STOC discharge, caffeine (10 mM) was externally applied to the cells and outward charge transfer was estimated from the drug-induced outward current response. Each point represents the mean; vertical lines indicate one s.e.mean. Numbers beside each point are the number of cells tested. With a combination of 0.01 mM GTPγS and 1 mM GDPβS in the pipette, STOC size did not decline to less than 50% of its maximum in 7 out of 10 cells (see Figure 7d) and thus the results on the remaining three cells were not plotted (also see text for detail). Asterisks indicate significant difference from the value for GTPγS alone ($P < 0.05$).

size to occur (Figure 7a,b and 10a). At 0.1 mM GTPγS with 1 mM GDPβS, STOC size did not decline by 50% in 3 out of 17 cells and these cells were omitted from the results. The mean value for the remaining 14 cells is shown (Figure 10a). In the case of caffeine (10^{-2} M) outward current, this was expressed as charge transferred (size of current × time) by measuring the area of the caffeine outward current. The inclusion of 1 mM GDPβS in the pipette reduced the effects of GTPγS on the caffeine outward current (Figure 10b) at all concentrations although GDPβS alone was without effect on caffeine outward current (Figure 9). In cells filled with GTPγS (0.1–1 mM), caffeine often produced rather than a brief outward current a small constant inward current which was of rapid onset and offset. Since the cells were held at 0 mV, close to E_{Cl}, this current could not be carried by chloride ions and seems most likely to be a sodium current or a reduction in outward current, although its nature was not further investigated.

Discussion

A single STOC seems to represent the discharge of an aliquot of stored calcium in relation to Ca-activated K-channels which are numerous in the smooth muscle cell membrane (Benham *et al.*, 1986; Benham & Bolton, 1986; Ohya *et al.*, 1987; Beech & Bolton, 1989). Why STOCs are of varying sizes and discharged sporadically rather than regularly is not completely understood, but may be related to the presence of several separate calcium storage sites of different size in a single cell and to differing degrees of loading of a store before discharge occurs (Benham & Bolton, 1986). Strong buffering of calcium within the cell abolishes STOCs, and their temporary abolition after application of caffeine which is a well-known calcium-store releasing agent, support the mechanism proposed. Similar STOCs have been described in ganglion cells (Brown *et al.*, 1983).

The large temporary outward current evoked by higher concentrations of caffeine, noradrenaline or carbachol suggests that adrenoceptor or muscarinic receptor activation can rapidly discharge a substantial part of the total calcium store. These three agents seem to act on the same store to the extent that release of calcium by one agent reduced release by another. Careful concentration-effect studies of the releasing properties of these agents were not performed but the impression was gained that caffeine was the most efficacious calcium store releaser and carbachol the weakest. The loss of STOCs following their application implies that the calcium released to produce a STOC comes from the same store.

Why caffeine introduced inside the cell by adding it to the pipette solution was without effect even at high concentrations is remarkable. It suggests that the site at which caffeine acts is on the external surface of the plasma membrane such that it is not easily reached by internally applied caffeine. However, the possibility cannot be completely discounted that the site of action of caffeine is internal but that caffeine added to the pipette solutions fails to reach this site in effective concentrations either because of metabolism or uptake en route. Caffeine is normally considered to permeate cell membranes easily and this may mean that it can escape relatively easily from the cell so that it is difficult to achieve adequate concentrations close to the internal surface of the cell membrane. Further work is needed to establish the reason for this intriguing observation.

Cyclic nucleotides introduced into the cell, or their membrane permeable analogues applied to the cells had only small effects on the frequency and size of STOCs but had a large effect on the response to caffeine after prior depletion of stores by noradrenaline. This result is consistent with earlier observations on whole smooth muscle tissues which led to the suggestion that β-adrenoceptor activation (which increases cyclic AMP) promotes calcium storage (Mueller & van Breeman, 1979; Itoh *et al.*, 1982; Bülbring & Tomita, 1987; Parker *et al.*, 1987). Rises in cyclic GMP have also been found to be associated with smooth muscle relaxation in this case caused by nitrosovasodilators (such as nitrites or sodium nitroprusside) nitric oxide, or endothelium-derived relaxing factor (EDRF) (Diamond & Holmes, 1975; Katsuki *et al.*, 1977; Murad *et al.*, 1985). The actions of cyclic nucleotides seem to be on a protein kinase which phosphorylates part of the sarcoplasmic Ca/Mg ATPase (Ca pump) or associated cofactor thus stimulating calcium accumulation (Nishikori & Maeno, 1979; Suematsu *et al.*, 1984). It was curious, however, that while cyclic nucleotides enhanced the caffeine outward current, STOCs were not restored in the presence of noradrenaline (Figure 5).

The alkaloid ryanodine abolished STOCs when introduced into the cell, presumably because of its calcium-store-releasing action (Hwang & van Breemen, 1987; Imagawa *et al.*, 1987). Outward current in response to caffeine took longer to be abolished than STOCs but this was also eventually lost. If ryanodine depletes calcium stores, then at the concentrations used the rate at which it released calcium must be insufficient to activate Ca-activated K-channels and no appreciable K-outward current developed during the release process although STOC discharge was stimulated (Figure 6b,c).

GTPγS had an action very similar to ryanodine except that lower concentrations of GTPγS took

longer to abolish STOCs and caffeine outward current. In skinned smooth muscle fibres, GTPγS had a pronounced calcium-store releasing action (Kobayashi *et al.*, 1988). The action of GTPγS on STOCs and caffeine outward current was antagonized by GDPβS which implies that an alpha subunit of a GTP-binding protein (G-protein) may be involved (Stryer & Bourne, 1986). GDPβS inhibited noradrenaline outward current although not abolishing it in all cells. This implies that there may be a G-protein step intervening between adrenoceptor activation and calcium store release. The calcium store release by the adrenoceptor is believed to be mediated by the formation of D-myo inositol 1,4,5,-trisphosphate from phosphatidylinositol 4,5-bisphosphate by the action of the enzyme phospholipase C (PLC) (see Bülbring & Tomita, 1987, for review). A G-protein has been implicated in the regulation of PLC activity (Cockcroft & Gomperts, 1985; Sasaguri *et al.*, 1986).

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Actions of constrictor (NPY and endothelin) and dilator (substance P, CGRP and VIP) peptides on pig splenic and human skeletal muscle arteries: involvement of the endothelium

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1 The effects of various vasoactive peptides and the involvement of the endothelium in these effects were studied on small human skeletal muscle arteries (SMA) and pig splenic arteries (SA) *in vitro*.

2 Under control conditions, neuropeptide Y (NPY) caused potent and strong contractions of both arteries. The maximal effect of NPY 500 nM was similar to that of phenylephrine and noradrenaline (10 μM). Endothelin was approximately 10 fold more potent than NPY in contracting SA, and the maximal response to endothelin 50 nM was 130% of that evoked by phenylephrine.

3 After removal of the endothelium (by rubbing the inner surface of the arteries) neither the maximal effect nor the EC_{50} value of NPY on SMA and SA or those of endothelin on SA were changed from control conditions.

4 The substance P (SP)-induced relaxation of precontracted SMA and SA during control conditions (80–90%) was abolished or greatly reduced after endothelium removal.

5 Under control conditions, calcitonin gene-related peptide (CGRP) was about 10 times more potent than vasoactive intestinal polypeptide (VIP) in relaxing SMA. After endothelium removal the relaxation induced by CGRP on SMA and SA and that of VIP on SMA were not changed from control conditions.

6 It is concluded that, in the SMA and SA, the potent vasoconstrictor effects of NPY and endothelin are mediated by direct actions on the vascular smooth muscle and not via a release of an endothelium-derived contracting factor. Relaxation induced by SP but not that of CGRP and VIP seems to be mediated via the endothelium.

Introduction

Many vasoactive substances are known to produce their effects via an action that is dependent on an intact endothelium. Endothelium-derived relaxing factors (EDRF) or contracting factors (EDCF) are presumed to be released and to mediate the relaxations and contractions, respectively (for reviews see Furchgott, 1984; Greenberg & Diecke, 1988).

In the present study the involvement of the endothelium in the vascular effects of various vasoactive peptides was investigated. It is well established that the relaxation of isolated arteries by substance P (SP) is strictly dependent on the presence of intact endothelial cells (see Furchgott, 1984). Concerning the action of the co-stored calcitonin gene-related peptide (CGRP) results are somewhat conflicting. Thus, previous observations suggest that CGRP induces endothelium-dependent relaxation of rat aorta (Brain *et al.*, 1985) whereas other reports describe the relaxation induced by CGRP in cat cerebral arteries (Edvinsson *et al.*, 1985) and pig coronary arteries (Franco-Cereceda *et al.*, 1987b) to be unaffected after removal of the endothelium (see also Luscher & Vanhoutte, 1988). A similar variability seems to exist regarding the involvement of the endothelium in the relaxant action of vasoactive intestinal peptide (VIP) (see Luscher & Vanhoutte, 1988). These differences in results obtained by different authors may imply that regional and/or species variations exist.

Little is known about the endothelium-dependence of vasoconstrictor peptides such as neuropeptide Y (NPY). Daly & Hieble (1987) reported that the potentiating effect of NPY on noradrenaline (NA)-induced contractions of the rabbit

ear artery was dependent on an intact endothelium, but it is at present unclear whether the contractile effect of NPY *per se* requires an intact endothelium and involves release of an EDCF such as the endothelium-derived vasoconstrictor peptide, endothelin (Yanagisawa *et al.*, 1988).

To investigate such questions the vascular effects of these peptides and the involvement of the endothelium in their responses were studied *in vitro* on two different arterial preparations from two different species i.e. human skeletal muscle arteries (SMA) and pig splenic arteries (SA).

Methods

Experimental procedures

The experiments were performed on small arteries obtained from the gluteus maximum muscle of 6 humans undergoing arthroplastic surgery of the hip and from the spleen of 5 pigs. The patients (age 48–65 years) were premedicated with morphine and scopolamine and received epidural anaesthesia with bupivacaine in combination with adrenaline or ephedrine. A piece of the gluteus maximus muscle was removed at the beginning of surgery, put in cold Krebs solution and brought to the laboratory where small (0.2–0.4 mm inner diameter) arterial segments were dissected out. Pieces of the spleen from pentobarbitone (20 mg kg^{-1}, i.v.) anaesthetized pigs were handled in a similar fashion. The splenic arteries were approximately 0.5 mm in diameter.

The arteries were cut in short (1–2 mm) segments and mounted on two metal holders in a 2 ml organ bath containing Krebs solution (see Högestätt *et al.*, 1983; Pernow *et al.*, 1987b). The solution was kept at 37°C and bubbled with 5% CO_2 in O_2 to maintain a pH of 7.4. One of the metal holders was connected to a force displacement transducer (Grass FT03 D) for recording isometric tension on a Grass polygraph (model 7D). The other holder was movable for application of a resting tension of 5 mN to the vessels. After an equilibration period of 30–60 min, noradrenaline (NA; 10 μM) was applied to the SMA and phenylephrine (PE; 10 μM) to the SA to obtain a control test contraction. After repeated rinsing the vasoconstrictor peptides were applied in cumulatively increasing concentrations. The effects of the relaxant peptides were studied by adding them to vessels precontracted with either NA (10 μM; SMA), PE (10 μM; SA) or NPY (500 nM). The effect of SP was only studied at one concentration (10 nM) since tachyphylaxis rapidly developed during cumulative addition of this peptide.

One group of vessels was rubbed against the metal holders in the organ bath in order to remove the endothelium. Successful removal of the endothelium was determined by absence of SP-induced relaxation. As shown in Figure 1, the relaxant effect of 10 nM SP was abolished or greatly reduced after this procedure, indicating adequate removal of endothelial cells. The presence of endothelium on SMA was also analyzed by application of acetylcholine (1 μM) which relaxed control vessels but not rubbed ones. A similar control procedure could not be performed with the SA, as acetylcholine produced contraction of these vessels. Control vessels were run in parallel experiments.

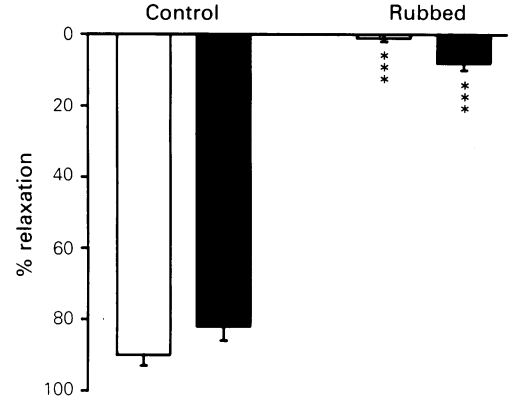

Figure 1 Relaxant effects of substance P (10 nM) on human isolated small skeletal muscle arteries (open columns) and pig splenic arteries (solid columns) during control conditions and after rubbing the inner surface of the vessels to remove the endothelium. The arteries were precontracted with 10 μM noradrenaline (skeletal muscle arteries) or phenylephrine (splenic arteries) and the effect of substance P is expressed as percentage relaxation of the precontracted tone. Mean values from 9–13 observations; s.e.mean shown by vertical bars. Significant differences between the effect in controls and in rubbed preparations are indicated, ***$P < 0.001$.

Calculations

The contractile effects of the peptides are expressed as percentage of those evoked by NA or PE in controls and rubbed preparations. In some preparations the contractile effects of NA and PE were slightly reduced after rubbing the endothelium but this was not a general phenomenon and was not correlated to the removal of the endothelium as assayed by absence of SP-induced relaxation. It is therefore assumed that the reduced effect was related to damage of the smooth muscle by the rubbing procedure. The effects of the relaxant peptides are expressed as percentage relaxation of the precontracted tone. The concentration that produced 50%

of maximal effect (EC_{50} values) were calculated from a lin-log paper.

Values in the text are given as means and s.e.mean. Significant differences between the effects on control and rubbed vascular segments as well as between the potency of different peptides were calculated with the Mann-Whitney U-test.

Drugs and solutions

The Krebs solution had the following composition (mM): NaCl 122, KCl 4.7, $NaHCO_3$ 15.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, glucose 11.5 and $CaCl_2$ 2.5. NA ((−)-arterenol) and phenylephrine (Sigma, St Louis, Mo, U.S.A.), porcine NPY and human CGRP (Peninsula, Belmont, Ca, U.S.A.), SP and VIP (CRB, Cambridge, U.K.) and porcine endothelin (Peptide Institute, Osaka, Japan) were used. All chemicals were dissolved in 0.9% NaCl and diluted in Krebs solution immediately before use.

Results

Effects of neuropeptide Y and endothelin on control arteries

NPY caused potent and strong contractions of both SMA and SA. The maximal effect of NPY (500 nM) was similar to that of NA and PE (10 μM) on the two arteries, respectively (Figures 2a and 3a,b). Endothelin induced a slight contraction of SA at 0.5 nM in most preparations and the maximal effect of 50 nM endothelin was 130 ± 11% ($n = 7$) of that induced by PE (10 μM) (Figures 2b and 3c). In comparison with NPY, endothelin was approximately 10 times more

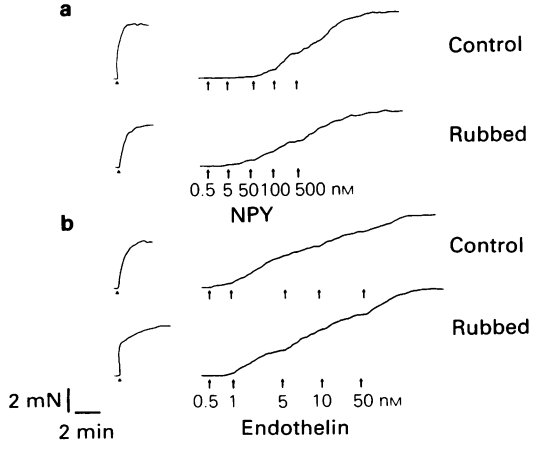

Figure 2 Recordings of the contractile effects of increasing concentrations of neuropeptide Y (NPY; a) and endothelin (b) on control and rubbed (to remove the endothelium) preparations of pig small splenic arteries. The response to phenylephrine (PE; 10 μM) is shown in the left column as a reference. Calibrations for tension and time are indicated in lower left corner.

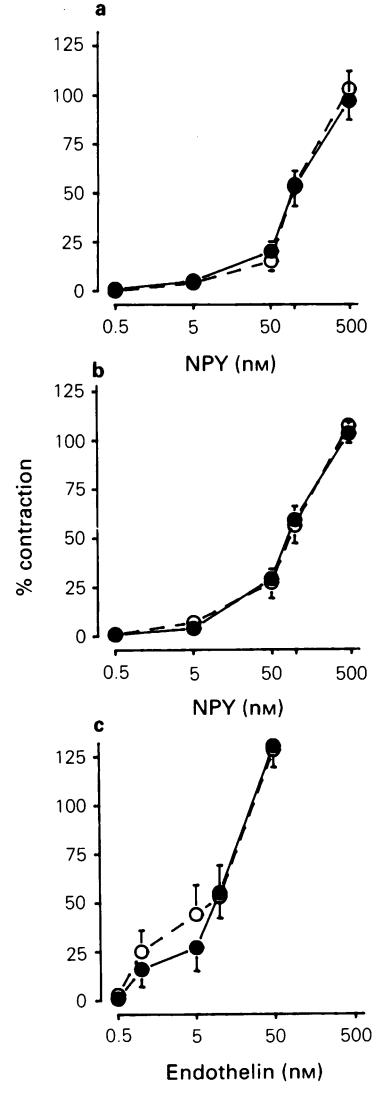

Figure 3 Concentration-response curves showing the contractile effects of neuropeptidey (NPY) on human skeletal muscle small arteries (a) and pig splenic arteries (b) and of endothelin on pig splenic arteries (c) during control conditions (●) and after removal of the endothelium by rubbing the inner surface of the vessels (○). The contractile effect is expressed as a percentage of that evoked by 10 μM noradrenaline (a) or phenylephrine (b, c) in each individual preparation. Mean values from 7–10 observations; s.e.mean shown by vertical bars.

Table 1 EC_{50} values of neuropeptide Y (NPY), endothelin, calcitonin gene-related peptide (CGRP) and vasoactive intestinal polypeptide (VIP)

	NPY		*Endothelin*	*CGRP*	*VIP*
	SMA	SA	SA	SMA	SMA
Control	7.0 ± 0.11	7.1 ± 0.07 *	8.0 ± 0.08	9.3 ± 0.16 **	8.3 ± 0.06
Rubbed	7.1 ± 0.03	7.1 ± 0.05	8.1 ± 0.11	9.5 ± 0.16	8.3 ± 0.21

The EC_{50} values are expressed as the negative logarithm of the concentration needed to produce 50% of the maximal effect on control and rubbed (to remove the endothelium) segments of small human skeletal muscle arteries (SMA) and pig splenic arteries (SA). There were no significant differences between the control and rubbed preparations. Significant differences between the EC_{50} values of NPY/endothelin and CGRP/VIP, respectively, are indicated, $^{**}P < 0.01$. Mean values and s.e.mean from 5–10 observations.

potent as a contractile agent in SA (Figure 3, Table 1).

Effects of neuropeptide Y and endothelin on rubbed arteries

The contractile effects of NPY on SMA and SA were not different in the rubbed preparations as compared with controls at any of the concentrations tested (Figures 2a and 3a,b). Furthermore, the EC_{50} values of NPY were not changed by endothelium removal (Table 1). Similar results were obtained with endothelin in SA. Thus, neither the maximal effect nor the EC_{50} value were altered in the absence of endothelium (Figures 2b and 3c, Table 1).

Effects of substance P, calcitonin gene-related peptide and vasoactive intestinal peptide on control arteries

SP (10 nM) relaxed precontracted control SMA and SA by 90 ± 3% ($n = 13$) and 82 ± 4% ($n = 12$), respectively (Figures 1 and 4). CGRP and VIP induced concentration-dependent relaxations of SMA with maximal effects at 10 and 100 nM, respectively (Figures 4 and 5). The EC_{50} values revealed that CGRP was approximately 10 times more potent than VIP (Table 1). In SA, CGRP (100 nM) caused a relaxation of 100% ($n = 5$; not shown).

Effects of substance P, calcitonin gene-related peptide and vasoactive intestinal peptide on rubbed arteries

The relaxant effect of SP (10 nM) on rubbed SMA and SA was reduced to 1 ± 1% ($n = 11$) and 8 ± 2% ($n = 9$), respectively (Figures 1 and 4). However, the effects of CGRP and VIP on SMA were not changed by endothelium removal. Thus, both the maximal relaxant effects and the EC_{50} values were similar in rubbed preparations and in controls (Figures 4 and 5, Table 1). The relaxant effect of CGRP (100 nM) on SA was also unchanged by endothelium removal (99 ± 1% relaxation, $n = 9$). Further CGRP relaxed rubbed SMA that were precontracted with NPY (500 nM) with an effect similar to that observed in preparations precontracted with NA (Figure 5a).

Discussion

The present results show that NPY causes potent and strong contractions of splenic arteries from the pig *in vitro* and as previously shown (Pernow *et al.*, 1987b) also of human skeletal muscle arteries. This is in accord with the large number of reports showing potent vasoconstrictor actions of NPY in various preparations *in vivo* (Lundberg & Tatemoto, 1982; Hellström *et al.*, 1985; Lundberg *et al.*, 1986; Pernow *et al.*, 1987a; 1988b). In several other blood vessels *in vitro* NPY causes weak or no contractions (see Pernow, 1988; Potter, 1988). In these latter cases relatively large arteries were investigated in contrast to the small ones used in the present study, indicating that NPY may exert its main effect on small

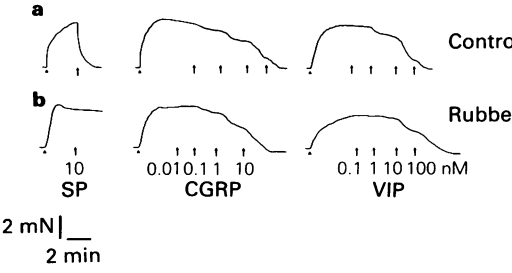

Figure 4 Recordings of the relaxant effects of substance P (SP), calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP) on control (a) and rubbed (to remove the endothelium) segments (b) of human skeletal muscle small arteries. The arteries were precontracted with 10 μM noradrenaline (arrow heads) and the peptides were added in the concentrations indicated in (b). Calibrations for tension and time are indicated in the lower left corner.

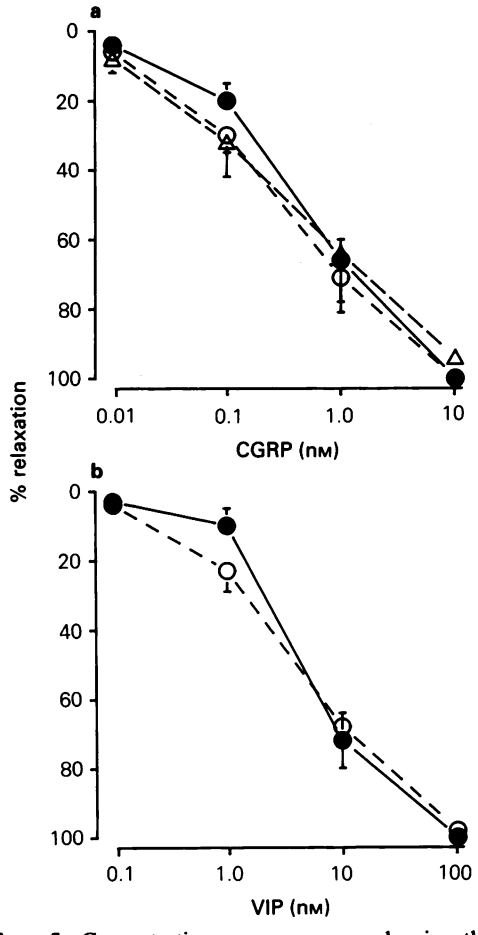

Figure 5 Concentration-response curves showing the relaxant effects of calicitonin gene-related peptide (CGRP; a) and vasoactive intestinal peptide (VIP; b) on human skeletal muscle arteries under control conditions (●) and after removal of the endothelium by rubbing the inner surface of the vessels (○). The arteries were precontracted with 10 μM noradrenaline and the effects of the peptides are expressed as percentage relaxation of the precontracted tone. In addition, the relaxant effect of CGRP on rubbed arteries precontracted with neuropeptide Y (500 nM, △) is shown in (a). Mean values from 5–9 observations; vertical bars show s.e.mean.

arteries and arterioles involved in blood flow regulation (see Pernow, 1988).

The contractile effect of NPY on both SMA and SA was found to be independent of an intact endothelial layer, indicating that NPY does not act via release of an EDCF such as endothelin, but rather has a direct effect on the vascular smooth muscle. This finding is in agreement with and supports the view that NPY released from sympathetic nerve terminals, which are located at the adventitio-medial junction, can be involved in vasoconstriction evoked by sympathetic nerve stimulation in both the spleen (Lundberg *et al.*, 1986) and skeletal muscle (Pernow *et al.*, 1988b).

It has previously been shown that in the rabbit ear artery, in which NPY does not produce any contractions *per se*, the potentiating effect of NPY on NA-induced contractions is dependent on an intact endothelium (Daly & Hieble, 1987). In the SMA and SA, where NPY itself caused potent and endothelium-independent contractions, no potentiating effects of NPY on NA-evoked contractions in SMA (Pernow *et al.*, 1987b) or PE-evoked contractions in SA (unpublished) are observed. These findings might suggest that NPY has different types of actions on different arteries and that the potentiation and contraction, respectively, induced by NPY are mediated via separate mechanisms.

Endothelin, which is a peptide isolated from porcine endothelial cells (Yanagisawa *et al.*, 1988), was found to be about 10 times more potent than NPY in contracting the SA. The EC_{50} value of endothelin in the SA (10 nM) is about 20 fold higher than that found in studies on isolated strips of pig coronary arteries (Yanagisawa *et al.*, 1988). This may indicate that regional variations exist regarding the potency of this novel peptide, which is supported by observations *in vivo* (Pernow *et al.*, 1988a). The contractile effect of endothelin was unchanged after removal of the endothelium, indicating that its receptors are not located on the endothelial cells, but rather that endothelin after release from the endothelium acts directly on the vascular smooth muscle.

The relaxant effect of SP was absent in rubbed arterial preparations which is in agreement with previous observations that the effect is mediated via release of an EDRF (see Furchgott, 1984). In contrast, the effect of CGRP on both arteries tested was found to be endothelium-independent which confirms and extends the findings from cat cerebral arteries (Edvinsson *et al.*, 1985) and from pig coronary arteries (Franco-Cereceda *et al.*, 1987b). In the rat aorta, however, the effect of CGRP has been described as endothelium-dependent (Brain *et al.*, 1985). In addition, the effect of CGRP on various large human arteries has been found to be endothelium-dependent (Hughes *et al.*, 1986). These apparent discrepancies might be due to species differences (see Luscher & Vanhoutte, 1988) and indicate that the mechanism of CGRP-induced vasodilatation varies between large and small arteries. Similar variations also seem to exist with regard to the action of VIP. In the present study VIP caused endothelium-independent relaxation of small SMA whereas in human splenic and traverse cervical

arteries the effect is endothelium-dependent (Hughes *et al.*, 1986).

The CGRP/SP-containing sensory nerves are generally located outside the media of the arteries (Franco-Cereceda *et al.*, 1987a). Since the effect of SP is mediated via the endothelium, this finding may imply that SP after release has to diffuse through the smooth muscle layer to the endothelium in order to produce its vascular effects. CGRP on the other hand can activate the smooth muscle directly, which seems more relevant from a functional viewpoint. It should, however, be emphasized that different conditions may exist in resistance vessels in an *in vivo* situation.

It has previously been reported that NPY inhibits vasodilatation induced by NA, adenosine and acetylcholine in rabbit large conduit coronary arteries *in vitro* (Han & Abel, 1987). In the present study, however, CGRP was found to relax the small SMA that were precontracted by NPY with a potency and efficacy similar to that observed in NA-precontracted arteries. This suggest that NPY does not exert any inhibitory action on the relaxant effect of CGRP on this vessel, despite the observations that these two peptides have opposite effects on the same second messenger system, as NPY inhibits (Fredholm *et al.*, 1985) and CGRP stimulates (Edvinsson *et al.*, 1985) cyclic adenosine monophosphate accumulation.

In conclusion, the present results describe the action of various contractile and relaxatory peptides on SMA and SA. NPY and endothelin caused potent endothelium-independent contractions, indicating that their effects are not mediated via release of an EDCF but rather via direct actions on vascular smooth muscle. The relaxant effect of SP required intact endothelial cells, whereas that of CGRP and VIP did not.

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23–25 July 1989

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7–12 August 1989

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1–3 September 1989

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17–20 September 1989

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19–21 September 1989

Electrochemical Detection, HPLC and *in vivo* monitoring in the Biosciences, Nottingham, U.K. (Dr I.A. MacDonald, Department of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham, U.K.)

20–21 September 1989

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28 September 1989

Leukotrienes. London, U.K. (Society for Drug Research, c/o Institute of Biology, 20 Queensbury Place, London SW7 2DZ, U.K.)

22–25 October 1989

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6–8 December 1989

Presynaptic Receptors — an Examination of Different Views, New York City, USA. (Conference Department, New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.)

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18–20 April 1990

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