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SPECIAL REPORT

5-Hydroxyindole slows desensitization of the 5-HT_3 receptor-mediated ion current in N1E-115 neuroblastoma cells

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Effects of 5-hydroxyindole (5-OHi) on 5-HT_3 receptor-operated ion current were investigated in voltage-clamped N1E-115 neuroblastoma cells. In the presence of 1 mM 5-OHi, the amplitudes of inward currents induced by the agonists 5-hydroxytryptamine (5-HT), 2-methyl-5-HT and dopamine were enhanced and desensitization of the responses was markedly slowed down. The results indicate that 5-OHi selectively modifies the desensitization of the 5-HT_3 receptor-mediated ion current.

Keywords: 5-Hydroxytryptamine; 5-HT_3 receptor; desensitization; 5-hydroxyindole; N1E-115 mouse neuroblastoma; whole-cell voltage clamp

Introduction Cells of the murine neuroblastoma clone N1E-115 express 5-hydroxytryptamine 5-HT_3 receptors (Hoyer & Neijt, 1988). The fundamental physiological and pharmacological properties of the 5-HT_3 receptor-operated ion current in whole-cell voltage-clamped neuroblastoma cells have been described in detail (Neijt *et al.*, 1986; 1988; 1989; Yakel & Jackson, 1988; Lambert *et al.*, 1989; Yang, 1990). The 5-HT_3 receptor appears to be a member of the ligand-gated ion channel family (Maricq *et al.*, 1991). Receptor occupation by 5-HT leads to a rapid increase of membrane inward current, followed by a decrease. The latter has been demonstrated to depend on agonist concentration and is caused by desensitization, which persists in the presence of the agonist. Desensitization is completely reversed after removal of the agonist (Neijt *et al.*, 1989). In N1E-115 cells, responses similar to those to 5-HT are evoked by the selective 5-HT_3 receptor agonist, 2-methyl-5-HT (2-Me-5-HT) and dopamine (Neijt *et al.*, 1988). Dopamine acts as a partial 5-HT_3 agonist. The response to dopamine in N1E-115 cells is completely blocked by the selective 5-HT_3 receptor antagonist, ICS 205–930 (Neijt *et al.*, 1986), and the agonists 5-HT and dopamine show full cross-desensitization (Neijt *et al.*, 1988). This demonstrates that the response to dopamine in N1E-115 cells is solely due to 5-HT_3 receptor activation.

We have investigated the effects of extracellular 5-hydroxyindole (5-OHi) in N1E-115 cells and show that this 5-HT moiety slows agonist-induced desensitization of the 5-HT_3 receptor-mediated inward current.

Methods Cell culture and electrophysiological techniques were identical to those previously described (Neijt *et al.*, 1989). Experiments were performed on dibutyryladenosine 3':5'-cyclic monophosphate (db-cyclic AMP) differentiated cells of passages 31–42 of the murine neuroblastoma clone N1E-115 (Amano *et al.*, 1972) under whole-cell voltage clamp, using the suction pipette technique (Lee *et al.*, 1978; Neijt *et al.*, 1989). Cells were voltage-clamped at a holding potential of −70 mV. Cells were continuously superfused with external solution and ion currents were evoked by changing to agonist- and, optionally, 5-OHi-containing external solution (Neijt *et al.*, 1989). 5-OHi was dissolved in dimethylsulphoxide as a 1 M stock solution, stored frozen at −20°C, and freshly thawed prior to the experiments. The composition of external and pipette solutions was the same as previously described (Neijt *et al.*, 1989). Cells were repeatedly exposed to agonist after an interval of at least 100 s in order to allow complete recovery from desensitization. All experiments were performed at room temperature (20–24°C). Values are presented as mean ± s.d.

Results The superfusion of N1E-115 cells under whole-cell voltage clamp ($V_m = -70$ mV) with external solution containing a maximally effective concentration of 10 μM 5-HT evoked a characteristic, transient 5-HT_3 receptor-mediated inward current (Figure 1a(i)). Superfusion with external solution containing 1 mM 5-OHi evoked no detectable electrophysiological response (not shown). However, the response evoked by superfusion with 10 μM 5-HT in the presence of 1 mM 5-OHi was greatly modified. The peak amplitude was enhanced and the desensitization was slowed down as compared to those of the control 5-HT response (Figure 1a(ii)). The same effects were observed irrespective of whether cells were already pre-exposed to 5-OHi or not, suggesting a rapid action. The effects of 5-OHi were rapidly reversed on washing with external solution (Figure 1a(iii)). Amplitude and desensitization of the 5-HT-induced inward current were hardly affected at a concentration of 10 μM 5-OHi (not shown). Similar reversible effects of 5-OHi were observed on inward currents evoked by superfusion with 50 μM of the selective 5-HT_3 agonist, 2-Me-5-HT (Figure 1b) or with 1 mM of the partial 5-HT_3 agonist, dopamine (Figure 1c). Responses in Figures 1b and 1c have been scaled to Figure 1a for matching control response amplitudes in order to demonstrate clearly the effects of 5-OHi. Average peak amplitudes of the 50 μM 2-Me-5-HT- and the 1 mM dopamine-induced control inward currents were 75 ± 7% ($n = 4$) and 35 ± 7% ($n = 17$) of that of the 10 μM 5-HT control responses in the same cells. To quantify the effects, the peak inward currents in the presence of 5-OHi and the fitted exponential time constants of desensitization have been normalized to those of control responses obtained from the same cells. The data presented in Table 1 show that 5-OHi enhanced the peak amplitude of inward currents induced by superfusion with the various 5-HT_3 receptor agonists by 30–94% and slowed desensitization 2.5–5 fold.

Discussion The mechanism of desensitization of the 5-HT_3 receptor-mediated ion current is still unresolved. The rate of desensitization has been shown to increase with agonist concentration in a sigmoid manner and it has been suggested that at saturating agonist concentration, desensitization is rate-limited by some intrinsic conformational change of the agonist receptor-ion channel complex (Neijt *et al.*, 1989). The present results show that 5-OHi, a moiety of the 5-HT molecule, dramatically slows the desensitization of inward

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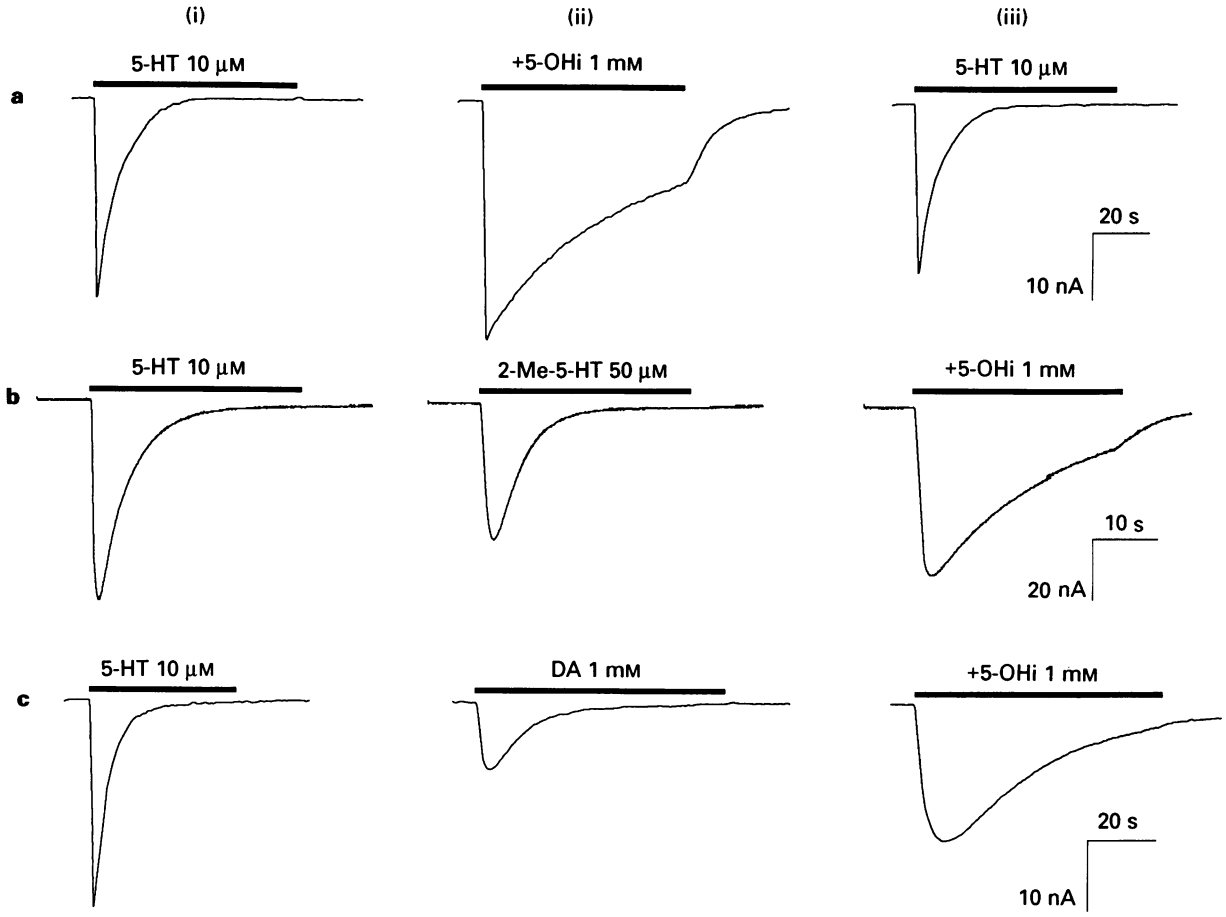

Figure 1 Effects of 5-hydroxyindole (5-OHi) on 5-HT_3 receptor-mediated inward current in N1E-115 cells voltage clamped at −70 mV. (a) Superfusion with external solution containing 10 μM 5-HT evokes a transient inward current, which rapidly desensitizes in the continued presence of 5-HT (a(i)). In external solution containing 1 mM 5-OHi the peak inward current induced by the same concentration of 5-HT is enhanced and the desensitization is markedly slowed (a(ii)). These effects of 5-OHi are rapidly reversed within 100 s of washing with external solution (a(iii)). (b), (c) Desensitization of inward currents induced by the selective 5-HT_3 receptor agonist 2-methyl-5-HT (b(ii)) and the partial 5-HT_3 receptor agonist dopamine (c(ii)) is also slowed and the peak amplitude of the inward currents is also enhanced in the presence of 5-OHi (b(iii) and c(iii)). Responses in (a), (b) and (c) are from three different cells. The responses of the different cells have been scaled for matching peak amplitudes of control inward currents evoked with 10 μM 5-HT.

Table 1 Effects of 1 mM 5-hydroxyindole on 5-hydroxytryptamine 5-HT_3 receptor-mediated inward currents in N1E-115 cells

Agonist	*Relative peak amplitude*	*Relative time constant*	n
5-HT	1.30 ± 0.10	3.27 ± 0.76	19
2-Me-5-HT	1.35 ± 0.10	4.75 ± 0.39	3
Dopamine	1.94 ± 0.15	2.73 ± 0.23	3

Peak amplitudes and time constants of desensitization are relative to the values obtained from control responses with the respective agonists in the same cell. Values represent mean ± s.d. obtained from *n* different cells.

currents induced by 5-HT_3 receptor agonists in N1E-115 cells. No effect of 5-OHi on the activation of inward current was observed. Simultaneously with the slowing of desensitization the amplitude of the inward current was enhanced. From the results in Table 1 there does not appear to be any relationship between the effects of 5-OHi on the amplitude and desensitization of currents evoked by 5-HT, 2-Me-5-HT and dopamine. Since all three agonists mediate their effects by 5-HT_3 receptors in N1E-115 cells, the independence of the effects of 5-OHi on amplitude and desensitization is most likely due to agonist-dependent differences in the coupling between receptor activation and desensitization.

The availability of a tool to modulate selectively the desensitization of the 5-HT_3 receptor-operated ion channel opens new perspectives for investigation of the mechanism of a functional process that is shared with other ligand-gated ion channels. In addition, the potential existence of endogenous modulators of desensitization becomes highly interesting from a pharmacological viewpoint. Although it is conceivable from the structural analogy between 5-HT and 5-OHi that the latter is able to interact with the agonist recognition site of the 5-HT_3 receptor, the precise nature of the interaction of 5-OHi remains to be established and is currently being investigated.

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SPECIAL REPORT

Induction of a calcium-independent NO synthase by hypercholesterolaemia in the rabbit

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Endothelium-dependent and -independent relaxation of aortic ring preparations was assessed and nitric oxide (NO) synthase activity measured in the lung, and cerebellum of cholesterol-fed and normal rabbits. Endothelium-dependent relaxation of acetylcholine and ATP was depressed while that to the calcium ionophore, A23187, was unaltered in the cholesterol-fed group. Relaxation to sodium nitroprusside was however greater in aortae from the cholesterol-fed animals. Neither Ca^{2+}-dependent nor Ca^{2+}-independent NO synthase activity could be detected in aortae or hearts taken from either group of animals. Activity of both enzymes was unaltered in cerebellae from both groups of animals. Activity of the Ca^{2+}-independent enzyme was however significantly greater (ca. 2 fold) in lungs from the cholesterol-fed rabbits though the activity of the Ca^{2+}-dependent NO synthase was not significantly altered. This finding may account for the increased production of nitrogen oxides previously observed in this model of hypercholesterolaemia.

Keywords: Hypercholesterolaemia; vascular relaxation; NO synthase activity; Ca^{2+}-dependence

Introduction Reduced activity of endothelium-derived relaxing factor (EDRF), recently identified as nitric oxide (NO, Palmer *et al.*, 1987), has been demonstrated in both large and small arteries in various models of hypercholesterolaemia (for review see Henderson, 1991). The mechanism underlying this inhibition is unclear (for review see Flavahan, 1992). A recent study by Minor and colleagues (1990) showed in bioassay experiments a decreased dilator activity of aortic effluent from cholesterol-fed rabbits which was associated with increased production of nitrogen oxides as measured by chemiluminescence. This study therefore suggests that while there is increased NO production as a result of hypercholesterolaemia, the reduced EDRF activity results either from increased inactivation of NO, or an increased release of vasoconstrictors. In an attempt to account for increased NO production, we have compared the constitutive (Ca^{2+}-dependent) and non-constitutive (Ca^{2+}-independent) NO synthase enzyme activities in various tissues from rabbits fed on a normal or a high-cholesterol diet.

Methods *Cholesterol feeding* Male New Zealand white rabbits (2–2.5 kg) were randomly assigned to two groups. One group received a standard diet and the other a diet uniformly enriched with 1% cholesterol for 8 weeks.

Tissue preparation Animals were killed by cervical dislocation. Blood samples were taken via cardiac punture into heparinised tubes, and plasma cholesterol levels assessed by a standard cholesterol oxidase method.

The thoracic aorta was removed into Krebs solution gassed with 95% O_2/5% CO_2 at room temperature and divided in half. One half of the cleaned aorta, the heart, one lung and cerebellum were removed into liquid nitrogen, and stored at −70°C for the measurements of NO synthase activity.

The remaining aortic tissue was cut into 3 mm wide rings for isometric tension recording.

Measurement of relaxation responses The 3 mm wide endothelium-intact aortic rings were mounted in tissue baths as described previously (Chappell *et al.*, 1987) and preconstricted to 50–70% of maximum with phenylephrine. Relaxation responses to cumulative addition of acetylcholine, ATP or sodium nitroprusside (SNP), or a single concentration of 3×10^{-7} M A23187 were measured and expressed as percentage relaxation of the phenylephrine-induced tone.

NO synthase assay The Ca^{2+}-dependent and -independent NO synthase activities present in the heart, lung, cerebellum and aorta were measured as previously described (Salter *et al.*, 1991). Briefly, tissues were homogenized in ice-cold lysis buffer containing protease inhibitors and centrifuged (4°C, 30,000 *g*, 10 min). An aliquot of the supernatant was added to the assay buffer containing L-[U-^{14}C]-arginine, NO-synthase co-factors with or without EGTA and an NO-synthase inhibitor. The reaction was terminated by addition of AG 50 W-X8 exchange resin (sodium form). The [^{14}C]-citrulline content of the supernatant was measured by standard liquid scintillation counting techniques.

Materials Phenylephrine hydrochloride, acetylcholine chloride, ATP, sodium nitroprusside and A23187 were obtained from Sigma Chemical Co, Dorset, UK. All other chemicals were Analar grade.

Statistics Results are expressed as mean ± s.e.mean. The concentration-response curves were fitted to log-logistic curves by a least squares numerical minimization procedure. By comparing two curves simultaneously with first separate and then combined curve parameters, variance ratio tests were performed to detect differences in the asymptotes, intercepts and slopes. Other comparisons were made by use of Student's *t* test for unpaired data and considered significantly different when $P<0.05$.

Results *Cholesterol feeding* Blood cholesterol levels were significantly increased in the cholesterol-fed rabbits (56.19 ± 3.81 mmol l^{-1}; $n=10$) compared to the normals (1.32 ± 0.13 mmol l^{-1}; $n=12$, $P<0.01$).

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Relaxation responses Phenylephrine-induced tone was not significantly different in rings from control or cholesterol-fed animals for each drug studied (ACh: 4.9 ± 0.28 g and 5.3 ± 0.4 g; ATP: 5.5 ± 0.28 g and 5.3 ± 0.45 g; SNP: 4.0 ± 0.13 g and 4.6 ± 0.31 g; A23187: 4.5 ± 0.23 g and 4.8 ± 0.34 g respectively; $n = 12$ for ACh, ATP and SNP and 6 for A23187). The concentration-response curve for acetylcholine-induced relaxation in aortae from the cholesterol-fed rabbits ($ED_{50} = 5.1 \times 10^{-7}$ M) was significantly shifted to the right when compared to that of aortae from normal-fed rabbits ($ED_{50} = 4.7 \times 10^{-8}$ M, $P<0.001$) (Figure 1a). Also, the peak relaxation to acetylcholine in aortae from cholesterol-fed rabbits (39.4 ± 7.0%) was significantly lower than those fed a normal diet (54.9 ± 2.9%; $P<0.01$). The ATP concentration-response curve (Figure 1b) was similarly significantly shifted to the right in tissues from cholesterol-fed animals compared to normal animals $EC_{50} = 5.1 \times 10^{-5}$ M and 1.7×10^{-5} M respectively ($P<0.001$). The peak relaxation achieved with ATP was not however significantly different between the two groups. Figure 1c by contrast shows that the concentration-response curve to SNP was significantly shifted to the left in aortae from the cholesterol-fed animals (EC_{50} 2.3×10^{-6} M) compared with control animals (EC_{50} 6×10^{-6} M; $P<0.001$). Peak relaxation to nitroprusside was not however significantly altered by cholesterol feeding. Relaxation to A23187 (3×10^{-7} M) was similar in aortae from both groups of rabbits (58.0 ± 1% ($n = 6$) in normals; 51.1 ± 5.9% ($n = 6$) in cholesterol-fed animals).

NO synthase activity Neither Ca^{2+}-dependent nor Ca^{2+}-independent NO synthase activity, could be detected in either the heart or aortic samples from either group of rabbits. Both enzymes were detectable in the cerebellar samples, but there was no significant differences between either the Ca^{2+}-dependent or -independent enzyme in the normal- (1.68 ± 0.16 and 0.02 ± 0.01 fmol min^{-1} μg^{-1} protein respectively) or the cholesterol-fed rabbits (1.72 ± 0.08 and 0.02 ± 0.01 fmol min^{-1} μg^{-1} protein respectively). As can be seen from Figure 2 however, the Ca^{2+}-independent NO synthase activity expressed in the lung of the cholesterol-fed rabbits was significantly greater than in the lungs of control rabbits. The Ca^{2+}-dependent NO synthase was slightly (but not statistically significantly) lower in the lungs from the cholesterol-fed animals compared to normal animals.

Discussion The data confirm previous studies in aortae from cholesterol-fed rabbits showing impaired endothelium-dependent responses to acetylcholine and ATP, whereas responses to the calcium ionophore, A23187, and sodium nitroprusside are unaltered or enhanced. The observation that the Ca^{2+}-independent NO synthase activity in the lungs of the cholesterol-treated animals was increased is a novel finding and if the findings can be extrapolated to the systemic vasculature may explain the observations by Minor and colleagues (1990) of an increased production of nitrogen oxides as a result of hypercholesterolaemia. The origin of the increased NO synthase in the lungs cannot be established with certainty however as lung tissue comprises many cell types. Blood vessels are one possible source, but other cell types like macrophages cannot be excluded.

The observation of reduced endothelium-dependent relaxation to ACh and ATP but unaltered relaxation to A23187 has been discussed elsewhere (Flavahan, 1992) and is unlikely to be due to altered receptor coupling between agonists and the endothelial cell. The fact that both NO synthase activity and nitrogen oxide production are increased in hypercholestrolaemia while endothelium-dependent relaxation is decreased, probably reflects increased destruction of NO, possible by free radicals. We were unable to detect any increase in activity of the Ca^{2+}-independent NO synthase in the heart or aortic tissue. The reason for this cannot be established with certainty though it is likely to be related to the sensitivity of the assay and also, at least in the case of the aorta, to the relatively small amount of tissue assayed. The source of Ca^{2+}-independent NO synthase activity in the lungs from control animals is not known.

The mechanism underlying the increase in inducible NO synthase activity is unknown. There is good evidence that cytokines are involved in the atherogenic process (Schwartz *et al.*, 1991) and are also known to induce the Ca^{2+}-independent NO synthase in endothelium and vascular smooth muscle (Busse & Mülsch, 1990; Lamas *et al.*, 1991). Evidence is also accumulating that oxidised low density lipoprotein may behave in a way similar to cytokines (for review see Rosenfeld, 1991), activating inflammatory and immune responses in cells in blood vessel walls and atherosclerotic plaques.

Interestingly no increase in the cerebellar activity of inducible NO synthase in the hypercholesterolaemia animals was found in the present study.

In conclusion, this study shows for the first time that hypercholesterolaemia in the rabbit increases expression of the Ca^{2+}-independent NO synthase enzyme in the lung and may account for increased production of nitrogen oxides observed previously in this model of hypercholesterolaemia.

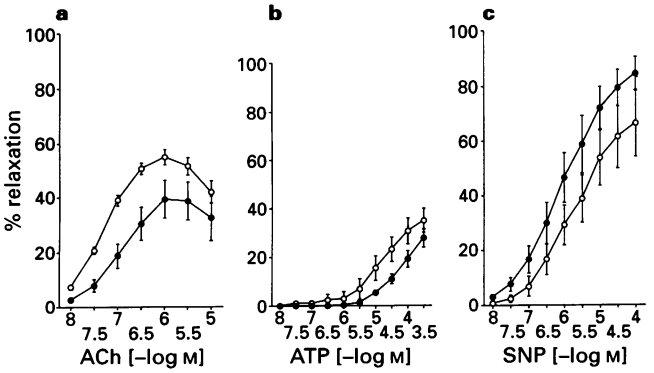

Figure 1 Concentration-responses to relaxation induced by acetylcholine (a), ATP (b) and sodium nitroprusside (c) in aortic rings from rabbits fed a normal diet (○) and a diet containing 1% cholesterol (●). Each point is the mean and vertical bars indicate the s.e.mean of 12 observations.

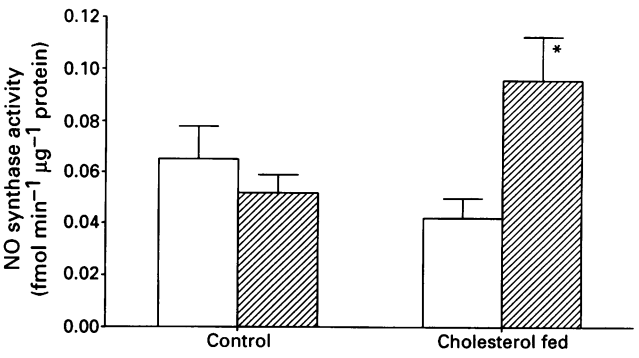

Figure 2 Diagram showing mean (± s.e.mean) Ca^{2+}-dependent (open columns) and -independent (hatched columns) NO synthase activity in lungs from rabbits fed a normal diet and a diet containing 1% cholesterol. (*$P<0.05$ cf. control group; $n = 8-11$ observations).

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SPECIAL REPORT

Ramiprilat increases bradykinin outflow from isolated hearts of rat

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To establish that bradykinin is formed in the heart we measured bradykinin in the venous effluent from rat isolated hearts perfused with Krebs-Henseleit buffer. In addition, we examined the effect on bradykinin outflow of the angiotensin converting enzyme (ACE) inhibitor, ramiprilat. From rat isolated normoxic hearts a bradykinin outflow of 0.85 ± 0.1 ng ml^{-1} perfusate g^{-1} wet weight was measured. Perfusion with ramiprilat increased the bradykinin concentration to 2.8 ± 0.3 ng ml^{-1} perfusate g^{-1} wet weight. During ischaemia bradykinin outflow maximally increased 8.2 fold to 7.0 ± 0.5 ng ml^{-1} perfusate g^{-1}, and in ramiprilat-perfused hearts 5.8 fold to 16.0 ± 1.8 ng ml^{-1} perfusate g^{-1}. In the reperfusion period bradykinin outflow normalized to values measured in the respective pre-ischaemic period.

The presents data show that bradykinin is continuously formed in the rat isolated heart. Ischaemia increases bradykinin outflow from the heart. Presumably by inhibiting degradation of kinins, ACE inhibition significantly increased the bradykinin concentration during normoxia, ischaemia and reperfusion.

Keywords: Bradykinin-formation; rat isolated hearts; ischaemia; angiotensin converting enzyme (ACE)-inhibition; ramiprilat

Introduction Angiotensin converting enzyme (ACE) inhibitors like ramiprilat act not only through inhibition of formation of angiotensin (AII) but also through inhibition of bradykinin degradation. Recent data from rat isolated working hearts showed that both ramiprilat and bradykinin improve myocardial metabolism during ischaemia and this was accompanied by a reduction in reperfusion injuries (Linz *et al.*, 1990). ACE inhibition and bradykinin perfusion evoked identical beneficial changes in cardiodynamics and metabolism in these hearts. The protective effects were abolished by the specific BK_2 bradykinin receptor antagonist Hoe 140 suggesting that bradykinin might be beneficial in myocardial ischaemia (Linz *et al.*, 1990). In the present study, we were interested in whether bradykinin is formed locally in rat isolated hearts. Therefore we measured bradykinin release in the venous effluent of rat isolated ischaemic hearts perfused with Krebs-Henseleit buffer, with and without the ACE inhibitor, ramiprilat.

Methods Male Wistar rats (weighing 280–300 g) Möllegaard, Skensved, Denmark) were given sodium heparin (500 ukg^{-1}) intraperitoneally (i.p.) 1 h before the animals were anaesthetized. Anaesthesia was induced by i.p. injection of 200 mg kg^{-1} hexobarbitone (Evipan). The hearts were excised and placed in ice-cold perfusion medium until contraction had ceased after approximately 5 s. Isolated hearts were perfused via the aorta at a constant perfusion pressure equivalent to 65 mmHg with Krebs-Henseleit solution gassed with 95% O_2 plus 5% CO_2 at 37°C and pH 7.4. The perfusate did not recirculate. Hearts were perfused with and without ramiprilat 1×10^{-7} mol l^{-1} for an initial 15-min control perfusion period. Then, acute regional myocardial ischaemia was produced by occlusion of the left coronary artery close to its origin. Arterial occlusion was maintained for 15 min after which the occlusion was removed and arterial reperfusion commenced.

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One ml of the venous effluent was directly collected into EDTA-solution (10^{-4} mol l^{-1}) after 5 and 10 min in the control perfusion period (pre-ischaemic period), after 5, 7.5, 10 and 15 min in the ischaemic period, and 1, 2, 3 and 4 min after reperfusion period. Subsequently the samples were lyophilized and resuspended in 0.2 M Tris/0.01 M EDTA/0.1% lysozyme, pH 6.4. Bradykinin concentrations were measured by use of a specific radioimmunoassay capable of detecting 20 pg ml^{-1} of kinin (Proud *et al.*, 1983). Bradykinin concentrations were expressed in ng ml^{-1} perfusate related to g wet weight of the whole heart (792 ± 160 mg) in the control and reperfusion period and of the right ventricle (132 ± 15 mg) in the ischaemic period. This procedure is valid because the rat heart has been shown to have few collaterals (Schaper *et al.*, 1988).

Statistical analysis was performed with ANOVA followed by the Bonferroni test when appropriate. Differences were considered significant if $P<0.05$. Results are given as mean ± s.e.mean.

Results In the control period, basal coronary flow in Krebs-Henseleit buffer perfused hearts was 13.9 ± 0.7 ml min^{-1}. During ischaemia, coronary flow was reduced by about 50% (7.6 ± 0.9 ml min^{-1}) and recovered in the reperfusion period (15.3 ± 1.1 ml min^{-1}). In Krebs-Henseleit buffer perfused hearts, basal bradykinin outflow in the control period was 0.85 ± 0.1 ng ml^{-1} perfusate g^{-1} wet weight after 10 min perfusion, increased during the ischaemic period to 7.0 ± 0.5 ng ml^{-1} perfusate g^{-1} wet weight after 7.5 min perfusion, and immediately returned to initial values in the reperfusion period (Figure 1).

In the presence of the ACE inhibitor, ramiprilat, coronary flow was not significantly changed when compared to Krebs-Henseleit buffer-perfused hearts. The respective values were 13.7 ± 0.6 ml min^{-1} (control period), 7.8 ± 1.0 ml min^{-1} (ischaemic period) and 14.1 ± 1.1 ml min^{-1} (reperfusion period). However, addition of the ACE inhibitor, ramiprilat, significantly enhanced the bradykinin concentrations in all three perfusion periods. In the control period the concentration of bradykinin in the venous effluent increased to

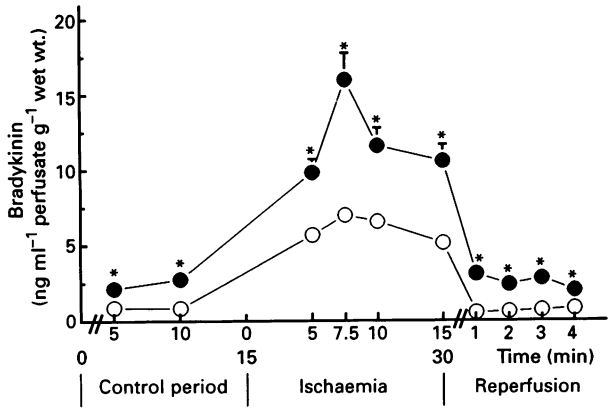

Figure 1 Bradykinin release from rat isolated hearts: (○) Krebs-Henseleit buffer-perfused hearts; (●) ramiprilat (1×10^{-7} mol l^{-1})-perfused hearts. Each value represents mean ± s.e.mean (vertical bars) of 12 control hearts and 12 hearts perfused with ramiprilat. *$P < 0.05$ vs Krebs-Henseleit buffer-perfused hearts (controls).

2.8 ± 0.3 ng ml^{-1} perfusate g^{-1} wet weight after 10 min perfusion. This was a 3.2 fold increase when compared with the Krebs-Henseleit buffer-perfused control group. In the ischaemic period, local ACE inhibition with ramiprilat induced a maximum increase of the bradykinin concentration in the venous effluent of 16.0 ± 1.8 ng ml^{-1} perfusate g^{-1} wet weight after 7.5 min perfusion, being back to initial values in the reperfusion period (Figure 1).

Discussion The present data show that bradykinin is released from rat isolated hearts perfused with Krebs-Henseleit buffer. This basal bradykinin release was increased 3.2 fold when the hearts were perfused with the ACE inhibitor ramiprilat, dissolved in Krebs-Henseleit solution. In comparison with normoxic controls, ischaemia increased bradykinin release 8.2 fold and 5.8 fold in the absence and presence of ramiprilat, respectively whereas in the reperfusion period in both groups, bradykinin release reached the values of the respective control perfusion period within 1 min. The missed overshoot of kinin release in the early reperfusion phase could be explained by the fact that the stimulus of ischaemia had been removed. Furthermore oxygen free radicals which occur in the early reperfusion phase might be responsible for an accelerated breakdown of kinins.

Increased bradykinin levels during ischaemia were observed by Wilkens *et al.* (1970) who reported that a small fall in cardiac tissue pH, which follows an ischaemic insult, induces an activation of the local kinin system. Hashimoto *et al.* (1977) found an increased concentration of bradykinin in coronary sinus blood after coronary occlusion in the dog. Also in the dog, myocardial ischaemia induced by coronary artery stenosis and sympathetic stimulation caused the heart to release kinins (Matsuki *et al.*, 1987). And lastly in man, kinin levels in peripheral blood were found to increase soon after myocardial infarction, which led Hashimoto *et al.* (1978) to suggest that kinins released in patients with infarction may have a compensatory cardioprotective effect.

Our own experiments on rat isolated hearts with post-ischaemic reperfusion injuries (Linz *et al.*, 1990) and dog experiments with myocardial infarction (Martorana *et al.*, 1991) support this hypothesis. With the BK_2-bradykinin receptor antagonist, Hoe 140, all observed bradykinin- or ramiprilat-mediated effects on the heart could be abolished (Linz *et al.*, 1990; Martorana *et al.*, 1991). Even in rats with aortic banding developing hypertension and left ventricular hypertrophy, the antihypertrophic effect of ramiprilat could be reversed by Hoe 140 (Linz & Schölkens, 1992).

A further argument for bradykinin-mediated actions of ACE inhibitors is the fact that ACE activity but also gene expression is increased during myocardial ischaemia as well as left ventricular hypertrophy (Schunkert *et al.*, 1990; Johnston *et al.*, 1991). Inhibition of local ACE (kininase II)-activity by ACE inhibitors reduces AII formation and bradykinin metabolism. In this way the difference in bradykinin release of Krebs-Henseleit buffer versus ramiprilat-perfused hearts is explained by the inhibition of the local ACE activity in the heart when the ACE inhibitor is perfused.

Preliminary studies where endothelium was destroyed by H_2O perfusion showed that bradykinin release in Krebs-Henseleit buffer and ramiprilat-perfused hearts was almost abolished indicating that bradykinin released from the heart mainly comes from endothelial cells (data not shown). This agrees with the results of Wiemer *et al.* (1991) showing that ramiprilat was capable of inhibiting the breakdown of endothelium-derived bradykinin in human cultured and bovine aortic endothelial cells.

Based on earlier findings with bradykinin and ACE inhibitors and on the present results with direct measurement of bradykinin in the venous effluent from rat isolated hearts perfused with Krebs-Henseleit buffer with or without ramiprilat, we suggest that ischaemia activates proteases in the heart resulting in continuous bradykinin generation. Furthermore local ACE inhibition in these isolated hearts reduces bradykinin breakdown with subsequent increased bradykinin outflow which is accompanied by an amelioration of ischaemia-reperfusion-induced injuries, including suppression of reperfusion arrhythmias (Linz *et al.*, 1990).

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SPECIAL REPORT

7-Nitro indazole, an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure

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7-Nitro indazole (7-NI) inhibits mouse cerebellar nitric oxide synthase (NOS) *in vitro* with an IC_{50} of 0.47 μM. Following i.p. administration in mice, 7-NI (10–50 mg kg^{-1}) produces dose-related anti-nociception as evidenced by an inhibition of late phase (15–30 min) but not early phase (0–5 min) hindpaw licking time following subplantar injection of formalin (10 μl, 5% v/v). The ED_{50} for this effect was 26 mg kg^{-1} (equivalent to 159.5 μmol kg^{-1}). Similar i.p. administration of 7-NI (20 and 80 mg kg^{-1}) in urethane-anaesthetized mice failed to increase MAP. Thus, 7-NI is a novel inhibitor of NOS which exhibits selectivity for the brain enzyme. Accordingly, 7-NI may be a useful starting point for the development of selective, centrally acting NOS inhibitors devoid of cardiovascular side effects and as a tool to study the central pharmacological effects of nitric oxide (NO).

Keywords: 7-Nitro indazole; L-N^{G}nitro arginine methylester (L-NAME); nitric oxide synthase; anti-nociception; formalin; blood pressure

Introduction L-N^G-nitro arginine methyl ester (L-NAME), a selective inhibitor of nitric oxide synthase (NOS), produces an opioid-independent anti-nociception in the mouse which is partially reversed by L-arginine (Moore *et al.*, 1991). That L-NAME is anti-nociceptive following i.c.v. administration in this species suggests a central mechanism of action. This conclusion is supported by electrophysiological studies in the rat which indicate a predominantly spinal site of action for L-NAME (Haley *et al.*, 1992).

L-NAME also inhibits vascular endothelial NOS resulting in a prolonged increase in blood pressure (e.g. Rees *et al.*, 1991). This action effectively precludes the use of L-NAME as an analgesic in man. In an attempt to identify selective inhibitors of brain NOS we have assessed the ability of a wide range of compounds to inhibit brain NOS *in vitro* and to increase mouse blood pressure. We describe here the results obtained using one such compound, 7-nitro indazole (7-NI).

Methods NOS activity was determined *in vitro* by the method of Dwyer *et al.* (1991). Mice (male, LACA, 28–32 g) were killed by cervical dislocation. Cerebella were removed, homogenized (1:10 v/v in 20 mM Tris buffer containing 2 mM EDTA, pH 7.4) and aliquots (25 μl) incubated (37°C) with L-arginine (120 nM) containing 0.5 μCi [^{3}H]-arginine (Amersham, sp. activity 66 Ci mmol^{-1}), NADPH (0.5 mM) and $CaCl_2$ (0.75 mM). Incubations also contained 7-NI (MIM, Research Chemicals Ltd.), L-NAME or L-N^G-monomethyl arginine (L-NMMA, Sigma) or an equal volume (5 μl) of 0.5% (w/v) sodium carbonate or distilled water as control. Final incubation volume was 105 μl. After 15 min, the reaction was stopped by addition of 3 ml HEPES buffer (20 mM containing 2 mM EDTA, pH 5.5) and the [^{3}H]-citrulline produced was separated by cation exchange chromatography on 0.5 ml columns of Dowex AG50-W8 Na^+ form (Sigma). [^{3}H]-citrulline was quantitated by liquid scintillation spectroscopy of duplicate 1 ml aliquots of the flow-through. In some experiments, mice were injected i.p. with 7-NI (25 mg kg^{-1}) or L-NAME (50 mg kg^{-1}) and killed 15 min thereafter. Cerebella were removed, homogenized and NOS activity determined as described above.

Anti-nociceptive activity of i.p. 7-NI was determined in mice by the formalin-induced hindpaw licking assay. Results show hindpaw licking time (s) in the early (0–5 min) and late phases (15–30 min) after subplantar injection of 10 μl formalin (5% v/v). In separate experiments, blood pressure of urethane (10 g kg^{-1})-anaesthetized mice was monitored for 45 min after i.p. administration of 7-NI. Full details of these methods have been published elsewhere (Moore *et al.*, 1991).

For *in vitro* experiments, 7-NI was dissolved in hot (80°C) sodium carbonate solution (0.5% w/v). 7-NI did not come out of solution on cooling. For *in vivo* experiments, 7-NI was suspended in arachis oil by sonication. Control animals received 10 ml kg^{-1} arachis oil or saline (0.9% NaCl, w/v). Results show mean ± s.e.mean. Statistical analysis was by Student's unpaired *t* test.

Results 7-NI potently inhibited mouse cerebellar NOS *in vitro* (IC_{50}, 0.47 ± 0.01 μM). For comparison, 7-NI was 1.8 times more potent than L-NAME (IC_{50}, 0.87 ± 0.02 μM) and 5 times more potent than L-NMMA (IC_{50}, 2.37 ± 0.03 μM) (Figure 1a). In separate experiments, administration of 7-NI (25 mg kg^{-1}, i.p.) decreased mouse cerebellar NOS activity measured 15 min thereafter by over 55% (3.9 ± 0.06 pmol citrulline mg^{-1} protein 15 min^{-1}, cf. 9.1 ± 0.26, arachis oil-injected controls, $n = 6$, $P < 0.01$). For comparison, a higher dose of L-NAME (50 mg kg^{-1}) produced only 46.2 ± 1.6% inhibition of this enzyme under identical conditions (4.46 ± 0.012 pmol citrulline mg^{-1} protein 15 min^{-1}, cf. 8.33 ± 0.15, saline-injected controls, $n = 6$, $P < 0.01$).

7-NI (10–50 mg kg^{-1}) also produced a dose-related inhibition of late phase formalin-induced hindpaw licking without influencing the early phase response (Figure 1b). The ED_{50} for 7-NI was 26.0 mg kg^{-1} (equivalent to 159.5 μmol kg^{-1}). In contrast, i.p. administration of 7-NI (25 and 80 mg kg^{-1}) did not increase MAP over the 45 min experimental period (e.g. 25 mg kg^{-1}, 47.4 ± 5.1 mmHg, cf. 51.6 ± 4.4 mmHg, $n = 4$, before 7-NI administration; 80 mg kg^{-1}, 43.9 ± 5.3 mmHg, cf. 49.5 ± 2.9 mmHg, $n = 4$, before 7-NI administration). In control experiments, i.p. administration of arachis oil failed to alter MAP.

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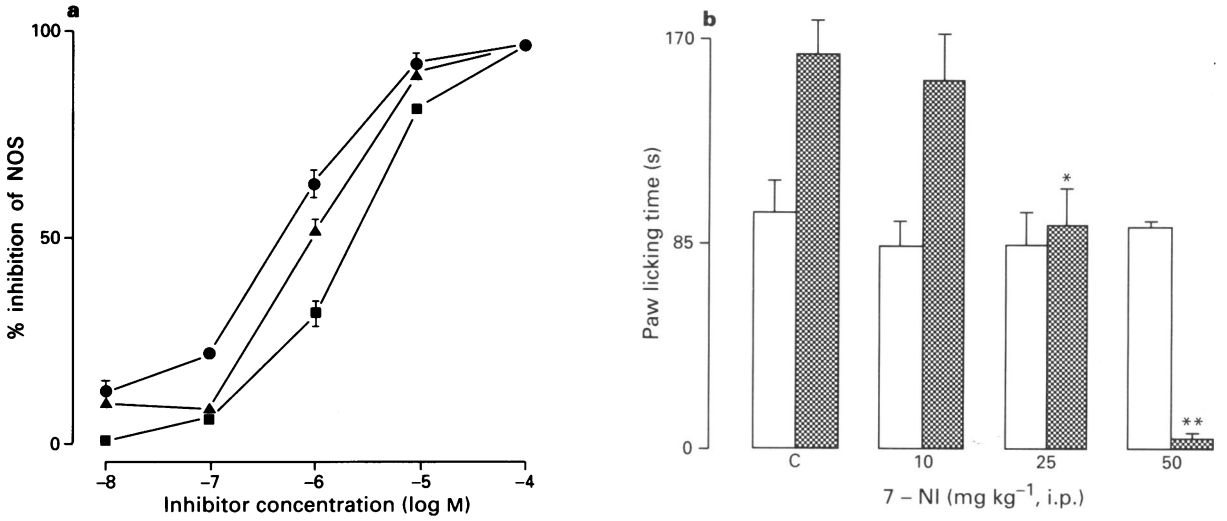

Figure 1 (a) Inhibition of mouse cerebellar nitric oxide synthase (NOS) by 7-nitro indazole (7-NI, ●), L-N^G-nitroarginine methyl ester (▲) and L-N^G-monomethyl arginine (■). Results show % inhibition of NOS and are mean ± s.e.mean, $n = 6$. Where no error bar is indicated error lies within dimensions of symbol. (b) Anti-nociceptive effect of 7-NI administered i.p. to mice 15 min before subplantar formalin injection. Open columns indicate early phase (0–15 min) whilst hatched columns indicate late phase (15–30 min) hindpaw licking times. Results show mean ± s.e.mean, $n = 6$–12, $^*P < 0.05$, $^{**}P < 0.01$. Control animals (labelled C) received 10 ml kg^{-1} arachis oil which, by itself, did not influence hindpaw licking time. (cf. saline-injected mice: early phase, 89.8 ± 7.0 s: late phase, 150.7 ± 11.5 s, $n = 15$).

Discussion We report here that 7-NI inhibits mouse cerebellar NOS. To the best of our knowledge 7-NI is the first potent inhibitor of this enzyme which is not a guanidino-substituted derivative of L-arginine. The chemical characteristics of 7-NI which confer NOS inhibitory activity remain to be determined although the presence of two adjacent nitrogen atoms in the pyrazole ring does bear some similarity to the arrangement of the guanidino terminus of L-arginine. However, pyrazole itself is inactive (Babbedge & Moore, unpublished) suggesting that the fused ring structure of the indazole nucleus is necessary for activity. We therefore propose that 7-NI may be useful as a tool to study the pharmacological actions of NO.

The finding that 7-NI, like L-NAME (Moore *et al.*, 1991), causes anti-nociception in the mouse provides further support for our hypothesis that NO plays a role in pain appreciation. In this context, it is of interest that 7-NI and L-NAME exhibit (a) similar anti-nociceptive potency in the mouse (ED_{50} for L-NAME, 186 μmol kg^{-1}; see Morgan *et al.*, 1992) and (b) similar ability to inhibit mouse cerebellar NOS. Interestingly, administration of 7-NI (25 mg kg^{-1}) to intact mice results in a greater inhibition of cerebellar NOS than does similar injection of a higher (50 mg kg^{-1}) dose of L-NAME. Further research to investigate the relative pharmacokinetic profiles of 7-NI and L-NAME following i.p. injection in mice are required.

Unlike, L-NAME, 7-NI does not cause an increase in MAP indicating a lack of effect on endothelial cell NOS activity in this species. These results show that 7-NI exhibits selectivity for the brain NOS enzyme thereby highlighting at least one difference between the brain and endothelial cell isoforms of this enzyme. It will clearly be of interest to determine the effect of 7-NI on other constitutive and inducible isoforms of NOS. The lack of cardiovascular side effects of 7-NI reported in this study, if confirmed in other species, is potentially of clinical interest. For example, the development of a selective inhibitor of brain NOS may be of therapeutic use not only for analgesia but for other central nervous system disorders in which over-production of NO is believed to play a causative role e.g. neurodegenerative disease (see Garthwaite, 1991).

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Enteral absorption of octreotide: absorption enhancement by polyoxyethylene-24-cholesterol ether

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1 The somatostatin octapeptide-analogue octreotide was absorbed as an intact peptide from the gastro-intestinal tract with an absolute bioavailability of about 0.3% in rats. Administration of octreotide in the presence of polyoxyethylene (24)-cholesterol-ether (POECE) resulted in an about 23 fold increase of bioavailability.

2 *In vitro* studies with Caco-2 cells showed a dose-dependent increase in octreotide permeation with increasing doses of coadministered POECE. The use of [^{3}H]-polyethyleneglycol (PEG) 4000 as an extracellular marker also indicated that higher doses of POECE may partly enhance paracellular transport of macromolecules.

3 By means of fluorescence microscopy it was shown that transepithelial transport of the fluorescent octreotide analogue (4-nitrobenzo-2-oxa-1,3-diazol [NBD] labelled octreotide) was enhanced by the addition of POECE. Besides an increased enterocyte uptake, there was evidence of enhanced partition of NBD-octreotide into the intercellular space between enterocytes after co-administration of POECE. In addition, there appeared to be changes in the hepatic topographic disposition of NBD-octreotide when it was given together with POECE compared with its administration alone.

4 In a study in healthy volunteers, 16 mg POECE significantly enhanced by 8 fold the absorption of octreotide after oral administration.

Keywords: Octreotide; intestinal; membrane transport; brush border membrane; polyoxyethylene-24-cholesterol ether

Introduction

Octreotide is a somatostatin analogue that is used for a variety of indications such as acromegaly, gastro-intestinal disorders and psoriasis (Battershill & Clissold, 1989; Katz & Erstad, 1989). However, its clinical use has been limited because up to now only subcutaneous or intravenous routes of administration are available (Lancet Editorial, 1990). Preliminary studies in healthy volunteers have demonstrated that oral administration of octreotide is possible with effective suppression of plasma insulin levels (Köhler *et al.*, 1987; Fuessl *et al.*, 1987); however, the overall bioavailability was poor (ca. 0.3%). In one study there was evidence that absorption preferentially takes place in the jejunal part of the small intestine, whereas it was much lower in the ileal part (Köhler *et al.*, 1987). These findings were confirmed in a recent study using *in situ* absorption experiments in rats and vesicles derived from rat small intestinal brush border membranes (Fricker *et al.*, 1992).

In the present study, the absorption enhancing effect of polyoxyethylene (24)-cholesterol-ether (POECE) on the absorption of octreotide and its fluorescent analogue (4-nitrobenzo-2-oxa-1,3-diazol-[NBD]-octreotide) was evaluated in an intestinal cell culture model (Caco-2), in animals and in man. Earlier studies showed an absorption enhancing effect of the nonionic detergent POECE on poorly absorbed drugs such as ergot alkaloids (Franz & Vonderscher, 1981) or antibiotics such as streptomycin or cephaloridine (Davis *et al.*, 1970). NBD-octreotide has been demonstrated to have comparable affinity to somatostatin-receptors and similar pharmacodynamic effects after oral administration to the parent peptide (Fricker *et al.*, 1991). In addition, the effect of POECE on the topographical distribution of NBD-octreotide in intestinal mucosal and liver tissue was investigated by fluorescence microscopy.

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Methods

Determinination of partition coefficients

One milligram of octreotide or NBD-octreotide was dissolved in 5 ml buffer (300 mM mannitol, 20 mM HEPES-Tris, pH 7.5). To this solution, 5 ml of octanol was added and rigorously shaken for 6 h at 37°C. Octanol and buffer phases were separated by centrifugation and drug content was determined in each phase by reversed-phase high performance liquid chromatography (h.p.l.c.) analysis.

In vitro *study in Caco-2 cells*

Caco-2 cells were maintained in Dubecco's modified Eagle medium (DMEM) containing 10% (w/v) foetal calf serum (Gibco) and 1% (w/v) non-essential amino acids. Cell medium was changed every third day and cells were used between passages 70 and 100. The cells were grown in 250 ml tissue culture flasks and detached from the flasks by treatment with 0.05% (w/v) trypsin/EDTA for 10 min at 37°C. Suspended cells were transferred onto collagen coated polycarbonate membrane filters (Nucleopore; pore size 3 μm; Cambridge, MA, U.S.A.). One milliliter cell suspension containing approximately 1.5×10^5 cells was placed on top of each filter positioned into tissue culture wells of 2.5 cm diameter. Each suspension was supplemented with 2 ml of DMEM. The integrity of the monolayers and the confluency of the cells were determined by measurement of the transepithelial resistance and the transmembrane transport of ^{3}H-labelled polyethyleneglycol (PEG) 4000 (NEN, Bad Homburg, Germany) as a macromolecular marker after placement of the filters into side-by-side diffusion chambers (Crown Glas, Sommerville, NJ, U.S.A.). Macromolecular PEG has been recently demonstrated to penetrate Caco-2 cell monolayers only to a neglible extent (<0.01% of the dose per hour [Hidalgo *et al.*, 1989; Artusson, 1990; Donovan *et al.*, 1990]). Microscopic control of monolayer integrity was performed after haematoxylin staining of the cells.

In vitro *transport assay*

The filters with the cell monolayers were placed into a side-by-side diffusion chamber and preincubated for 10 min with 3 ml DMEM on each side. The cell monolayers were then incubated with 10 μM ^{14}C-labelled octreotide in the absence or presence of 0.1, 0.5, or 1.0% (w/v) POECE on the apical side. After addition of the peptide, aliquots of the medium were taken at 15 min intervals for up to 120 min from the basolateral side of the monolayer and the peptide concentration was determined by liquid scintillation counting. The apparent permeability coefficient (P_{app}) was calculated by the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0} \quad (cm\ h^{-1}),$$

where C_0 is the concentration of the administered peptide at the apical cell surface, A is the surface area of the cell layer, and dQ/dt is the permeability rate (μg h^{-1}). When NBD-octreotide was used as substrate, the cell monolayer was incubated with 100 μM peptide. After 30 min of incubation, the cells were washed with supplemented DMEM and subjected to fluorescence microscopic examination.

In situ *absorption study in rats*

Studies in rats were performed with both octreotide and its NBD analogue. This analogue binds to the somatostatin receptors, inhibits growth hormone secretion in cultured pituitary cells and shows a high proteolytic stability during incubation with mucosal scrapings (Fricker *et al.*, 1991).

All animal studies were approved by the Committee of the Swiss Cantonal Agency for Animal Protection. Male Wistar rats (BRL, Füllinsdorf, CH), weighing approximately 300 g were kept without food, but with free access to water, for one day prior to the experiment. The animals were anaesthetized by intra-peritoneal injection of urethane (1 g kg^{-1}). The peritoneum was opened by a midline incision and 5 cm segments of the desired intestinal area were ligated in order to prevent transit of the administered peptide down the gut maintaining normal blood supply. The beginning of the jejunum was localised 5 cm distal to the ligamentum of Treitz. Octreotide (100 μg) or its NBD derivative dissolved with or without 1% (w/v) POECE in 0.5 ml 0.9% saline was injected into the jejunal segment. For a reference for absorption, 2 μg octreotide or its NBD derivative was dissolved with or without 1% (w/v) POECE in 100 μl ml 0.9% saline prior to injection into the portal vein. Blood samples were taken by puncture of the jugular vein 10 min before and 20, 30, 60, 120, 180 and 300 min after drug administration and immediately centrifuged at 10000 *g* for 5 min at 4°C. The plasma was kept frozen until the concentration of octreotide was determined by a radioimmunoassay (Marbach *et al.*, 1985). The rabbit antiserum was found to recognise only intact peptide with a very low cross-reactivity to peptide fragments, somatostatin-14 or somatostatin-28. The area under the plasma curve (AUC) was estimated by the trapezoidal rule. The absorption efficiency F_{abs} was calculated following the equation:

$$F_{abs}(\%) = [(AUC_{intra\text{-}intestinal} \times dose_{i.v.})/(AUC_{i.v.} \times dose_{intra\text{-}intestinal})] \times 100$$

Fluorescence microscopy

One milligram of NBD-labelled octreotide was dissolved in 50 μl ethanol and diluted to a final concentration of 100 μmol l^{-1} with 0.9% saline in the absence or in the presence of 1% (w/v) POECE. One milliter of this solution was injected into a jejunal loop, which was ligated at both ends, thereby maintaining normal blood supply. Subsequently, tissue biopsies from the ligated intestine and from different lobes of the liver were performed 30 min after the injection. Snips of liver (3 × 3 × 3 mm) and jejunal tissue were placed on small pieces of cork, fixed by addition of cryomedium (Reichert-Jung, Nussloch, Germany) and deep frozen in liquid nitrogen. Tissues were cut into 5 μm sections with a microtome-cryostat (model 2700 Frigocut; Reichert-Jung) at −20°C. The slices were directly examined by fluorescence microscopy after embedding with tissue medium (Entellan; Merck, Darmstadt, Germany).

A polyvar microscope (Reichert-Jung) with incident fluorescence (exciting wavelength between 450 and 490 nm, emitted light > 520 nm) was used to visualize the tissue distribution of fluorescent peptide. Ektachrome-400 films were used for photographic documentation. The films were exposed and developed from 400–1600 ASA to demonstrate the rapidly bleaching fluorescence in its original brightness.

In vivo *absorption study in man*

The study was carried out in 10 healthy male volunteers (mean age 25 years, range 22 to 36 years) in accordance with the guidelines of the declaration of Helsinki as revised in Tokyo (1975) and in Venice (1983), approved by the Ethics Committee of the University of Basel/Kantonsspital. Written informed consent was obtained from all subjects. Fasted healthy human volunteers swallowed four different oral capsules or received an intravenous infusion according to an open randomized five-period latin-square design. The washout period between consecutive administrations was at least 3 days.

Octreotide preparations were prepared by Sandoz Ltd., Basel, Switzerland: (Form A) capsule containing 8 mg octreotide, 16 mg POECE, and 100 mg microcrystalline cellulose (MC; used as inert additive for galenical optimisation); (Form B) capsule containing 8 mg octreotide and 100 mg MC; (Form C) capsule containing only 8 mg octreotide; (Form D) capsule containing 8 mg octreotide and 16 mg POECE; (Form E) ampoule containing 100 μg 1 ml^{-1} for i.v. infusion.

Administrations of capsules was after an overnight fast of at least 10 h. Capsules were swallowed with 50 ml of water. The solution for the intravenous infusion was prepared as follows: two ampoules containing 100 μg octreotide ml^{-1} each were added to 98 ml 0.9% NaCl solution; 50 ml of this solution was then infused over 30 min into each subject through a catheter placed into an arm vein amounting to 100 μg octreotide per subject. Blood samples for octreotide determination were drawn up to 8 h (infusion) and 12 h (capsules).

Subjects abstained from eating until lunch (4 h) and were not allowed to consume more than 50 ml of water per hour. A standardised liquid lunch (500 ml Ensure, Abbott Lab.) was taken 4 h after drug administration. This lunch had a caloric content of 2090 kJ (500 kcal). It was swallowed by the subjects in less than 5 min. During the 12 h after drug administration, there were no xanthine or alcohol containing beverages allowed. Subjects abstained from smoking during the study.

Materials

[^{14}C]-octreotide with a specific activity of 40.99 Ci mg^{-1}, unlabelled peptide and NBD-labelled octreotide were synthesized in the Preclinical Research Department, Sandoz Pharma Ltd., Basel, Switzerland. POECE was produced by Chemical Development, Sandoz Pharma Ltd., Basel. The radiochemical purity of labelled peptide was checked by high-performance thin-layer chromatography (h.p.t.l.c.) analysis before the experiments and was found to be greater than 98%. Caco-2 cells, originally derived from a human colorectal carcinoma, were obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A. All other chemicals were purchased in reagent grade from commercial sources.

Statistical analysis

All results are reported as mean (s.d.). For comparison of parameters, samples were tested by two-way analysis of variance (ANOVA) using the GLM procedure of the SAS software package (SAS software package, 1988). In case of significant differences, ANOVA was followed by the Newman-Keuls multi-comparison test for pairwise comparisons (SAS software package, 1988).

Results

Characterization of the cell monolayers

Transport experiments were performed between days 11 and 14 after placement of the cells onto the filters. By this time, a transepithelial resistance of $280 \pm 20\ \Omega\ cm^2$ was maintained, indicating confluency of the monolayer. The amount of [^{3}H]-PEG 4000 permeating the cell monolayers at the same time varied between 0.01 and 0.15% per hour of an administered tracer dose. The functional integrity of the cells was monitored by the determination of taurocholate or phenylalanine transport as recently described (Hidalgo & Borchardt, 1990a,b). Both model compounds were taken up by the cells in a saturable process from the apical surface. A significantly lower permeation rate of taurocholate was observed when the bile acid was presented from the basolateral cell surface, indicating the formation of polar-organised cells, which exhibit active transport of bile salts predominantly from the apical to the basolateral side. This corresponds to directed transport mechanisms occurring in intestinal cells (Osiecka *et al.*, 1985; Marcus *et al.*, 1991). In the presence of octreotide, no significant alteration of the morphological features of the cells or the transepithelial resistance was observed.

Transport of octreotide through the monolayers

The permeability coefficient of octreotide varied in single experiments between 0.005 and $0.012\ cm\ h^{-1}$. As shown in Figure 1, the addition of POECE in concentrations between 0.1 and 1% (w/v) at the apical surface resulted in a significant dose-dependent increase of the monolayer permeability for octreotide. In the presence of 1% (w/v) POECE, the octreotide permeation rate increased 4 fold. Control experiments using [^{3}H]-PEG 4000 as an extracellular marker demonstrated that 1% (w/v) POECE enhanced both transcellular and to some extent paracellular permeation as indicated by a 2 to 3 fold increased extent of permeation of [^{3}H]-PEG 4000. This hypothesis was also supported by the observation of a drop in transepithelial resistance in the presence of POECE. It decreased within 2 h from 280 ± 20 to $200 \pm 15\ \Omega\ cm^2$.

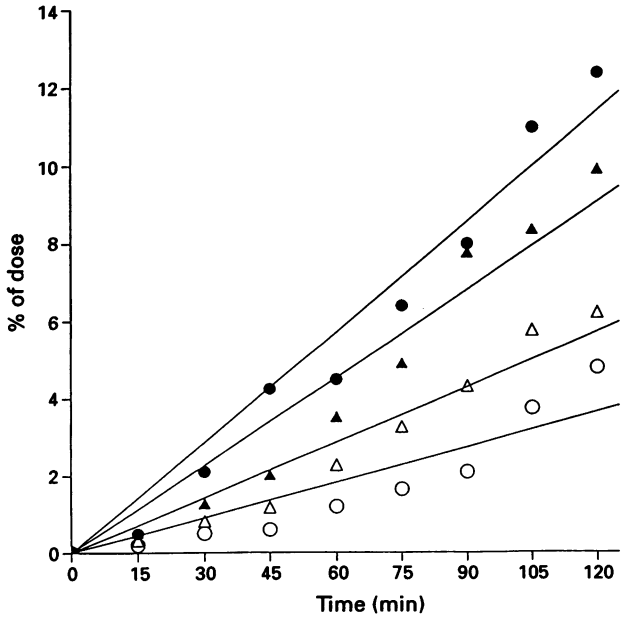

Figure 1 Dose-dependent octreotide permeation through Caco-2 cell monolayers as a function of polyoxyethylene-24-cholesterol ether (POECE) content: (○) control; (△) 0.1% (w/v) POECE; (▲) 0.5% (w/v) POECE; (●) 1.0% (w/v) POECE

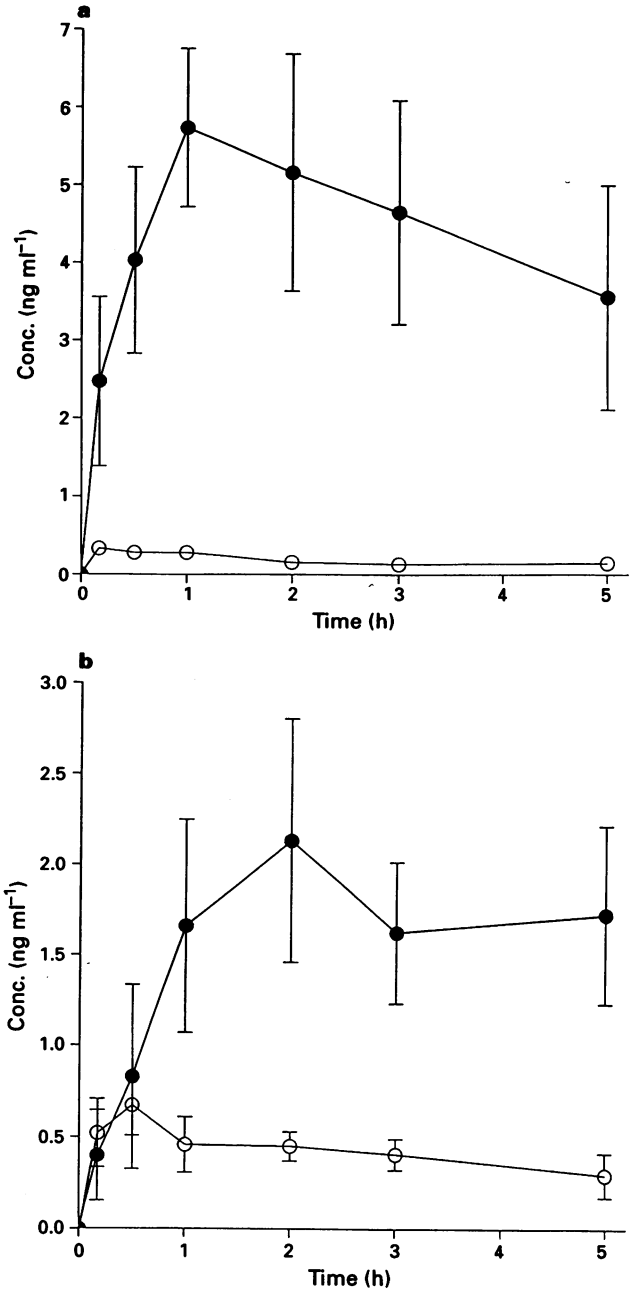

Figure 2 Mean (± s.e.mean, vertical bars) octreotide (a) and NBD-octreotide (b) plasma concentrations after intra-jejunal injection of 100 μg octreotide or NBD-octreotide with and without co-administration of 1% (w/v) polyoxyethylene-24-cholesterol ether (POECE) in 6 rats: (○) without POECE, (●) with POECE

In situ *absorption study in rats*

In situ experiments with rats were performed to evaluate the effect of POECE on the absorption rate and absorption efficiency of octreotide and NBD-octreotide as well as the

Table 1 Pharmacokinetic parameters after *in situ* i.p. or i.j. administration of octreotide and NBD-octreotide with and without co-administration of 1% (w/v) POECE in 0.9% saline solution in rats

	Octreotide			
	(2 μg i.p.)	(2 μg i.p. + POECE)	(100 μg i.j.)	(100 μg i.j. + POECE)
AUC (0–5 h) ($ng\ h^{-1}\ ml^{-1}$)	6.5 ± 3.0	13.6 ± 4.0	0.9 ± 0.2	22.3 ± 14.3
F_{abs} (%)[1]	100	210.5	0.3	6.9
C_{max} ($ng\ ml^{-1}$)	5.9 ± 1.0	8.1 ± 0.3	0.4 ± 0.2	6.6 ± 2.6
T_{max} (h)	0.17 ± 0.0	0.17 ± 0.0	1.92 ± 2.4	1.6 ± 1.7
	NBD-octreotide			
	(2 μg i.p.)	(2 μg i.p. + POECE	(100 μg i.j.)	(100 μg i.j. + POECE
AUC (0–5 h) ($ng\ h^{-1}\ ml^{-1}$)	3.2 ± 0.7	4.4 ± 1.3	2.1 ± 0.8	7.6 ± 5.1
F_{abs} (%)[1]	100	138.1	1.3	4.9
C_{max} ($ng\ ml^{-1}$)	3.2 ± 1.0	6.1 ± 1.0	0.7 ± 0.4	2.6 ± 1.7
T_{max} (h)	0.17 ± 0.0	0.17 ± 0.0	2.3 ± 1.8	2.3 ± 1.5

Data are mean values ± s.d.; $n = 6$; POECE = polyoxyethylene-24-cholesterol ether; i.p. = intra-portal injection; i.j. = intra-jejunal injection; [1]calculated on basis of mean values

tissue distribution of NBD-octreotide. One-hundred microgram of octreotide or NBD-octreotide was administered into the jejunum in the absence or presence of 1% (w/v) POECE. As reference, 2 μg of each peptide was administered by i.p. injection to control animals. As shown in Table 1, lower plasma concentrations were observed after the administration of NBD-octreotide than after administration of the same dose of octreotide (this may reflect the higher lipid solubility of NBD-octreotide [octanol/water coefficient 27.0] compared with octreotide [octanol/water coefficient 0.6] and the resulting differences in tissue binding and disposition). On average, intra-jejunal co-administration of POECE resulted in an approximately 24 fold increase of octreotide absorption (Table 1 and Figure 2a). The maximum plasma concentrations of octreotide were 6.6 $ng\ ml^{-1}$ compared with 0.4 $ng\ ml^{-1}$ in the control animals, resulting in an AUC (0–5 h) of 22.3 $ng\ h^{-1}\ ml^{-1}$ and 0.9 $ng\ h^{-1}\ ml^{-1}$, respectively. Interestingly, after intra-portal administration, the plasma levels of octreotide were also increased when POECE was co-administered (AUC (0–5 h) of 13.6 $ng\ h^{-1}\ ml^{-1}$ versus 6.5 $ng\ h^{-1}\ ml^{-1}$) indicating

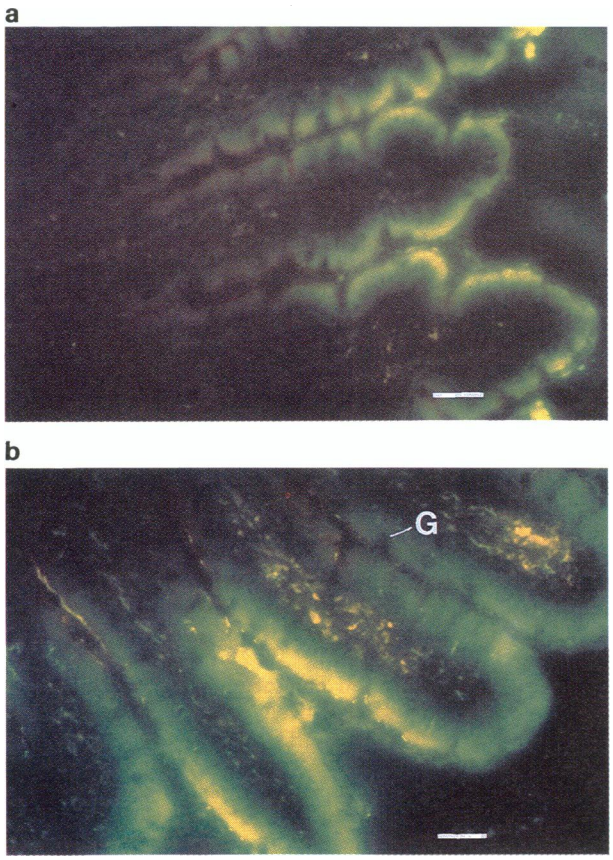

Figure 3 Fluorescence microscopy of jejunal mucosa 10 min after local injection of 100 $\mu mol\ l^{-1}$ NBD-octreotide without (a) or with (b) co-administration of 1% (w/v) polyoxyethylene-24-cholesterol ether (magnification: 253 fold, bar = 0.5 mm). G = goblet cells

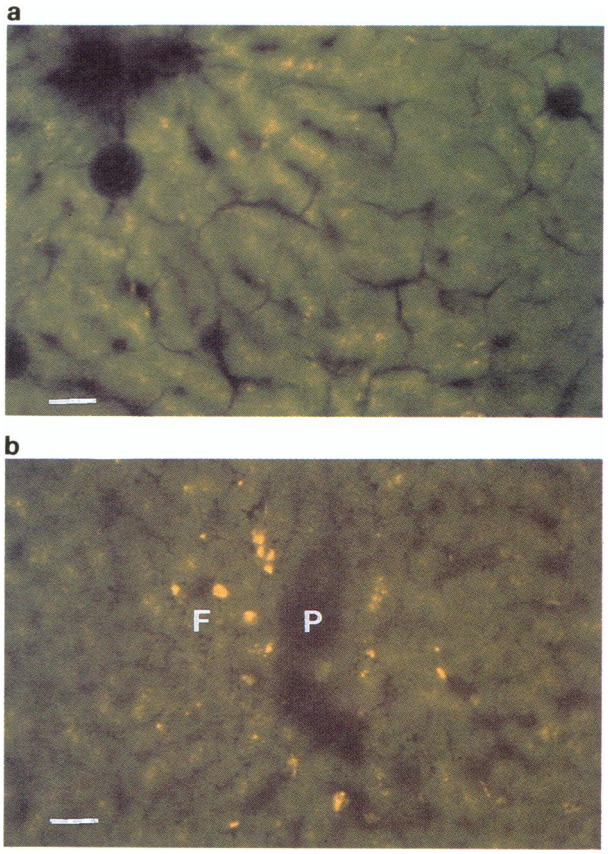

Figure 4 Fluorescence microscopy of hepatic tissue 15 min after local injection of 100 $\mu mol\ l^{-1}$ NBD-octreotide without (a) or with (b) co-administration of 1% (w/v) polyoxyethylene-24-cholesterol ether (magnification: 253 fold, bar = 0.5 mm). P = portal vein, F = focal fluorescence

Table 2 Pharmacokinetic parameters after peroral administration of 8 mg octreotide or 100 μg octreotide intravenous infusion to healthy male human volunteers

	Octreotide				
	(8 mg + 16 mg POECE + 100 mg MC)	(8 mg + 100 mg MC)	(8 mg)	(8 mg + 16 mg POECE)	(100 μg i.v. over 30 min)
AUC (0–12 h) (ng h^{-1} ml^{-1})	24.5 ± 28.6	3.4 ± 3.0	4.7 ± 2.9	16.5 ± 23.3	9.7 ± 2.3
F (%)	3.3 ± 3.7	0.4 ± 0.3	0.6 ± 0.4	2.4 ± 3.3	100
C_{max} (ng ml^{-1})	8.8 ± 10.8	0.8 ± 0.8	1.5 ± 2.1	5.1 ± 7.1	9.5 ± 2.0
T_{max} (h)	1.1 ± 1.1	2.7 ± 1.5	2.3 ± 1.6	1.7 ± 1.4	0.5 ± 0.1

Data are mean values ± s.d., $n = 10$; POECE = polyoxyethylene-24-cholesterol ether; MC = microcrystalline cellulose; i.v. = intravenous infusion

that POECE influences not only absorption but also the systemic distribution and/or elimination of octreotide. The pharmacokinetic data obtained with NBD-octreotide were qualitatively similar but quantitatively lower than those expressed by octreotide. Co-administration of 1% (w/v) POECE with NBD-octreotide led to a 3.6 fold increase in absorption (Table 1, Figure 2b).

Fluorescence microscopy

Within 10 min after intra-intestinal application of 100 μmol l^{-1} NBD-octreotide with and without co-administration of 1.0% (w/v) POECE, fluorescence was observed at the surface of the intestinal villi and within the enterocytes (Figure 3a). After 30 min incubation, intensive fluorescence could be detected at the basolateral side of the enterocyte and in the microvessels of the intestinal villi. There was evidently no fluorescence in the goblet cells. Co-administration of POECE enhanced the extent of absorption as indicated by a bright intracellular fluorescence already after 10 min of incubation (Figure 3b). In the liver, a significantly different tissue distribution of NBD-octreotide was observed after administration of NBD-octreotide alone or in the presence of 1% (w/v) POECE: in the absence of POECE most of the fluorescence occurred in the precanalicular region of the hepatocytes within 10 to 15 min after administration. But, after coadministration of 1% (w/v) POECE, additional focal fluorescence was found in the cells lining the sinusoidal space such as the Kupffer cells and/or the endothelial cells. This indicates a different intra-hepatic disposition of NBD-octreotide in the presence of POECE (Figures 4a and b).

When Caco-2 cells were incubated with NBD-octreotide, almost no fluorescence could be observed inside the cells. However, in the presence of 1% (w/v) POECE a bright intracellular fluorescence could be observed, indicating not only paracellular but also transcellular transport.

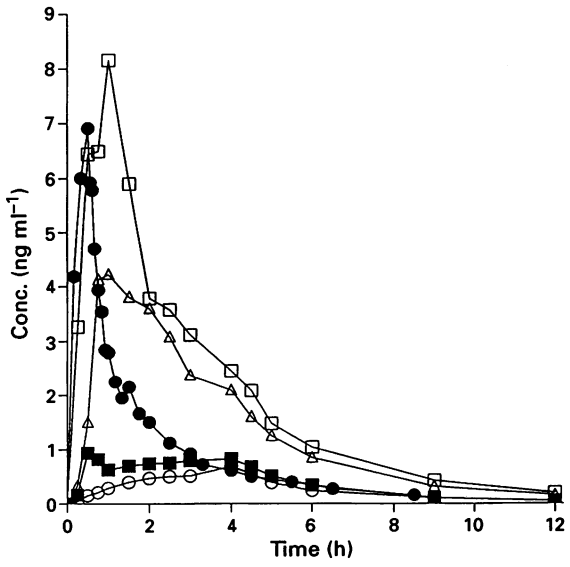

Figure 5 Mean octreotide plasma concentrations after oral administration of different formulations of octreotide (8 mg) in comparison with an intravenous infusion of 100 μg to 10 healthy male volunteers: (□) 8 mg octreotide capsule containing 16 mg polyoxyethylene-24-cholesterol ether (POECE) and 100 mg microcrystalline cellulose (MC); (○) 8 mg octreotide capsule containing 100 mg MC; (■) 8 mg octreotide capsule; (△) 8 mg octreotide capsule containing 16 mg POECE; (●) intravenous infusion of 100 μg octreotide over 30 min

In vivo *absorption study in man*

The enhancement of octreotide absorption by POECE observed in the *in situ* experiments were confirmed in healthy human volunteers. As shown in Table 2 and depicted in Figure 5, after all oral administrations of octreotide, plasma concentrations of the peptide were observed that are known to suppress prolactin secretion (Köhler *et al.*, 1987; Fuessl *et al.*, 1987). Addition of POECE enhanced octreotide absorption 4 to 8 fold. This resulted in maximum plasma concentrations comparable to that of the 100 μg intravenous infusion.

Discussion

Therapeutic application of the octapeptide somatostatin analogue octreotide by the oral route is feasible (Fuessl *et al.*, 1987). However, its low overall bioavailability (less than 0.3%) after oral administration as well as the observations of preferential absorption sites in the gastro-intestinal tract (Köhler *et al.*, 1987; Fricker *et al.*, 1991; 1992) suggest the use of enhancers to promote enteral absorption.

In the present study, POECE was evaluated as an absorption enhancing agent for octreotide. Furthermore, the underlying mechanisms were investigated. Both, *in vitro* experiments using the filter grown enterocyte-like colon carcinoma cell line Caco-2 as well as *in vivo* studies in rats and man demonstrated the potential capacity of POECE to enhance enteral absorption of octreotide. In all systems used, co-administration of POECE resulted in at least a 4-fold increased absorption of octreotide or its fluorescence labelled analogue NBD-octreotide.

Recently, the absorption enhancing properties of anionic polyoxyethylene ethers have been reported (Touitou *et al.*, 1980; Brookes & Marshall, 1981; Morimoto *et al.*, 1985). However, the mechanisms by which POECE exerts its effect are not completely defined. Results with the fluorescence-labelled NBD-octreotide showed an increased intracellular fluorescence which indicates that POECE enhanced the transcellular route of peptide absorption in both Caco-2 cells and enterocytes. These findings confirm recent studies that demonstrate the uptake of octreotide and its NBD analogue by a membrane-mediated mechanism (Fricker *et al.*, 1991; 1992).

The present *in vitro* results also indicated that, in addition to this pathway, POECE has an effect upon the tightness of the junctions between the enterocytes, thereby opening the paracellular route for octreotide absorption. This hypothesis is supported by three observations: Firstly, permeation of the extracellular marker [^{3}H]-PEG 4000 through the Caco-2 cells was increased after incubation of the cells with POECE. Secondly, the transepithelial resistance significantly decreased in presence of POECE, and thirdly, NBD-octreotide showed an altered hepatic disposition after intra-jejunal co-administration of POECE in rats, which indicates the formation of mixed micelles in the systemic circulation. These results may be interpreted as absorption of intact mixed POECE/NBD-octreotide micelles from intestinal lumen through paracellular spaces or the reconstitution of the separately absorbed constituents. Micelle formation between the highly lipophilic POECE and octreotide has been shown *in vitro* by laser light scattering measurements (unpublished observations).

As demonstrated in Table 1, both octreotide and NBD-octreotide showed an increased systemic availability after intraportal injection together with POECE. This may also be explained by the formation of mixed micelles and subsequently altered hepatic extraction. Some of these micelles may be recognized by macrophage type cells (e.g. Kupffer cells) in the sinusoidal space, while the remaining micelles may enter the systemic circulation, thus preventing the enclosed octreotide from rapid hepatic extraction. In this concept, the stability of the mixed micelles will be the major determinant and the lower systemic availability of NBD-octreotide compared with the bioavailability of octreotide itself may be caused by a decreased micelle stability resulting from the higher lipophilicity and therefore greater tissue affinity. Alternatively, POECE might inhibit octreotide elimination or have a positive influence on hepatic blood flow. However, based on plasma profiles of octreotide or NBD-octreotide the present data do not allow an answer to the question whether co-administration of POECE results in an increased half-life of octreotide in rats. A possible effect of POECE on hepatic circulation has not yet been rigorously studied. First experiments with isolated perfused liver preparations do not support this hypothesis.

The results of the human study confirm that POECE resulted in higher octreotide plasma concentrations in man, reaching concentrations that were comparable to levels obtained after a 100 μg intravenous infusion. The latter dose is regarded as clinically sufficient for many octreotide indications (Battershill & Clissold, 1989).

In summary, the results of this study indicate that POECE is a promising absorption enhancing agent for the enteral absorption of octreotide. There is evidence suggesting that POECE enhances both transcellular and, to a certain extent, also paracellular pathways of absorption. By using POECE, clinically significant plasma concentrations of octreotide can be obtained in man after oral administration.

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Localization and characterization of neuropeptide Y binding sites in porcine and human colon

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1 We have used quantitative receptor autoradiography to investigate the localization and characteristics of binding sites for ^{125}Iodine-Bolton Hunter-labelled human neuropeptide Y ([^{125}I]-BH-NPY) in porcine and human colon, and compared the binding characteristics with those found in porcine spleen.

2 Saturable, specific, high affinity [^{125}I]-BH-NPY binding was localized to myenteric ganglia in porcine and human colons, and to submucosal ganglia in porcine colon.

3 Specific [^{125}I]-BH-NPY binding to porcine myenteric ganglia was reversible in the presence of guanosine 5'-O-(3-thiotriphosphate) and was inhibited by related peptides with the rank order of potency; porcine NPY = human NPY = peptide tyrosine tyrosine (PYY) >> pancreatic polypeptide.

4 The Y_2 selective analogue, NPY(13–36), competed for [^{125}I]-BH-NPY binding to porcine myenteric ganglia with greater potency than the Y_1 selective analogue, [Leu^{31}, Pro^{34}]NPY, the difference being small, but significant.

5 The characteristics of [^{125}I]-BH-NPY binding to porcine myenteric ganglia were similar to those observed concurrently to porcine splenic red pulp.

6 The small difference in inhibitory potencies between NPY(13–36) and [Leu^{31}, Pro^{34}]NPY observed in this study in comparison with previous studies was not explained by differential ligand depletion during incubations, but may be due to differences in methodology between binding studies performed on tissue sections and on membranes.

7 We conclude that specific [^{25}I]-BH-NPY binding sites are present in the myenteric and submucosal ganglia of the colon and that these sites may act as functional receptors by which NPY and PYY modulate colonic motility and electrolyte transport.

Keywords: Neuropeptide Y; myenteric plexus; colon; receptors; peptide YY; autoradiography; spleen

Introduction

Neuropeptide Y (NPY) is a 36 amino acid regulatory peptide initially isolated from brain as a consequence of its C-terminal amide residue (Tatemoto *et al.*, 1982). NPY-like immunoreactivity (NPY-LI) is widely distributed in peripheral tissues. In human and porcine colon, NPY-LI is present in sympathetic nerves, particularly around blood vessels, and also in a proportion of intrinsic nerves in the myenteric and submucosal plexi (Ferri *et al.*, 1984). In the spleen, NPY-LI has similarly been demonstrated in perivascular sympathetic nerves (Lundberg *et al.*, 1988a).

NPY is a member of a family of regulatory peptides which includes the structurally related pancreatic polypeptide (PP) and peptide tyrosine tyrosine (PYY). The peptides NPY and PYY share many biological actions (Sheikh, 1991), but have markedly different distributions in peripheral tissues. PYY-like immunoreactivity (PYY-LI) has been demonstrated in endocrine cells in both human and porcine colon, but not in sympathetic or intrinsic neurones (Lundberg *et al.*, 1982), whereas NPY-LI has not been demonstrated in intestinal endocrine cells.

PYY-LI is released into the systemic circulation in mammals following feeding (Adrian *et al.*, 1985; Taylor, 1985), and may therefore act as a circulating hormone. In addition, local release of NPY and PYY may serve paracrine, autocrine or neurotransmitter functions. NPY and PYY each affect intestinal smooth muscle motility, probably by modifying neurotransmitter release from myenteric neurones rather than by a direct effect on intestinal smooth muscle (Lundberg *et al.*, 1982; Hellstrom *et al.*, 1985; 1989; Hellstrom, 1987; Cadieux *et al.*, 1990). NPY and PYY inhibit secretion in both the small and large intestines (Okuno *et al.*, 1992).

NPY and PYY act through specific cell surface, G-protein-linked receptors. Pharmacological and molecular biological techniques have indicated the existence of at least 3 subclasses of NPY receptor, referred to as Y_1, Y_2 and Y_3 (Wahlestedt *et al.*, 1990; Rimland *et al.*, 1991). The analogue [Leu^{31}, Pro^{34}]NPY has been proposed as a specific ligand and agonist at the Y_1 receptor (Fuhlendorff *et al.*, 1990), while Y_2 receptors bind and can be activated by long C-terminal fragments of NPY, including the fragment NPY(13–36). The recently cloned Y_3 receptor demonstrates a higher affinity for NPY than PYY, by contrast to the Y_1 and Y_2 receptors which exhibit equal affinities for these two endogenous peptides, or a slightly higher affinity for PYY (Rimland *et al.*, 1991).

Quantitative *in vitro* receptor autoradiography allows the pharmacological characterization of anatomically localized binding sites (Palacios & Dietl, 1989). We have now used this technique to investigate the localization and characterization of binding sites for ^{125}Iodine-Bolton Hunter-labelled human NPY([^{125}I]-BH-NPY) in human and porcine colon in an attempt to characterize NPY receptors involved in the modulation of colonic motility and secretion. We have, furthermore, compared the characteristics of [^{125}I]-BH-NPY binding in porcine colon with that in porcine spleen, the latter having been extensively characterized previously in membrane preparations as a Y_2 subclass of receptor (Lundberg *et al.*, 1988a,b; Modin *et al.*, 1991).

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Methods

Tissues

Porcine spleens ($n = 12$) and colons ($n = 12$) were obtained fresh from a local abattoir within 15 min postmortem. Normal human colonic tissues ($n = 7$) were obtained from the resection edge of colonic carcinomata and human spleens ($n = 6$) were obtained post-mortem or at operative splenectomy. Rat spleens ($n = 6$) were obtained from adult Wistar rats immediately after they had been killed by an overdose of phenobarbitone. All tissues were snap frozen in melting dichlorodifluoromethane (Arcton-12, ICI), and stored at −70°C until cut to 10 μm sections and thaw-mounted on Vectabond treated slides (Vector Laboratories, Peterborough, UK). Mounted sections were dried at 4°C for 2 h then used immediately or stored with desiccant at −20°C until use.

Binding conditions

For binding experiments, sections were preincubated twice for 15 min in buffer A (10 mM HEPES, 130 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, pH 7.4). Excess buffer was removed and consecutive sections incubated for 2 h in 45 μl (colons) or 70 μl (spleens) buffer B (buffer A plus 1% bovine serum albumin) containing 0.2 nM [^{125}I]-BH-NPY alone (total binding), or with an excess (1 μM) of unlabelled human NPY (hNPY) (non-specific binding). Sections were washed twice for 5 min in buffer A then rinsed in distilled water and dried in a stream of cold air. Incubations, were performed in humid chambers at room temperature, which was 22°C except for the duration of analogue rebinding experiments, when the ambient room temperature was 27 to 29°C. In addition, association time course experiments were also undertaken at 37°C.

Binding conditions were optimised by comparing different buffers, incubation and wash times and ligand concentrations. Association time courses were obtained with 0.2 nM [^{125}I]-BH-NPY and dissociation time course experiments were performed following 2 h incubation with 0.2 nM [^{125}I]-BH-NPY, by the addition of unlabelled hNPY to a final concentration of 1 μM, or by transfer of sections to a bath containing 300 ml of buffer A without NPY. Saturation studies were performed with 0.05 nM to 0.8 nM [^{125}I]-BH-NPY, and also by incubation with 0.2 nM [^{125}I]-BH-NPY plus increasing concentrations of unlabelled human NPY in the range 31 pM to 4000 pM. Binding inhibition studies used 2 h incubations with 0.2 nM [^{125}I]-BH-NPY plus various concentrations of unlabelled ligand. Binding inhibition with [Leu^{31}, Pro^{34}]NPY was performed on 2 groups of 6 cases for each of porcine spleen and colon, using [Leu^{31}, Pro^{34}]NPY from 2 different sources (see below). The inhibitory potencies of the 2 batches of [Leu^{31}, Pro^{34}]NPY were directly compared on paired samples from 6 porcine spleens.

Tissue and ligand stability

The stability of [^{125}I]-BH-NPY binding sites in tissue sections, and of [^{125}I]-BH-NPY and competing ligands in supernatants, during the incubations, were assessed in order to help validate calculated kinetic, equilibrium and inhibition constants.

Possible loss of specific [^{125}I]-BH-NPY binding in tissue sections during incubations was assessed with extended preincubations. Sections of porcine colon were preincubated in buffer A for 0.5 to 5 h before incubation with 0.2 nM [^{125}I]-BH-NPY for a further 2 h.

Ligand stability was initially assessed by rebinding experiments. [^{125}I]-BH-NPY (100 μl, 0.2 nM) in buffer B, with or without the addition of peptidase inhibitors, was incubated for 0, 2 or 4 h on 10 μm sections of porcine colon. Supernatant (70 μl) was then transferred to fresh, preincubated consecutive sections of porcine colon and incubated for a further 2 h, before washing and exposure of sections to film. Separate experiments demonstrated that increasing incubation volumes from 70 μl to 100 μl did not significantly affect binding.

Possible causes of depletion of [^{125}I]-BH-NPY binding activity in supernatants during incubation with porcine colon were further investigated. Sections of porcine colon (10 μm) were incubated with 100 μl 0.2 nM [^{125}I]-BH-NPY and 25 μl aliquots of supernatant were collected before and after 2 and 4 h incubation. Aliquots were then diluted 1:19 (v/v) in incubation buffer (B).

To assess possible adherence of ligand to tissue sections or slides, 200 μl of each sample ($n = 6$ paired samples before and after incubation) was further diluted to 2 ml in acetic acid:water (5:95 v/v), and counted for radioactivity in a NE1600 multiwell counter (Nuclear Enterprises, Edinburgh).

Reverse-phase, high performance liquid chromatography (h.p.l.c.) was used to assess possible degradation of ligand; h.p.l.c. was performed on 200 μl aliquots of 6 samples (3 following preincubation with porcine colon, and 3 control samples of [^{125}I]-BH-NPY in buffer B, without preincubation) obtained as above, using a Waters dual pump system with a μBondapack C_{18} column (30 × 0.8 cm, Waters Ass., Chester, U.K.). Elution was carried out isocratically at 2 ml min^{-1} in acetic acid:water (5:95 v/v) followed by a 15 min linear gradient to propan-1-ol:acetic acid:water (40:3:57 v/v/v). One minute fractions were counted for radioactivity. Percentage yields of radioactivity are expressed as means of 3 samples. The albumin peak was identified by difference in ultraviolet absorption at 280 nm between eluates from incubation buffer (B) and preincubation buffer (A).

The major peak of radioactivity in each of 4 samples (2 before and 2 following 2 h incubation with porcine colon) were reanalysed by h.p.l.c. Solvent was removed under vacuum and each sample dissolved in acetonitrile:water:HFBA (20:100:0.08 v/v/v) and subjected to h.p.l.c. on a μBondapak C_{18} column (30 × 0.8 cm). Elution was carried out isocratically at 2 ml min^{-1} in acetonitrile:water:HFBA (20:100:0.08 v/v/v) followed by a 15 min linear gradient to acetonitrile:water:HFBA (70:100:0.08 v/v/v). One minute fractions were again counted for radioactivity.

Attempts were made to reduce ligand depletion by adding the following peptidase inhibitors to the incubation buffer; captopril (1 μM), phosphoramidon (1 μM), bestatin (20 μM), a combination of leupeptin (10 μM), bacitracin (1.5 mM) and chymostatin (5 mg l^{-1}), or all 6 antipeptidases.

Depletion of inhibitory potency of the analogues NPY (13–36) and [Leu^{31}, Pro^{34}]NPY by porcine colon and spleen, and of unlabelled hNPY by porcine colon was assessed by a modification of the method used in rebinding experiments. Buffer B (70 μl) containing unlabelled analogue (0.1 μM for colon, 0.2 μM for spleen) or hNPY (2 nM) was incubated with each tissue section for 2 h at room temperature. Supernatant from each section was then mixed with an equal volume of 0.4 nM [^{125}I]-BH-NPY and transferred to fresh, consecutive 10 μm sections of the corresponding tissue and further incubated for 2 h at room temperature. In parallel, further consecutive sections were incubated with 0.4 nM [^{125}I]-BH-NPY mixed with an equal volume of buffer B, 1 μM unlabelled hNPY, either of the analogues without prior incubation with tissue sections (final concentrations 0.5 μM and 0.1 μM for colons and spleens respectively), or unlabelled NPY (final concentration 1 nM). Sections were then washed and apposed to film as described below. The concentrations of hNPY and analogues were selected to give approximately 50% inhibition of [^{125}I]-BH-NPY binding.

Quantitation

Unfixed sections were apposed to Hyperfilm-^{3}H (Amersham, UK) and exposed for 3 days at 4°C. Films were subsequently developed in D-19 developer (Kodak) for 3 min at 20°C and fixed in Amfix (Amersham, UK). Autoradiograms were

quantified on an IBAS 2000 image analysis system (Kontron, Watford, UK). Standard curves relating grey values to log concentration of bound [^{125}I]-BH-NPY (amol mm^{-2}) were obtained for each film using sections of radiolabelled polymer standards (American Radiolabelled Chemicals Inc., St. Louis, U.S.A.). Specific binding was calculated as total minus non-specific binding. Each experiment was performed on tissue from 5 to 12 cases.

The kinetic constants for association (k_{+1}) and dissociation (k_{-1}) were derived from association and dissociation time course experiments. The rate of loss of specific [^{125}I]-BH-NPY bind-ings in tissue sections was estimated as a monoexponential decay constant from specific binding data following extended preincubations.

Equilibrium dissociation constants (K_D) and maximal binding to myenteric ganglia (B_{max}) for each of 5 porcine colons were derived from saturation experiments by non-linear regression. In addition, values were also calculated from data from kinetic experiments, K_D being k_{-1}/k_{+1}, and $B_{max} = B_{eq}$ $(K_D + L)/L$, where B_{eq} is the equilibrium binding with 0.2 nM [^{125}I]-BH-NPY, and L is the free concentration of [^{125}I]-BH-NPY (= 0.2 nM).

The concentrations of unlabelled ligands producing 50% inhibition of binding of 0.2 nM [^{125}I]-BH-NPY to myenteric ganglia (IC_{50}) were calculated for each case from binding inhibition experiments. Inhibition constant (K_i) values were derived from IC_{50} values according to the formula; $K_i = IC_{50}/(1 + L/K_D)$. For porcine colon, the K_D value used was that derived from saturation experiments. For porcine spleen, K_D was estimated from binding inhibition experiments using unlabelled NPY and the formula $K_D = IC_{50} - L$.

Curve fitting and experimental derivation of constants were performed by iterative non-linear regression using GraphPAD Inplot 3.1 (GraphPAD, San Diego). Single-site models were used for saturation and kinetic data, and a multisite model for fitting sigmoid curves to binding inhibition data. Values are expressed as arithmetic or geometric means as appropriate with 95% confidence intervals. Between groups comparisons were made on the original geometric data by paired or unpaired Student's t test or by ANOVA, as appropriate. Values of $P<0.05$ were taken as significant.

Histochemistry and microautoradiography

For microscopic localization of binding, sections with bound [^{125}I]-BH-NPY were fixed in Bouin's fixative for 60 min at 22°C, washed in distilled water and dried in cold air, then dipped in photographic emulsion (Ilford K5) and exposed for 7 days at 4°C. Dipped sections were then developed as for films (above) and counterstained with haematoxylin and eosin. In addition, for each case of porcine colon, consecutive sections to those used for the production of autoradiographic images on film were fixed in formol saline and stained for acetylcholinesterase according to the method of El-Badawi & Schenk (1967). Autoradiograms were then compared with acetylcholinesterase stained sections to establish the localization of binding of [^{125}I]-BH-NPY to cholinesterase positive ganglia.

Chemicals

[^{125}I]-BH-NPY was obtained from Amersham International plc, UK, with a specific activity of 2000 Ci mmol^{-1}. [^{125}I]-PYY (porcine) was obtained from Du Pont (UK) Ltd., Stevenage, specific activity 2200 Ci mmol^{-1}. NPY (13–36) (porcine) and [Leu^{31}, Pro^{34}]NPY (porcine) were obtained from Peninsula Laboratories plc, U.K. A further supply of [Leu^{31}, Pro^{34}]NPY was obtained from Sigma Chemical Co., Poole, UK. Human and porcine NPY, porcine peptide YY, human pancreatic polypeptide, β-cyclic calcitonin gene-related peptide, substance P, neurokinin A, guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S), bacitracin, bestatin, captopril, chymostatin, leupeptin and phosphoramidon, and enzyme-free bovine serum albumin were each obtained from Sigma Chemical Co., Poole, UK. H.p.l.c. solvents were obtained from Rathburn Chemicals, Peebleshire, Scotland.

Results

Specific [^{125}I]-BH-NPY binding sites were detected in porcine colon, localized on autoradiography films to the region of the myenteric plexus and on similar structures in the submucosal region (Figure 1a,b). [^{125}I]-PYY gave an identical distribution of specific binding. Low density non-specific binding was demonstrated in other regions of smooth muscle, submucosa and mucosa, and high density, non-specific binding at the luminal surface. Light microscopic examination of counterstained, emulsion-dipped sections of porcine colon confirmed specific binding of [^{125}I]-BH-NPY to ganglia in the myenteric and submucosal plexuses (Figure 2). Silver grains were densely distributed over a majority of ganglion cells. Comparison of autoradiography films with light microscopy of corresponding sections histochemically stained for acetylcholinesterase confirmed that specific [^{125}I]-BH-NPY binding corresponded to acetylcholinesterase-positive ganglia in both the myenteric and submucosal plexi. Specific binding of [^{125}I]-BH-NPY was not identified on vascular smooth muscle under these conditions. In 5 of 7 cases of normal human

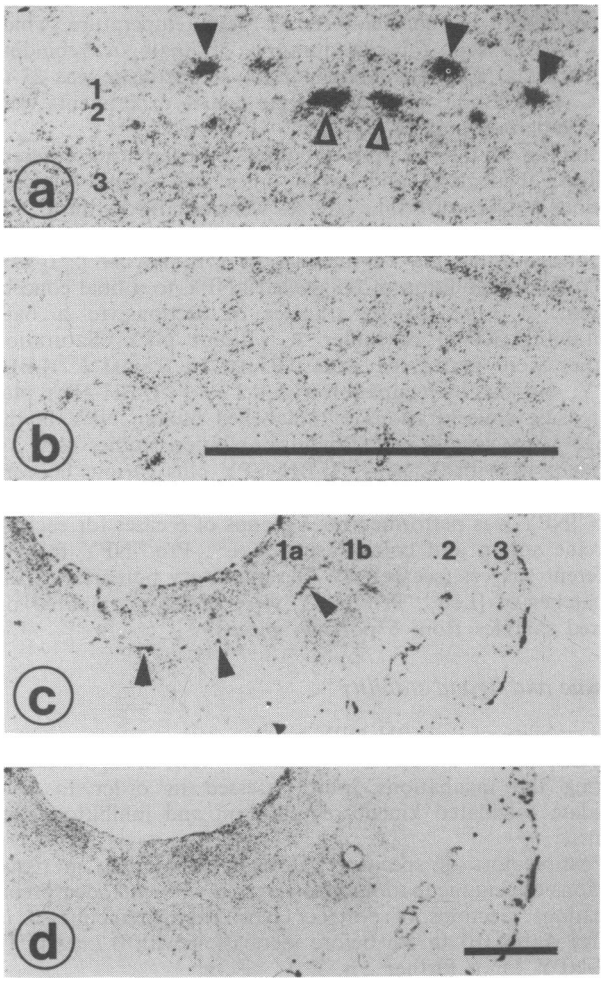

Figure 1 Film autoradiographs of [^{125}I]-Bolton Hunter-labelled human neuropeptide Y binding to porcine (a,b) and human (c,d) colon: total binding (a,c); non-specific binding (b,d); closed arrow-heads myenteric ganglia; open arrow-heads submucosal ganglia; (1) muscle ((1a) longitudinal muscle, (1b) circular muscle); (2) submucosa; (3) mucosa. Bars = 2 mm.

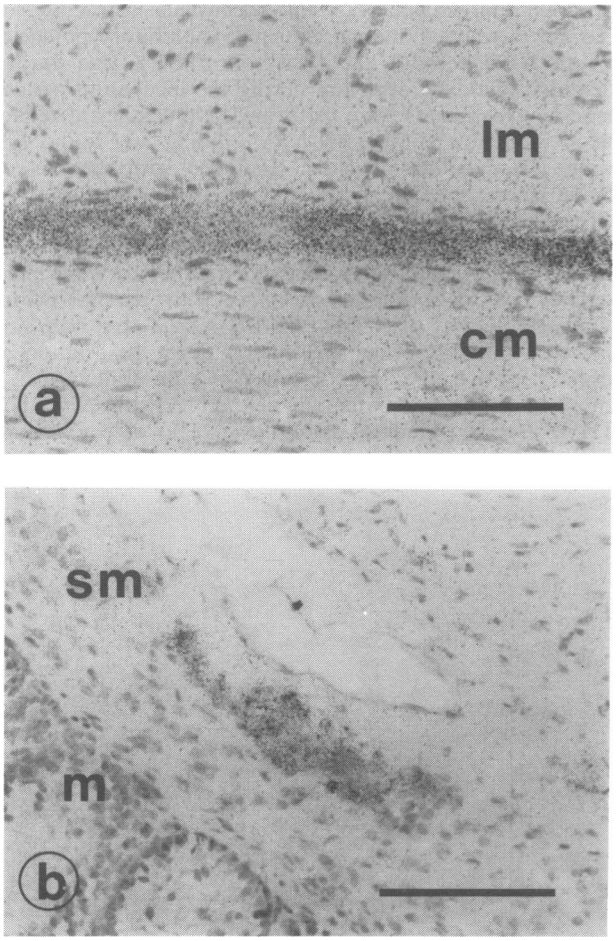

Figure 2 Photomicrographs of emulsion-dipped sections of porcine colon: myenteric ganglion (a); submucosal ganglion (b); (lm) longitudinal muscle, (cm) circular muscle, (sm) submucosa, (m) mucosa. Haematoxylin and eosin counterstained. Bars = 100 μm.

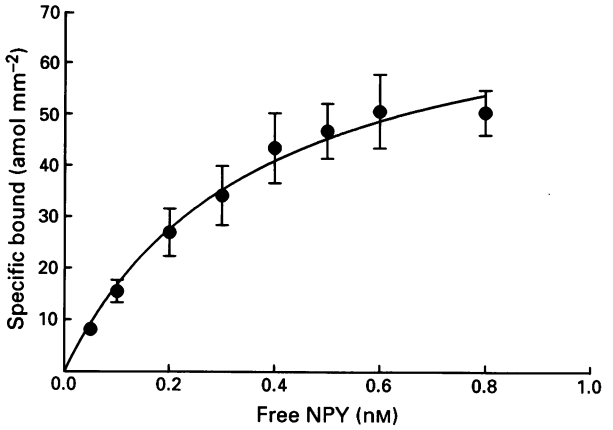

Figure 3 Saturation of specific [^{125}I]-Bolton Hunter-labelled neuropeptide Y binding to porcine colonic myenteric ganglia. Each point represents the mean (s.e.mean, vertical bars) of 5 cases.

colon similar specific binding of [^{125}I-BH-NPY was localized to ganglia in the myenteric plexus (Figure 1c,d).

Equilibrium studies indicated that binding of [^{125}I]-BH-NPY to porcine colonic myenteric ganglia was saturable, and of high affinity (Table 1, Figure 3). High affinity binding was confirmed in kinetic studies (Table 1), which revealed that specific binding of 0.2 nM [^{125}I]-BH-NPY to myenteric ganglia reached a maximum within 2 h at room temperature (Figure 4). At 37°C specific binding reached a maximum by 30 min but rapidly declined with increasing incubation times beyond 50 min. All subsequent experiments were therefore performed at room temperature.

Dissociation of ligand from the myenteric plexus following the addition of 1 μM unlabelled pNPY was slow, with > 50% of equilibrium binding remaining after 5 h (Figure 4). Similar results were obtained by transferring labelled sections to a bath containing 300 ml buffer A without NPY. The addition of the non-hydrolysable GTP analogue, GTP-γ-S to the dissociation buffer greatly increased dissociation of specific [^{125}I]-BH-NPY binding from the myenteric plexus. After 2 h in the presence of GTP-γ-S (2 μM) specific binding was reduced to 9% (7–12%) of equilibrium binding, as compared with 77% (69–85%) in the absence of GTP-γ-S.

There was a gradual decline in specific binding of [^{125}I]-BH-NPY to myenteric ganglia with increasing preincubation times. The rate of decline in specific binding was similar to that observed following the addition of unlabelled NPY in the absence of GTP-γ-S (decay constant = 5.5 (2.5–12.0) $\times 10^{-5}$ s^{-1}). Furthermore, a similar decline in specific binding to myenteric ganglia was observed with prolonged incubations with [^{125}I]-BH-NPY beyond 2 h.

The binding activity of 0.2 nM [^{125}I]-BH-NPY was reduced following a 2 h incubation with sections of porcine colon when compared with that of fresh ligand. Specific binding to porcine colon myenteric ganglia was reduced from 104 (83–130) amol mm^{-2} to 54 (48 to 61) amol mm^{-2} ($P < 0.0005$). Addition of phosphoramidon, captopril, bestatin, leupeptin, chymostatin or bacitracin, alone or in combination, to the incubation buffer did not significantly increase specific binding to porcine myenteric ganglia, nor did it prevent the decline in NPY binding activity in the supernatants.

Radioactivity counts in supernatants from this experiment, representing total ^{125}I not bound to the solid phase, did not reveal any significant decline during the 2 h incubation, from 1740 (1588 to 1892) c.p.m. to 1588 (1524 to 1654) c.p.m. ($P > 0.05$).

H.p.l.c. on a propan-1-ol:acetic acid gradient of control, standard samples not preincubated with tissue sections (Figure 5a) resulted in a single peak of radioactivity with a retention time of 12 min (h.p.l.c. fractions 15 and 16) appearing shortly after the albumin peak (retention time 11 min, fraction 14). Radioactivity in this peak accounted for 91% of that injected.

Table 1 Kinetic and equilibrium constants for [^{125}I]-Bolton Hunter-labelled neuropeptide Y binding to porcine colon myenteric ganglia

Experimental design	k_{+1} ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	K_D (pM)	B_{max} (amol mm^{-2})
Saturation	–	–	409 (220–760)	81 (70–93)
Kinetic	9 (4–20) $\times 10^5$	3.2 (1.9–5.5) $\times 10^{-5}$	60 (20–230)	128 (60–279)

Values are expressed as geometric means (95% CI), $n = 5$.

Following 2 h incubation with porcine colon, h.p.l.c. again produced a major peak of radioactivity with a retention time of 12 min, accounting for 83% of that injected, as well a minor peak with a retention time of 9 min, (h.p.l.c. fractions 12 and 13) accounting for a further 6% of injected radioactivity (Figure 5b). Further h.p.l.c. of the major peaks from 2 samples with and 2 without incubation with porcine colon using an acetonitrile gradient revealed a single peak of radioactivity in all cases with a retention time of 10 min (fractions 13 and 14) and no minor peaks, with a yield of 88%.

Binding inhibition analysis on 6 porcine colons demonstrated relative affinities for the [^{125}I]-BH-NPY binding sites in myenteric ganglia of pNPY = hNPY = pYY >> NPY (13–36) ≥ [Leu^{31}, Pro^{34}] NPY >> PP (Table 2, Figure 6). The unrelated peptides substance P (1 μM), neurokinin A (1 μM) and calcitonin gene-related peptide (1 μM) did not significantly inhibit [^{125}I]-BH-NPY binding to myenteric ganglia.

To investigate further the relative potencies of the selective Y_1 and Y_2 ligands, [Leu^{31}, Pro^{34}]NPY and NPY (13–36) in inhibiting binding of [^{125}I]-HB-NPY to porcine colon, binding inhibition studies were performed on samples from an additional 6 pigs using [Leu^{31}, Pro^{34}]NPY obtained from Sigma Chemical Co., Poole, UK, as well as NPY (13–36). Results from this experiment were closely similar to those obtained previously, and combining the results from colons of all 12 pigs revealed NPY (13–36) to be slightly, but significantly, more potent at inhibiting specific [^{125}I]-BH-NPY binding to porcine colonic myenteric ganglia (Table 2) with a mean difference in log IC_{50} = 0.4 (0.04 to 0.75, P = 0.032). None the less, NPY (13–36) and [Leu^{31}, Pro^{34}]NPY were each able to inhibit completely specific [^{125}I]-BH-NPY binding to porcine myenteric ganglia, with Hill coefficients near unity.

To assess whether the closely similar inhibitory potencies of both NPY (13–36) and [Leu^{31}, Pro^{34}]NPY was due to differential analogue depletion during the incubation period, rebinding experiments were performed. No significant reduction in inhibition of [^{125}I]-BH-NPY binding was found following preincubation of either analogue with sections of porcine colon.

Specific [^{125}I]-BH-NPY binding sites were also demonstrated on porcine spleen, located over red pulp. Only non-specific binding could be demonstrated either to white pulp arterioles, or to splenic arteries. As with porcine colon, equilibrium binding of 0.2 nM [^{125}I]-BH-NPY to red pulp was achieved within 2 h. There was no significant dissociation of specific [^{125}I]-BH-NPY binding in red pulp following 2 h incubation in buffer containing neither [^{125}I]-BH-NPY nor GTP-γ-S (specific binding 103% (85–123%) of equilibrium binding). Addition of 2 μM GTP-γ-S to the dissociation

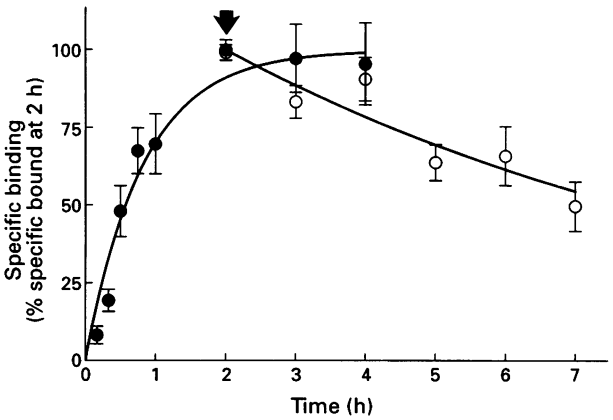

Figure 4 Association (●) and dissociation (○) time courses of specific binding of 0.2 nM [^{125}I]-Bolton Hunter-labelled neuropeptide Y to myenteric ganglia in porcine colon at room temperature. Arrow; addition of 1 μM unlabelled NPY for dissociation time course. Each point represents the mean (± s.e.mean, vertical bars) of 5 cases.

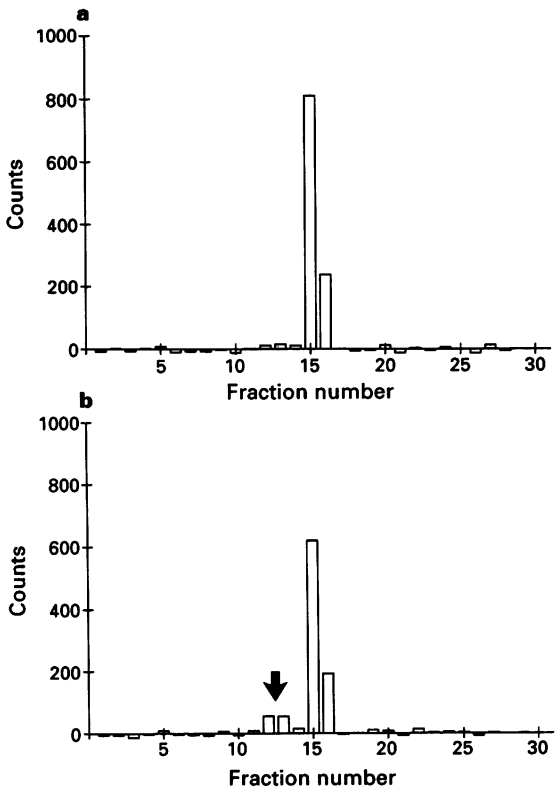

Figure 5 High performance liquid chromatography of [^{125}I]-Bolton Hunter-labelled neuropeptide Y on a propanol/acetic acid gradient (a) before, and (b) after 2 h incubation with sections of porcine colon. One minute samples of eluent were counted for 30 s and background subtracted. Arrow; second peak of radioactivity appearing after incubation.

Table 2 Inhibition of binding of 0.2 nM [^{125}I]-Bolton Hunter-labelled neuropeptide Y ([^{125}I]-BH-NPY) to porcine colon by unlabelled peptides

Ligand	*IC_{50}* (nM)		*K_i* (nM)		*Hill coefficient*	
pNPY	0.29	(0.19–0.44)	0.19	(0.12–0.30)	1.29	(0.75–2.22)
hNPY	0.35	(0.21–0.60)	0.24	(0.14–0.40)	1.56	(1.16–2.08)
PYY	0.44	(0.27–0.74)	0.30	(0.18–0.50)	1.65	(1.29–2.10)
[Leu^{31}, Pro^{34}]NPY	27*	(18–42)	18	(12–28)	1.14	(1.03–1.26)
NPY (13–36)	11*	(6–21)	7	(4–14)	1.13	(0.88–1.46)
PP	670	(410–1060)	450	(275–712)		

Values are expressed as geometric means (95% CI). *NPY (13–36) more potent than [Leu^{31}, Pro^{34}]NPY, P = 0.032, paired t test, n = 12. p = porcine; h = human; PYY = peptide tyrosine tyrosine; PP = pancreatic polypeptide

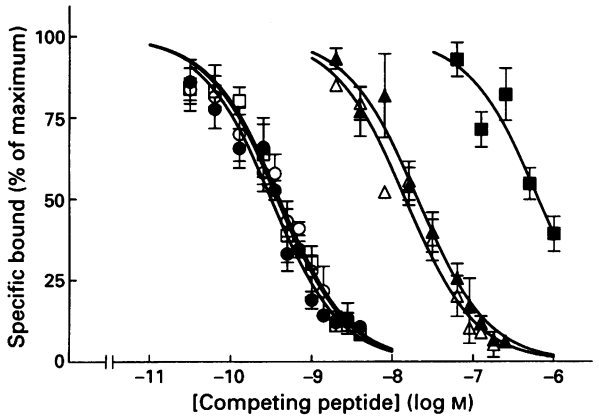

Figure 6 Competitive inhibition of 0.2 nM [^{125}I]-Bolton Hunter-labelled neuropeptide Y ([^{125}I]-BH-NPY) binding to porcine colon myenteric plexus by unlabelled peptides: (●) porcine NPY; (○) human NPY; (□) porcine peptide tyrosine tyrosine; (▲) [Leu^{31}, Pro^{34}] NPY; (△) NPY (13–36); (■) pancreatic polypeptide. Each point represents the mean (± s.e. mean, vertical bars) of 6 cases.

buffer significantly decreased specific binding to 15% (10–24%) of equilibrium binding after 2 h.

Binding inhibition studies on 12 porcine spleens indicated that, as with porcine colon, NPY (13–36) was slightly but significantly, more potent than [Leu^{31}, Pro^{34}]NPY at inhibiting specific [^{125}I]-BH-NPY binding to porcine splenic red pulp (Table 3, mean difference in log IC_{50} values = 0.53 (0.09–0.96), $P = 0.02$). Two separate batches of [Leu^{31}, Pro^{34}]NPY were tested on consecutive sections of 6 porcine spleens. Only small differences in inhibitory potencies were observed between batches with IC_{50} values of 1.0 (0.8–1.4) $\times 10^{-7}$ M and 3.5 (1.6–7.4) $\times 10^{-7}$ M respectively.

Rebinding experiments were performed on sections of porcine spleen to assess possible differential analogue depletion during incubations. No significant diminution in inhibitory potency was observed for either [Leu^{31}, Pro^{34}]NPY or NPY (13–36) following 2 h incubation with porcine spleen.

In contrast to the porcine spleen, specific [^{125}I]-BH-NPY binding was not demonstrable in human spleens, nor in normal rat spleen under the conditions tested.

Discussion

We have demonstrated specific, saturable, high affinity binding sites for [^{125}I]-BH-NPY in ganglia of the myenteric and submucosal plexuses of normal porcine colon. Specific binding showed similar high affinity for both NPY and PYY suggesting that these sites may represent shared NPY/PYY receptors. NPY-like immunoreactivity (NPY-LI) is present in nerve fibres and occasional ganglion cells of both myenteric and submucosal plexi in the colon of most mammalian species so far studied, including pig and man (Furness *et al.*, 1983; Ferri *et al.*, 1984). PYY-LI is localized to mucosal endocrine cells in mammalian colon (Lundberg *et al.*, 1982) and is released into the circulation following the ingestion of a meal (Taylor, 1985; Greeley *et al.*, 1989).

Both NPY and PYY have pharmacological actions in mammalian colon. Both were shown in the cat and guinea-pig to reduce colonic motility, induce colonic relaxation, and also to reduce tone in precontracted colon (Lundberg *et al.*, 1982; Hellstrom *et al.*, 1985; Hellstrom, 1987; Wiley & Owyang 1987). Subsequent studies have shown that NPY and PYY increase rectal tone in the cat and, similarly, increase basal tone in the rat distal colon (Hellstrom *et al.*, 1989; Cadieux *et al.*, 1990). Each of these effects of NPY may be mediated by actions on myenteric neurones, rather than by a direct action on colonic smooth muscle (Hellstrom, 1987; Wiley & Owyang, 1987; Cadieux *et al.*, 1990). NPY inhibits calcium currents in rat cultured myenteric plexus neurones, providing further evidence of functional NPY receptors in the myenteric plexus (Hirning *et al.*, 1990). The specific binding sites for [^{125}I]-BH-NPY and [^{125}I]-PYY observed in this study in myenteric ganglia may represent functional receptors through which locally released NPY and PYY may regulate colonic motility.

NPY and PYY inhibit electrolyte secretion by colonic mucosa (Cox *et al.*, 1988; Okuno *et al.*, 1991). In contrast to binding studies in small intestinal mucosal membranes in rats (Nguyen *et al.*, 1990), we were unable to demonstrate specific binding of either [^{125}I]-BH-NPY or [^{125}I]-PYY to porcine colonic mucosa. However, specific binding of both ligands was observed to ganglia in the colonic submucosal plexus, suggesting that effects of NPY on colonic secretion may, at least in part, be indirectly mediated through submucosal nerves.

In addition to effects on intestinal motility and secretion, NPY induces mesenteric vasoconstriction and potentiates noradrenaline-induced vasoconstriction, both probably post-junctional effects on vascular smooth musle (Hellstrom *et al.*, 1985; Andriantsitohaina & Stoclet, 1990) as well as reducing noradrenaline release by a prejunctional action (Westfall *et al.*, 1987). Despite these known pharmacological effects of NPY on mesenteric vasculature, we were unable to detect specific binding to blood vessels in any of our tissues. Specific binding of [^{125}I]-BH-NPY to colonic vessels may be present, but at a low density by comparison with non-specific binding. Modin *et al.* (1991) found that specific binding of iodinated NPY to splenic arteries represented only 10% of total binding, despite demonstrating splenic vasoconstriction by NPY. Such low specific to non-specific ratios are not amenable to detailed study by quantitative *in vitro* receptor autoradiography. Other possibilities include blocking of binding by endogenous ligand, perhaps released when the animals were killed, or differences in affinity for Bolton Hunter-labelled NPY of vascular smooth muscle as compared with myenteric ganglia and splenic red pulp.

H.p.l.c. revealed only a small degree of ligand degradation following 2 h incubation of [^{125}I]-BH-NPY with porcine colon. The significant loss of [^{125}I]-BH-NPY binding ability during the 2 h incubation period therefore appears to be multifactorial, and only partially explained by ligand degradation. Other possibilities include the release of inhibitory factors such as GTP or endogenous NPY from tissue sections, or the inactivation of components of buffer B required

Table 3 Binding inhibition of 0.2 nM [^{125}I]-Bolton Hunter-labelled neuropeptide Y ([^{125}I]-BH-NPY) to porcine spleen by unlabelled peptides

Ligand	*IC_{50} (nM)*	*K_i (nM)*	*Hill coefficient*
pNPY	1.5 (0.6–3.5)		1.9 (1.5–2.4)
[Leu^{31}, Pro^{34}]NPY	112* (42–291)	97 (37–252)	1.1 (0.8–1.5)
NPY (13–36)	34* (27–42)	29 (23–36)	1.7 (1.3–2.0)

Values are expressed as geometric means (95% CI). *NPY (13–36) more potent than [Leu^{31}, Pro^{34}]NPY at inhibiting binding of [^{125}I]-BH-NPY to porcine splenic red pulp ($P = 0.02$, paired t test, $n = 12$)

for binding, such as the albumin.

Nonetheless, even a 50% depletion of binding activity during the incubation, would not be expected to affect substantially calculated K_D and K_i values. It is apparent, therefore, that, in the absence of GTP, [^{125}I]-BH-NPY binds to myenteric ganglia with high affinity. The markedly increased dissociation observed in the presence of GTP-γ-S indicates the presence of a second (low) affinity state for these binding sites which is characteristic of G protein-linked receptors.

Kinetic studies and competition with unlabelled ligands indicate that, under identical conditions, porcine myenteric ganglion and splenic red pulp binding sites for [^{125}I]-BH-NPY share many characteristics. In particular, both are sensitive to GTP-γ-S, and both [Leu^{31}, Pro^{34}]NPY and NPY (13–36) completely inhibited specific binding with similar potencies, NPY (13–36) being slightly, but significantly more potent than [Leu^{31}, Pro^{34}]NPY. These findings suggest that porcine colonic myenteric ganglia and splenic red pulp each bear the same subclass of NPY receptor. The closely similar potencies of the two analogues observed in this study contrast, however, with results from membrane preparations of porcine spleen described by Modin *et al.* (1991), who found NPY (13–36) to be approximately 100 times more potent than [Leu^{31}, Pro^{34}]NPY in inhibiting specific binding of iodinated NPY. However, the same authors also found that *in vivo*, both analogues were approximately equipotent at increasing porcine splenic vascular resistance, each being one tenth as potent as intact NPY. The relative potencies of NPY and NPY (13–36) at inhibiting [^{125}I]-BH-NPY binding to myenteric ganglia observed in our study are consistent with studies *in vitro* on intestinal muscle strips which indicate that long C-terminal fragments of NPY inhibit muscle tone, but are less active than the intact peptide (Allen *et al.*, 1987).

We considered whether a differential depletion of the two analogues could explain their apparently similar inhibitory potencies in porcine colon and spleen, but no significant loss of inhibitory potency was observed for either NPY (13–36), or [Leu^{31}, Pro^{34}]NPY following preincubation with porcine spleen or colon.

It remains possible that the process of membrane preparation can affect binding characteristics of membrane G protein-receptor complexes, or that specificity may be influenced by the use of different incubation conditions. Such factors may be particularly important in studies using receptor agonists, the binding of which to receptors is dependent on the integrity of receptor-G protein interactions. We have demonstrated two affinity states of [^{125}I]-BH-NPY binding sites in porcine colon and spleen, depending on the presence of GTP analogues. Y_1 and Y_3 receptor cDNA sequences have recently been cloned (Rimland *et al.*, 1991; Herzog *et al.*, 1992; Larhammer *et al.*, 1992), and it is hoped that hybridization techniques using specific nucleotide probes will provide a means of distinguishing the expression of receptor subtypes not dependent on relative differences in binding of agonists.

Specific binding sites for [^{125}I]-BH-NPY were identified on the myenteric ganglia of normal human colon in 5 of 7 cases, suggesting that NPY may be a modulator of human colonic motility, and that porcine colon may be an appropriate model for studying the colonic actions of NPY and PYY. By contrast to the red pulp of porcine spleen, however, specific [^{125}I]-BH-NPY binding sites were not identified in any of 6 specimens of normal human spleen, or in normal rat spleens. These findings may represent interspecies differences in the distribution of NPY receptors, and are similar to comparative studies performed on the kidney (Leys *et al.*, 1987).

It remains to be determined whether abnormalities of NPY release or receptor expression underlie diseases characterized by disordered gut motility, such as the irritable bowel syndrome and idiopathic megacolon. Similarly, abnormalities of NPY/PYY systems in disorders of colonic secretion such as inflammatory bowel disease await full investigation.

In conclusion, we have demonstrated specific [^{125}I]-BH-NPY binding sites in human and porcine colonic myenteric ganglia, and in porcine colonic submucosal ganglia. These binding sites have the characteristics of high affinity, G protein-linked receptors and show similar kinetic and specificity characteristics to [^{125}I]-BH-NPY binding sites in porcine splenic red pulp, when studied under identical conditions. This distribution of [^{125}I]-BH-NPY binding sites suggests that NPY/PYY may modulate colonic motility and secretion by regulating colonic neuronal activity. NPY and PYY have been implicated in a variety of disorders of the human gastrointestinal tract (Sheikh, 1991) and the development of metabolically stable compounds which interact with colonic NPY/PYY receptors may offer novel therapeutic approaches to these diseases.

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Stimulation of insulin secretion by imidazoline compounds is not due to interaction with non-adrenoceptor idazoxan binding sites

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1 The potency of interaction of several imidazoline compounds with non-adrenoceptor idazoxan binding sites (NAIBS) in rat liver membranes was compared with their ability to alter insulin secretion from rat pancreatic islets.

2 NAIBS could be labelled specifically with [^{3}H]-idazoxan in both rat liver membranes and in rat islet homogenates. Liver binding sites exhibited a K_D for [^{3}H]-idazoxan of 24 nM and a B_{max} of 264 fmol mg^{-1} protein.

3 Binding of [^{3}H]-idazoxan to NAIBS in rat liver membranes was displaced effectively by unlabelled idazoxan (IC_{50} 0.1 μM) and by UK14304 (IC_{50} 0.5 μM). However, two other imidazoline compounds efaroxan and RX821002, which are related in structure to idazoxan, were much less effective as displacers.

4 In insulin secretion experiments, the ATP-sensitive potassium channel agonist diazoxide (250 μM) was able to suppress the rise in insulin secretion induced by 20 mM glucose. Both efaroxan and RX821002 (100 μM) antagonized the inhibitory effect of diazoxide on glucose-induced insulin secretion. By contrast, neither idazoxan (100 μM) nor UK14304 (50 μM), was able to overcome significantly the inhibitory effect of diazoxide.

5 The ability of 100 μM efaroxan to antagonize the suppression of insulin secretion mediated by diazoxide, was not prevented by idazoxan (up to 100 μM) or by UK14304 (up to 50 μM).

6 The results indicate that the stimulatory effects of imidazoline compounds on insulin secretion are not due to interaction with NAIBS similar to those present in rat liver.

Keywords: Islets of Langerhans; α_2-adrenoceptors; insulin secretion; idazoxan; efaroxan; RX821001

Introduction

Recent studies have shown that certain α_2-adrenoceptor antagonists, including phentolamine (Chan *et al.*, 1988; Smith & Furman, 1988; Schulz & Hasselblatt, 1988) DG-5128 (Kameda *et al.*, 1981; 1982; Kawazu *et al.*, 1987; Chan *et al.*, 1991b) and efaroxan (Berridge *et al.*, 1989; Chan *et al.*, 1990; 1991a) are able to enhance the rate of insulin secretion when administered to experimental animals or human volunteers *in vivo*, or when added to isolated islets of Langerhans incubated *in vitro*. These effects have been attributed to blockade of islet α_2-adrenoceptors (Kameda *et al.*, 1982; Broadstone *et al.*, 1987) leading to an increase in insulin secretion by virtue of the relief of a tonic inhibitory tone mediated by endogenous catecholamines (Robertson *et al.*, 1976). However, this mechanism does not offer a satisfactory explanation for the stimulatory response, since some α_2-antagonists (such as yohimbine) are very effective blockers of sympathetic inhibition of insulin secretion (Langer *et al.*, 1983; Morgan & Montague, 1985) but do not elicit any direct increase in secretion in the absence of added agonist (Chan *et al.*, 1988; Garrino & Henquin, 1990).

One common feature which is shared by the α_2-antagonists capable of stimulating insulin secretion, is the possession of an imidazoline ring within their structure. Indeed certain imidazolines which do not bind to α_2-adrenoceptors are also able to stimulate insulin secretion (Schulz & Hasselblatt, 1989) suggesting that possession of an imidazoline ring may be an important determinant of secretogogue activity. The reasons for this are not clear at present, but it is noteworthy that binding sites have recently been identified on cells, that are able to bind some α_2-selective imidazoline compounds (e.g. [^{3}H]-idazoxan) with high affinity, but do not bind non-imidazoline α_2-selective ligands such as yohimbine and rauwolscine (Coupry *et al.*, 1987; Hamilton *et al.*, 1988; Langin & Lafontan, 1989). These sites appear, therefore, to be distinct from α_2-adrenoceptors and, in support of this, the two can be separated by affinity chromatography (Parini *et al.*, 1989). Because of their pharmacological characteristics, the imidazoline binding sites have been termed 'non-adrenergic (non-adrenoceptor) idazoxan binding sites' (NAIBS) and have now been identified in a wide range of tissues (reviewed by Atlas, 1991; Kilpatrick *et al.*, 1992).

At present there is considerable interest in defining a functional role for imidazoline binding sites, and it has been postulated that they may be involved in control of neurotransmitter secretion (Gothert & Molderings, 1991) and in regulation of membrane K^+ permeability (Zonnenschein *et al.*, 1990). This is significant since we (Chan *et al.*, 1990; 1991a,b) and others (Plant & Henquin, 1990; Dunne, 1991; Plant *et al.*, 1991) have presented evidence suggesting that those imidazolines which stimulate insulin secretion, may do so by inducing closure of ATP-sensitive K^+ channels in the β-cell membrane. This response has been seen in both normal rat islets and in the rat insulinoma cell line, RINm5F (Chan *et al.*, 1991a) which has recently been shown to express non-adrenoceptor binding sites labelled by [^{3}H]-idazoxan (Remaury & Paris, 1992).

In view of this evidence, the present study was undertaken to examine whether the stimulatory effects of imidazoline compounds on insulin secretion can be attributed to interaction with NAIBS. To address this question, we have compared the ability of certain imidazoline compounds to bind to NAIBS present on rat liver membranes with their capacity to

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modulate insulin secretory responses in rat islets of Langerhans.

Methods

Isolation of islets of Langerhans

Islets of Langerhans were isolated from the pancreata of male Wistar rats (180–250 g body weight) allowed free access to food and water, by collagenase digestion (Montague & Taylor, 1968). The isolation medium was a bicarbonate-buffered physiological saline solution (Gey & Gey, 1936) equilibrated to pH 7.4 by gassing with O_2:CO_2 (95:5). The buffer was supplemented with 4 mM glucose and 1 mM $CaCl_2$. Islets were selected individually under a binocular dissecting microscope and were used within 2 h of isolation.

Preparation of islet homogenates

Approximately 1000 isolated islets were hand picked as described above. Islets were then re-selected individually to optimize purity, and placed in 100 µl of Tris (50 mM)/EDTA (1 mM)/$MgCl_2$ (10 mM) buffer (TEM) pH 7.5. The islets were disrupted with ultrasound (low power for 5 s) and the resulting suspension was briefly centrifuged (5000 *g*; 5 s) in a microfuge to pellet any remaining intact islets. The supernatant containing the membranes was then used directly in radioligand binding assays.

Insulin secretion experiments

For insulin secretion studies, islets were either incubated under static conditions or in a perfusion system, as described previously (Morgan & Montague, 1984). Briefly, for static experiments, groups of three isolated islets were incubated in bicarbonate buffered medium, supplemented with 1 mg ml^{-1} bovine serum albumin. Islets were incubated for 1 h at 37°C in the presence of appropriate test reagents, and samples of the medium were then removed for measurement of insulin by radioimmunoassay. In perifusion studies, islets were perfused at a flow rate of 1 ml min^{-1} for 30 min before the start of the experiment. Thereafter, samples of perifusion medium were collected at 1 min intervals and their insulin content measured.

Preparation of rat liver membranes

Membranes were prepared from the livers of male Wistar rats allowed free access to food and water. The tissue was excised, finely minced with scissors, and homogenized in a power-driven Teflon/glass Potter Elvjhem homogenizer (6 passes) in TEM buffer (10 ml buffer per g tissue). The homogenate was centrifuged at 1000 *g* for 10 min (4°C) and the resulting supernatant then centrifuged at 40000 *g* for 20 min (4°C). The pellet was washed by resuspension in 40 ml TEM buffer and re-centrifuged at 40000 *g* for 20 min (4°C). The final pellet was resuspended in TEM buffer, and stored at −80°C until required.

Radioligand binding experiments

[^{3}H]-idazoxan (specific activity 40 Ci $mmol^{-1}$) was used to label NAIBS. Membranes (approx. 300 µg protein), [^{3}H]-idazoxan and competing drugs were incubated in a final volume of 0.25 ml TEM buffer, pH 7.5, for 1 h at 25°C. In saturation studies [^{3}H]-idazoxan was used over the concentration range 0.1–100 nM. In all experiments 100 µM yohimbine was used to block α_2-adrenoceptors, and non-specific binding was determined in the presence of 100 µM unlabelled idazoxan. In competition studies, inhibition curves were obtained by incubating membranes with 20 nM [^{3}H]-idazoxan, and a range of concentrations of competing compounds (1 nM–10 µM). Incubations were terminated by rapid vacuum filtration through Whatman GF/B filters. Filters were washed with 10 ml of ice cold Tris (50 mM)/EDTA (5 mM) buffer, pH 7.4, and their radioactivity measured after addition of 4 ml of scintillant.

Protein assays

The protein content of liver membrane and islet homogenate preparations was measured by the bicinchoninic acid method of Smith *et al.* (1985).

Statistical analysis

Data were analysed by Student's *t* test and differences were considered significant if the *t* value corresponded to a probability of 1:20 or less ($P<0.05$).

Materials

[^{3}H]-idazoxan (specific activity 40 Ci $mmol^{-1}$) was purchased from Amersham International, and [^{125}I]-insulin (specific activity 2000 Ci $mmol^{-1}$) was purchased from ICN-Flow. UK14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline) was a gift from Pfizer Research, Sandwich, Kent. Idazoxan, efaroxan and RX821002 (2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline) were provided by Reckitt and Colman Products, Hull. Diazoxide was obtained from Glaxo Pharmaceuticals. All other reagents were of analytical reagent grade.

Results

In order to compare the receptor binding profiles of imidazoline compounds with their ability to stimulate insulin secretion, both radioligand binding studies and insulin secretion experiments were performed. Radioligand binding studies were carried out by use of [^{3}H]-idazoxan to label sites in rat liver membranes and in rat islet homogenates. Insulin secretion experiments were performed with rat isolated islets of Langerhans.

Binding of [^{3}H]-idazoxan to rat liver membranes

In the presence of 100 µM yohimbine (to prevent binding of ligand to α_2-adrenoceptors) [^{3}H]-idazoxan still labelled a binding site in rat liver membranes, consistent with the presence of NAIBS in this tissue. Binding of [^{3}H]-idazoxan was saturable (Figure 1a) and Scatchard analysis revealed a single class of high affinity sites (Figure 1b). In three experiments, the K_D varied between 19–33 nM (mean 24 nM), and the B_{max} value averaged 264 ± 52 fmol mg^{-1} protein.

Competition studies were performed to determine the order of binding potency of various compounds at rat liver NAIBS, by assessing their ability to displace [^{3}H]-idazoxan. These experiments revealed that both idazoxan itself and the α_2-agonist UK14304 were effective in displacing [^{3}H]-idazoxan binding (Figure 2). The IC_{50} values were approximately 0.1 µM for idazoxan and 0.5 µM for UK14304 (Figure 2). By contrast, two other imidazolines, efaroxan and RX821002 (Stillings *et al.*, 1985) were only weakly effective as displacers of [^{3}H]-idazoxan binding (Figure 2). Indeed, at a concentration of 10 µM, RX821002 only displaced the binding of 20 nM [^{3}H]-idazoxan by approximately 10%. These results suggest that idazoxan and UK14304 interact strongly with NAIBS, whereas efaroxan and RX821002 show only very weak interaction at this site. This is consistent with previous data (Langin & Lafontan, 1989; Atlas, 1991; Remaury & Paris, 1992).

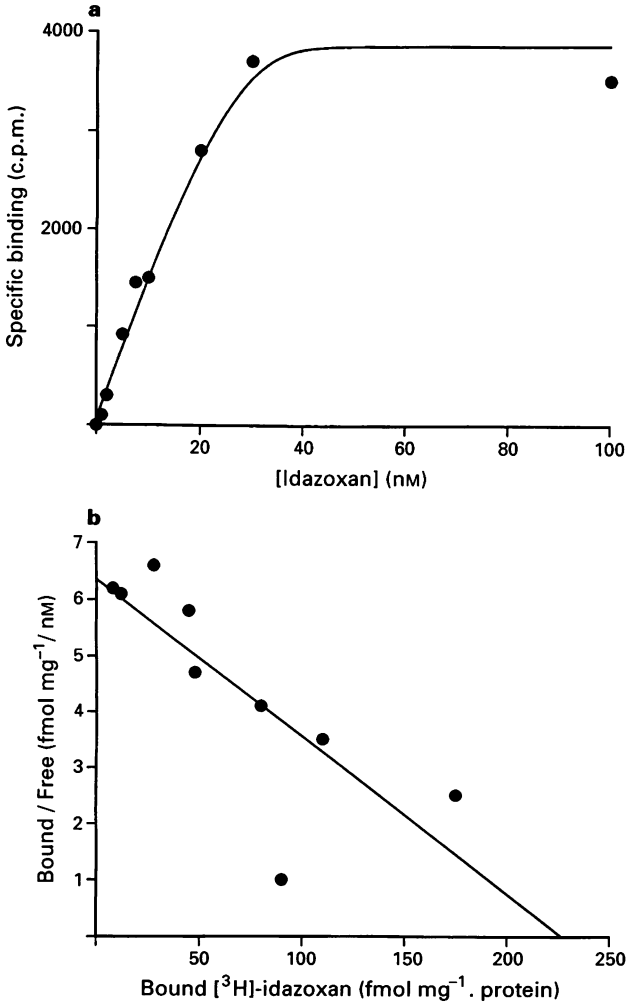

Figure 1 Binding of [^{3}H]-idazoxan to rat liver membranes. Liver membranes were incubated with [^{3}H]-idazoxan, over the concentration range 0.1 to 100 nM, for 1 h at 25°C. Non-specific binding was determined in the presence 100 μM unlabelled idazoxan. In all experiments 100 μM yohimbine was included to block binding of [^{3}H]-idazoxan to α_2-adrenoceptors. Data represent triplicate determinations from an experiment that is representative of three: (a) shows the saturation binding curve and (b) the data obtained after transformation of the results to generate a Scatchard plot. The line was fitted by linear regression analysis ($r = -0.79$).

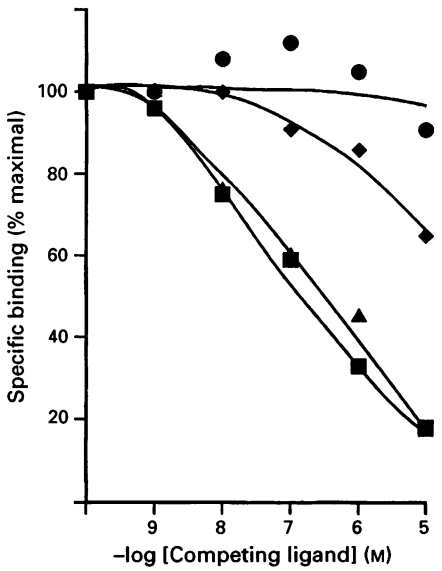

Figure 2 Effects of imidazoline compounds on the binding of [^{3}H]-idazoxan to NAIBS in rat liver membranes. Competition binding asays were performed on rat liver membranes with a single concentration (20 nM) of [^{3}H]-idazoxan. Competing agents were added over the concentration-range 1 nM to 10 μM. Competing agents were idazoxan (■), UK14304 (▲), efaroxan (◆) and RX821002 (●). Tubes were incubated for 1 h at 25°C, then bound and free ligand were separated by rapid vacuum filtration. Non-specific binding was determined in the presence of 100 μM idazoxan. Yohimbine (100 μM) was included in all tubes to prevent binding of [^{3}H]-idazoxan to α_2-adrenoceptors. Results are expressed as percentage of total specific binding measured in the absence of competing agent.

Insulin secretion studies

In the light of the above results we next proceeded to study the ability of these same imidazoline compounds to interact with rat islets of Langerhans. Initially, a limited number of ligand binding experiments were performed to determine whether rat islets express [^{3}H]-idazoxan binding sites. These experiments were very difficult to perform due to the small amounts of tissues obtained from a preparation of rat islets and to the low specific activity of the ligand. Nevertheless, in the presence of 100 μM yohimbine, [^{3}H]-idazoxan specifically labelled sites present in homogenates of rat islets. In three separate experiments the extent of binding was found to be 139, 70 and 86.5 fmol mg^{-1} protein (mean = 98.5 fmol mg^{-1} protein).

In order to assess the effect of imidazoline compounds on islet function, their ability to reverse the inhibitory effect of 250 μM diazoxide on insulin secretion was studied. Diazoxide is an agonist of ATP-sensitive potassium (K^+-ATP) channels (Dunne & Petersen, 1991) and inhibits glucose-induced insulin secretion by opening K^+-ATP channels. This leads to hyperpolarization of the cell, closure of voltage-sensitive calcium channels and a decrease in the rate of insulin release. We have previously reported that imidazoline compounds which can stimulate insulin secretion directly, are also able to overcome the inhibitory effects of diazoxide (Chan *et al.*, 1990; 1991a,b). Thus the use of diazoxide provides a convenient assay system for determining the effectiveness of imidazolines in interacting functionally with islet cells.

In support of previous evidence (Chan *et al.*, 1990; 1991a) efaroxan (100 μM) significantly reversed the inhibitory effect of diazoxide on glucose-induced insulin secretion in rat islets (Table 1). Similar effects were obtained with 100 μM RX821002 (Table 1). Lower concentrations of each compound were also effective, although at least 10 μM was required to elicit a response (not presented; Chan *et al.*, 1990). By contrast idazoxan (100 μM) did not produce any significant reversal of diazoxide-induced inhibition of glucose-induced insulin secretion (Table 1). Furthermore, in perifusion experiments, carried out under conditions of α_2-blockade, the effects of diazoxide were not reversed by 25 μM UK14304 (Figure 3).

One potential complication in these latter studies, is that UK14304 is an α_2-agonist (Cambridge, 1981) and can, thus, directly inhibit glucose-induced insulin secretion in its own right (Morgan *et al.*, 1989). However, the possibility that any tendency of UK14304 to antagonize the effect of diazoxide had been masked by its α_2-agonist properties, was excluded in control experiments (Figure 3). These confirmed that the regime of α_2-blockade employed (continuous infusion of the non-competitive antagonist phenoxybenzamine) was effective in preventing the interaction of UK14304 with islet α_2-receptors (Figure 3). These experiments also demonstrated that glucose stimulation of secretion was maintained throughout the perifusion period when diazoxide was not introduced (Figure 3).

Thus, it appears that those compounds which bind with high affinity to NAIBS in rat liver, are ineffective in reversing the inhibitory effects of diazoxide when used in insulin secre-

Table 1 Effects of imidazoline compounds on the inhibitory response to diazoxide in rat islets

Incubation conditions			
[Glucose] (mM)	*Diazoxide* (250 μM)	*Test reagents*	*Insulin secretion* (ng per islet h^{-1})
20	–	–	2.1 ± 0.15
20	+	–	0.5 ± 0.09
20	+	Idazoxan 100 μM	1.1 ± 0.20
20	+	Efaroxan 100 μM	2.0 ± 0.21*
20	+	RX821002 100 μM	1.7 ± 0.30*

Groups of three isolated islets were incubated for 1 h at 37°C, in the presence of test reagents as shown. After this time, samples of medium were removed for measurement of insulin levels by radioimmunoassay. Data are presented as mean values ± s.e.mean for 12–18 observations.
*$P < 0.001$ relative to 20 mM glucose plus diazoxide

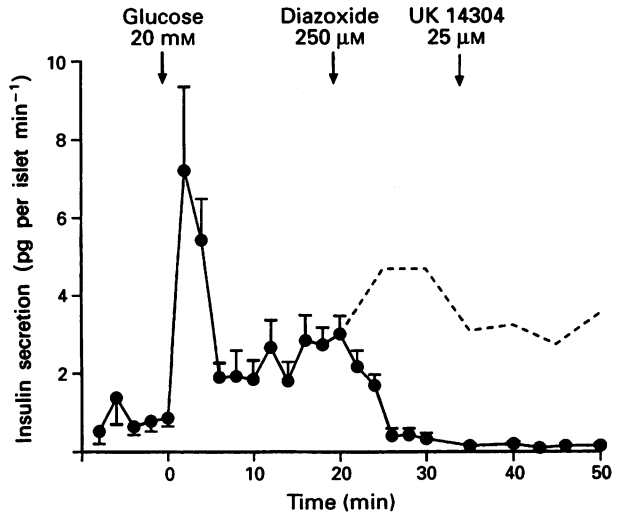

Figure 3 Effects of UK14304 on the inhibition of glucose-induced insulin secretion mediated by diazoxide. Groups of 100 isolated islets from rat were perifused with medium containing 4 mM glucose and 5 μM phenyoxybenzamine for 30 min. After this time ($t = 0$) 20 mM glucose was introduced. Diazoxide (250 μM) was added after a further 20 min (solid line) and UK14304 (25 μM) infused during the final 15 min of perifusion. Phenoxybenzamine infusion was maintained throughout the experiment. Control experiments in which diazoxide was omitted are shown by the dotted line. Samples of the perifusion medium were collected and their insulin content measured by radioimmunoassay. Data are presented as mean rates of insulin secretion (± s.e.mean, vertical bars) from three separate islet preparations.

Table 3 Effects of UK14304 on the ability of efaroxan to antagonize the inhibition of glucose-induced insulin secretion due to diazoxide

Incubation conditions				
[Glucose] (mM)	*Diazoxide* (250 μM)	*Efaroxan* (100 μM)	*[UK14304]* (μM)	*Insulin secretion* (ng per islet h^{-1})
4	–	–	–	0.61 ± 0.10
20	–	–	–	4.29 ± 0.53
20	+	–	–	0.99 ± 0.12
20	+	+	–	2.74 ± 0.53
20	+	+	0.1	2.81 ± 0.35^{NS}
20	+	+	1	2.51 ± 0.21^{NS}
20	+	+	10	2.74 ± 0.24^{NS}
20	+	+	50	2.25 ± 0.30^{NS}

Groups of three isolated islets from rats were incubated for 1 h at 37°C under the conditions shown, insulin levels were measured by radioimmunoassay and 100 μM yohimbine was also included in all tubes to provide more effective α_2-adrenoceptor blockade. Data are presented as mean values ± s.e.mean from 12 observations.
NS: Not significantly different from efaroxan in the absence of UK 14304

tion experiments. Conversely, compounds which antagonize the inhibitory effect of diazoxide in insulin secretion experiments, bind only very weakly to NAIBS.

To examine these relationships further, we investigated whether UK14304 or idazoxan was able to antagonize the effects of efaroxan on insulin secretion. The differences in affinity displayed by these three compounds at NAIBS is such that efaroxan should be readily displaced by either idazoxan or UK14304 if NAIBS represent its site of action in the endocrine pancreas. In initial studies we made the assumption that the presence of 100 μM efaroxan (a potent α_2-antagonist; Chapleo *et al.*, 1984) would be sufficient to block any effects mediated by α_2-receptors. Under these conditions 50 μM UK14304 was able to reduce the level of insulin secretion measured in the combined presence of efaroxan and diazoxide (Table 2) giving the impression that antagonism of the response mediated by efaroxan had occurred. However, further addition of 100 μM yohimbine completely abolished this effect (Table 3) demonstrating that the antagonistic effect of UK14304 was mediated by α_2-adrenoceptors and not by direct competition with efaroxan at NAIBS.

In support of this evidence, 100 μM idazoxan was also unable to antagonize the effect of efaroxan in insulin secretion experiments (Table 2).

Table 2 Effects of UK14304 and idazoxan (Idz) on the ability of efaroxan to antagonize the inhibition of glucose-induced insulin secretion mediated by diazoxide

Incubation conditions					
[Glucose] (mM)	*Diazoxide* (250 μM)	*Efaroxan* (100 μM)	*[UK]* (μM)	*Idz* (100 μM)	*Insulin secretion* (ng per islet h^{-1})
4	–	–	–	–	1.16 ± 0.16
20	–	–	–	–	3.37 ± 0.32
20	+	–	–	–	1.19 ± 0.14
20	+	+	–	–	3.79 ± 0.67
20	+	+	1	–	2.46 ± 0.39
20	+	+	50	–	0.69 ± 0.08*
20	+	+	–	+	3.50 ± 0.30^{NS}

Groups of three isolated islets from rats were incubated for 1 h at 37°C in the presence of test reagents as shown. Samples of medium were removed at the end of the incubation period and their insulin levels measured by radioimmunoassay. Data are presented as mean values ± s.e.mean for 12 observations.
*$P < 0.001$ relative to efaroxan in the absence of UK14304
NS: Not significantly different from efaroxan in the absence of idazoxan

Discussion

The major aim of this study was to establish whether a correlation exists between the binding profile of imidazoline compounds at NAIBS, and the ability of these same compounds to modulate insulin secretion from rat isolated islets of Langerhans. NAIBS have been described in a range of tissues (reviewed by Atlas, 1991; Kilpatrick *et al.*, 1992) but seem to have variable pharmacology suggesting that they may not represent a single molecular entity (Wikberg *et al.*, 1991; Kilpatrick *et al.*, 1992). Despite this, all sites defined to date exhibit a high affinity for idazoxan, and we chose to use this ligand in order to compare the pharmacology of rat liver NAIBS with effects of imidazoline compounds on insulin secretion.

The data from binding experiments showed that, in rat liver membranes, [^{3}H]-idazoxan labelled NAIBS in a saturable manner, with a K_D of 24 nM and B_{max} of 264 fmol mg^{-1} protein. This correlates well with previous findings (Zonnenschien *et al.*, 1990). With rat islets, the limited amount of tissue available prohibited the construction of a full saturation curve, but we were able to demonstrate that [^{3}H]-idazoxan bound to non-adrenoceptor sites. Thus, at least some of the cells present in rat islets appear to express NAIBS, although the data do not permit the conclusion that these sites are necessarily present on insulin secreting β-cells. However the demonstration that membranes derived from two cultured β-cell lines, RINm5F (Remaury & Paris, 1992) and HIT-T15 (N.G. Morgan, unpublished observations) also express NAIBS, is consistent with this possibility.

In competition binding studies, idazoxan and UK14304 each displaced 20 nM [^{3}H]-idazoxan from rat liver membranes very effectively. By contrast, efaroxan and RX821002 were only very weakly effective even at concentrations up to 10 μM. Thus for NAIBS present in rat liver, the rank order of potency is idazoxan > UK14304 >> efaroxan > RX821002. This is in good agreement with previous data (Langin & Lafontan, 1989; Atlas, 1991; Remaury & Paris, 1992).

Our previous work on the effects of efaroxan in the endocrine pancreas revealed that high concentrations of the agent (> 10 μM) are required to elicit insulin secretion directly or to antagonize the inhibitory effect of diazoxide on secretion (Chan *et al.*, 1990; 1991a). The present results show that this is also true for RX821002. Since neither efaroxan nor RX821002 bind to NAIBS with high affinity, the observation that high concentrations of each compound are required to elicit responses in insulin secretion experiments, could be consistent with the possible involvement of NAIBS. Furthermore the evidence that control of insulin secretion by imidazolines may relate to alterations in the K^+ permeability of the β-cell membrane (Chan *et al.*, 1990; 1991a,b; Plant & Henquin, 1990; Dunne, 1991; Plant *et al.*, 1991) is also consistent with previous reports implicating NAIBS in inhibition of K^+ channel opening in vascular smooth muscle (McPherson & Angus, 1989).

However, the possible involvement of NAIBS in control of insulin secretion is called into question by data which reveal that neither idazoxan nor UK14304 (at concentrations up to 100 μM) were able to reverse significantly the inhibitory effect of 250 μM diazoxide in islets. Since idazoxan and UK14304 each have a higher affinity for NAIBS than either efaroxan or RX821002, it might be expected that the former compounds would be more potent in islets. This was not the case. However, it is also possible that the lack of stimulation in response to idazoxan or UK14304 might reflect a failure of these compounds to interact with the NAIBS in an agonistic manner. In this context, there is evidence that idazoxan can behave as an antagonist at NAIBS in some tissues (Olmos *et al.*, 1992), although this is apparently not a universal property (Gothert & Molderings, 1991). Thus, to exclude the possibility that efaroxan/RX821002 and idazoxan/UK14304 might exert opposite agonistic and antagonistic activities respectively, in pancreatic islets, we examined the ability of idazoxan and UK14304 to compete with efaroxan in insulin secretion experiments.

Initially it was observed that UK14304 (50 μM) blocked the effects of 100 μM efaroxan in islets (Table 2). Thus, it appeared that UK14304 was able to compete with efaroxan in an antagonistic fashion. However, this result is misleading since, under conditions of increased adrenoceptor blockade (inclusion of 100 μM yohimbine) UK14304 no longer antagonized the response to efaroxan (Table 3). Taken together, these data show that the antagonistic effect of UK14304 was mediated by α_2-adrenoceptor activation and not by direct competition with efaroxan for NAIBS.

In support of the evidence that UK14304 did not compete with efaroxan in islets, addition of 100 μM idazoxan was also unable to reverse the effect of 100 μM efaroxan.

In conclusion, the present results support our previous suggestions (Chan *et al.*, 1990; 1991a,b) that the stimulatory effects of imidazolines on insulin secretion do not result from binding of these compounds to islet cell NAIBS. In drawing this conclusion, we wish to emphasize that our results do not exlude the possibility that the effects of these compounds might be mediated by some type of 'imidazoline binding site' in islets. However, if this is the case, then this site must represent a class of binding site which has low affinity for idazoxan. This differentiates it from NAIBS which, by definition, have high affinity for idazoxan.

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Protective effects of bradykinin on the ischaemic heart: implication of the B_1 receptor

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1 We studied the role of bradykinin (BK) and its active metabolite Des-Arg^9-BK on noradrenaline release in association with the incidence of ventricular arrhythmias at reperfusion of the ischaemic myocardium.

2 Experiments were performed in Langendorff perfused isolated hearts of rats subjected to 30 min no flow followed by 5 min reperfusion. The electrocardiogram was monitored continuously and noradrenaline was measured in the effluent as well as in the myocardial tissue.

3 In untreated hearts, cumulative noradrenaline overflow following global ischaemia reached 226 ± 35 pmol g^{-1} of heart ($n = 8$, $P < 0.05$) during the 5 min of reperfusion along with ventricular tachycardia and/or fibrillation. A decrease in myocardial noradrenaline (-31%) was also observed.

4 Bradykinin perfused at concentrations between 0.01 and 1 μM, 10 min before flow was stopped and at reperfusion, inhibited noradrenaline overflow in a concentration-dependent manner. At a concentration of 1 μM, bradykinin completely abolished noradrenaline overflow. For the same concentration of bradykinin, myocardial noradrenaline contents were significantly higher ($n = 5-8$, $P < 0.05$). Ventricular fibrillation but not ventricular tachycardia was also prevented.

5 Des-Arg^9-BK (0.1 μM) in the same experimental conditions had similar effects. While Hoe 140, a selective antagonist at B_2 receptors, did not abolish the effects of bradykinin, Lys [Leu^8] Des-Arg^9-BK, an antagonist at B_1 receptors, abolished the effects of both Des-Arg^9-BK and bradykinin.

6 These results suggest that the cardioprotective action of bradykinin in the preparation may be mediated partially by an inhibitory effect on noradrenaline liberation which could be mediated by the activation of B_1 receptors.

Keywords: Myocardial ischaemia-reperfusion; bradykinin; Des-Arg^9-BK; noradrenaline; arrhythmias; Hoe 140; Lys [Leu^8] Des-Arg^9-BK

Introduction

Myocardial ischaemia is defined as an insufficient blood supply to cardiac tissue. If the blood supply is not restored promptly, the affected tissue will die. Reperfusion is thus the most important way to salvage the ischaemic tissue. However, reperfusion causes damage such as morphological alterations, increased resting tension, increased enzyme leakage (Hearse, 1977; Jennings *et al.*, 1981) and it can also precipitate severe ventricular arrhythmias, including lethal ventricular fibrillation (Corr & Witkowski, 1983).

The sympathetic nervous system is potentially involved in reperfusion-induced arrhythmias. In fact, reperfusion is accompanied by a massive liberation of noradrenaline (NA) into the coronary effluent, the amount of which is probably related to the occurrence of sustained ventricular tachycardia (Godin *et al.*, 1985).

Bradykinin (BK), a well known vasodilator peptide via B_1 and B_2 receptors (Regoli & Barabe, 1980) has been shown to be effective against ischaemia/reperfusion injuries. Its cardioprotective effects have been attributed to improvement in cardiac performance, preservation of high energy rich phosphate, abolition of reperfusion arrhythmias (Linz *et al.*, 1990), as well as an increase in nutritional flow across the capillary wall increasing thereby glucose uptake and utilization (Rösen *et al.*, 1983). A reduction of noradrenaline overflow (Carlsson & Abrahamsson, 1989), and an increase in prostacyclin and nitric oxide release (Van Gilst *et al.*, 1987; Palmer *et al.*, 1987) are other potential effects of bradykinin on the ischaemic phenomenon.

Des-Arg^9-BK, the active metabolite of bradykinin and the typical B_1 receptor agonist (Regoli & Barabe, 1980) has been shown to exhibit some significant effects on vascular tone (Churchill & Ward, 1986; Deblois & Marceau, 1987). However, normal tissues do not generally respond to Des-Arg^9-BK (Regoli & Barabe, 1980). In fact, different authors have shown the induction of this B_1 receptor in pathological circumstances such as tissue trauma, inflammation or anoxia (Bouthillier *et al.*, 1987; Marceau & Regoli, 1991).

In the present study we have shown that B_1 receptors could be involved in the cardioprotective action of bradykinin in rat isolated hearts submitted to reperfusion after acute global ischaemia.

Methods

Perfusion procedure

Male Wistar rats (250–275 g) were anaesthetized with diethylether and 200 iu of heparin was injected intravenously. Hearts were removed rapidly and perfused according to the Langendorff mode at a constant pressure of 100 cmH_2O with a modified Krebs-Henseleit (KH) solution continuously gassed with O_2/CO_2 (95:5) at a temperature of 37°C as previously described (Chahine *et al.*, 1991). The composition (mM) of the solution was: NaCl 118.7, KCl 1.8, $CaCl_2$ 3, $MgSO_4$ 0.85, KH_2PO_4 1.2, $NaHCO_3$ 25, ascorbic acid 0.057, EDTA 0.027, and glucose 5.5. The low concentrations of K^+ and Mg^{2+} and high Ca^{2+} were chosen for their contribution to increase arrhythmias (Lubbe *et al.*, 1978; Keren *et al.*, 1988).

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Global ischaemia was produced by interrupting aortic flow for 30 min followed by 5 min reperfusion. During the stop flow period, hearts were superfused with the same KH solution to prevent an important temperature drop. Under such conditions, hearts continue to contract very slowly and do not stop beating completely. The epicardial electrogram (EPI-ECG) and heart rate were recorded on a Grass polygraph model 50 with 2 electrodes positioned, one above the aorta and the other at the apex of the heart.

Quantification of arrhythmias

Ventricular fibrillation (VF) was assessed when the ECG recording showed chaotic activity with an amplitude less than that of the normal ECG. When at least six consecutive rapid regular beats occurred, this was considered as ventricular tachycardia (VT).

Experimental protocol

Hearts were allowed to equilibrate for 15 min. (i) In the control group (C; $n = 5$), they were perfused for 35 min without any intervention. (ii) In the ischaemic group (ISC; $n = 15$) a stop flow of 30 min was followed by 5 min of normal reperfusion. (iii) In the treated groups ($n = 6$ to 10), KH solution containing bradykinin (1 to 0.01 μM) or Des-Arg^9-BK (0.1 μM) was added to the perfusion medium 10 min before stop flow and continued to the end of the experiment. The maximum dose of Des-Arg^9-BK was 0.1 μM since a higher concentration of this peptide induced depression of heart contractility. (iv) In two groups ($n = 8$ each), Hoe 140 (1 μM) or Lys [Leu^8] Des-Arg^9-BK (1 μM), the antagonists of B_2 and B_1 receptors respectively, were perfused 10 min before bradykinin (0.1 μM) and remained in the tissue to the end of the experiment. (v) In the last group ($n = 8$) Lys[Leu^8] Des-Arg^9-BK was perfused 10 min before Des-Arg^9-BK (0.1 μM) and to the end of the experiment. ECG was monitored continuously. The effluent perfusate was collected before ischaemia and at reperfusion for determination of coronary flow and biochemical parameters. At the end of the experiments hearts were frozen at − 80°C until noradrenaline content determination.

Biochemical assays

Noradrenaline output Samples for catecholamine assay were collected from the coronary effluent before stop flow and during the 5 min reperfusion. The samples (1 ml each) were preserved on ice, immediately stabilized by the addition of a preservation solution (0.025 mM EGTA + 0.02 mM glutathione) and stored at − 80°C until assay. A radioenzymatic determination of noradrenaline was performed (Peuler & Johnson, 1977) after testing the absence of cross reactivity of all pharmacological agents used. Results were corrected by coronary flow and heart weight.

Myocardial noradrenaline was determined by high performance liquid chromatography (h.p.l.c.) using about 100 mg of myocardial tissue from heart apex according to the method previously described (Chahine *et al.*, 1991) and the amounts expressed as nmol mg^{-1} protein after the determination of heart protein content according to Lowry *et al.* (1951).

Bradykinin was also quantified in the perfusate fluid by a radioimmunoassay (RIA) method. Briefly, Tyr^8 BK was iodinated with Na ^{125}I (IMS 30, Amersham) and purified by h.p.l.c. on a ODS C18 column using a linear gradient of acetonitrile in formic acid. RIA was performed as previously described (Adam *et al.*, 1989) using a rabbit polyclonal antibody specific to the carboxy terminal Arg; the sensitivity of the method was 6 pg ml^{-1}.

Drugs

All the compounds of the Krebs-Henseleit solution, bradykinin and Des-Arg^9-bradykinin were purchased from Sigma (St. Louis, MO, U.S.A.). Hoe 140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7, Oic] bradykinin) was generously supplied by Hoechst AG, Germany and Lys [Leu^8] Des-Arg^9-BK was prepared in Dr D. Regoli's laboratory. All solutions were freshly prepared on the day of experimentation.

Statistics

To assess the incidence of VF and VT as well as to compare the two events, Fisher's exact test was used. Results are expressed as mean ± s.e.mean. Statistical significance between groups was determined by Dunnett's test. In all cases, a $P < 0.05$ was considered to be significant.

Results

Arrhythmias and cardiodynamics

After stopping flow, all ischaemic untreated hearts developed bradycardia; furthermore, between 20 and 30 min sino-atrial and high degree AV block were also noted. At reperfusion, VF and/or VT were observed (Table 1). In these hearts, coronary flow decreased by only 20% after 5 min reperfusion (Table 2). In the bradykinin pretreated hearts, a concentration of 1 μM abolished sino-atrial and AV block completely, but not bradycardia, during the stop flow period; at this concentration bradykinin completely prevented the development of VF at reperfusion (Table 1). A slight decrease in heart rate (data not shown) and a slight increase in coronary flow were observed immediately after bradykinin (1 μM) perfusion and remained moderate for 10 min. While Hoe 140 1 μM was not able to antagonize the protective effect of 0.1 μM bradykinin against VF, Lys [Leu^8] Des-Arg^9-BK seemed to abolish partially the effects of bradykinin. Des-Arg^9-BK (0.1 μM) in the same experimental conditions significantly diminished the incidence of VF (Table 1); however, a slight decrease in coronary flow was observed (Table 2). Lys [Leu^8] Des-Arg^9-BK was effective in antagonizing the effects of Des-Arg^9-BK. It is important to mention that antagonists alone reduced the coronary flow slightly, however, they had no influence on the incidence of arrhythmias.

Biochemical parameters

Noradrenaline overflow In control hearts there was a basal release of noradrenaline (3.6 ± 0.22 pmol min^{-1} g^{-1} of heart) which did not change significantly after 35 min of normal perfusion. In untreated hearts submitted to 30 min stop flow,

Table 1 Effects of bradykinin (BK) and Des-Arg^9-BK on the incidence of ventricular tachycardia (VT) and fibrillation (VG) at reperfusion post ischaemia in the absence and presence of antagonists

	VT	*VF*
Control	0	0
Ischaemia	15/15	13/15
BK 0.01 μM	5/6	5/6
BK 0.1 μM	7/10	4/10*
BK 1 μM	4/6	0/6*
Hoe 140 1 μM + BK 0.1 μM	6/8	3/8*
Lys [Leu^8] Des-Arg^9-BK 1 μM + BK 0.1 μM	7/8	6/8
Des-Arg^9-BK 0.1 μM	5/8	3/8*
Lys [Leu^8] Des-Arg^9-BK 1 μM + Des-Arg^9-BK 0.1 μM	7/8	7/8

Control: hearts not submitted to ischaemia.
*$P < 0.05$ compared to ischaemia.

Table 2 Coronary flow (ml min^{-1}) in control, ischaemic untreated and treated hearts

	− 20	− 1	2	4	5 (min)
Control	15.2 ± 1.4	15.0 ± 1.34	–	–	14.5 ± 1.25
Ischaemia	15.0 ± 1.2	15.2 ± 1.36	12.8 ± 2.50	12.5 ± 2.80	12.0 ± 1.7*
BK 0.01 μM	14.8 ± 1.70	14.6 ± 0.98	13.2 ± 1.52	13.0 ± 1.40	11.8 ± 1.00*
BK 0.1 μM	14.5 ± 0.80	14.4 ± 0.86	13.3 ± 0.66	12.5 ± 0.75	12.5 ± 0.87
BK 1 μM	14.4 ± 0.75	15.8 ± 1.04	14.6 ± 1.24	13.8 ± 1.20	13.0 ± 1.04
Hoe 140 1 μM + BK 0.1 μM	15.2 ± 1.57	13.8 ± 1.60	12.7 ± 0.97	12.5 ± 0.85	12.7 ± 0.96
Lys [Leu^8] Des-Arg^9-BK 1 μM + BK 0.1 μM	16.1 ± 1.0	14.0 ± 1.14	12.1 ± 1.12*	11.5 ± 0.98*	11.0 ± 0.88*
Des-Arg^9-BK 0.1 μM	15.3 ± 1.60	13.9 ± 1.83	12.8 ± 2.00	12.5 ± 1.55	11.8 ± 1.22*
Lys [Leu^8] Des-Arg^9-BK 1 μM + Des-Arg^9-BK 0.1 μM	15.8 ± 0.95	14.3 ± 1.12	12.3 ± 1.00*	11.4 ± 0.90*	10.8 ± 1.05*

− 20: before drug administration; − 1: before 30 min stop flow; 2, 4, and 5 min reperfusion.
*$P<0.05$ compared with the corresponding basal value at − 20 min, $n = 5-8$.
BK: bradykinin; Hoe 140: B_2 antagonist; Lys [Leu^8] Des-Arg^9-BK: B_1 antagonist.

noradrenaline overflow reached 226 ± 35 pmol g^{-1} during the 5 min of reperfusion (Figure 1). Pretreatment with bradykinin caused a concentration-dependent decrease in the ischaemia-induced release of noradrenaline at reperfusion with a complete abolition at 1 μM. Des-Arg^9-BK (0.1 μM) also reduced noradrenaline overflow to 80% of the control value. Antagonists (Hoe 140 and Lys [Leu^8] Des-Arg^9-BK) given alone before agonists, had no effect on noradrenaline release; only the latter blocked the protective effects of Des-Arg^9-BK as well as partially those of bradykinin.

Myocardial noradrenaline Tissue noradrenaline stocks were reduced to 31% in ischaemic untreated hearts. Pretreatment with 1 μM bradykinin preserved the myocardial noradrenaline content and to a lesser extent this effect was observed also with 0.1 μM bradykinin and 0.1 μM Des-Arg^9-BK; the antagonists were inactive on their own and partially prevented the effects of the kinins (Figure 2).

Bradykinin As shown in Table 3, the concentrations of bradykinin measured in the coronary effluent were significantly lower than the original concentrations added to the KH buffer solution. A recovery between 25 and 50% of the initial value was observed.

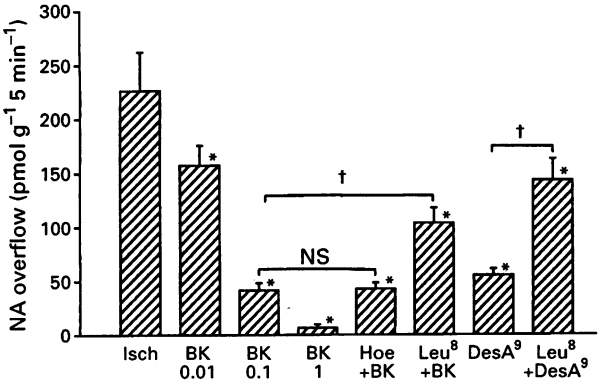

Figure 1 Cumulative noradrenaline (NA) overflow occuring above the basal release during 5 min reperfusion following 30 min stop flow in Isch: ischaemic untreated hearts; BK 0.01: ischaemic hearts pretreated with 0.01 μM bradykinin; BK 0.1: pretreated with 0.1 μM bradykinin; BK 1: pretreated with 1 μM bradykinin; Hoe + BK: ischaemic hearts pretreated with 1 μM Hoe 140 before 0.1 μM bradykinin; Leu^8 BK: pretreated with Lys [Leu^8] Des-Arg^9-BK 1 μM before 0.1 μM bradykinin; DesA^9: ischaemic hearts pretreated with 0.1 μM Des-Arg^9-BK; Leu^8 + DesA^9: pretreated with Lys [Leu^8] Des-Arg^9-BK 1 μM before 0.1 μM Des-Arg^9-BK. Results are expressed as means ± s.e.mean (vertical bars) in pmol g^{-1} 5 min^{-1} of heart, $n = 5-8$. *$P<0.05$ compared with Isch; †$P<0.05$ compared with BK 0.1 μM or Des-Arg^9-BK; NS = not significant.

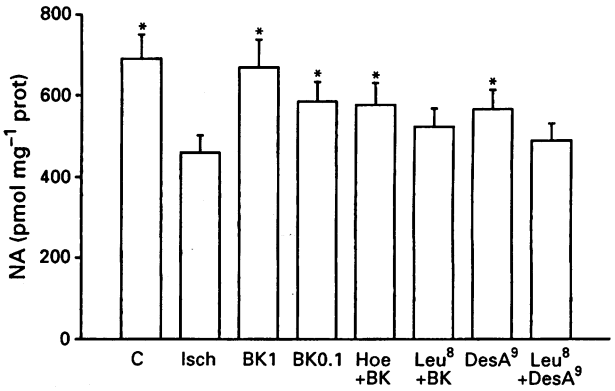

Figure 2 Myocardial noradrenaline (NA) content in isolated hearts submitted to 5 min reperfusion following 30 min stop flow. C: control hearts perfused during 35 min; Isch: ischaemic untreated hearts; BK 0.1: ischaemic hearts pretreated with 0.1 μM bradykinin; BK 1: pretreated with 1 μM bradykinin; Hoe + BK: ischaemic hearts pretreated with 1 μM Hoe 140 before 0.1 μM bradykinin; Leu^8 BK: pretreated with Lys [Leu^8] Des-Arg^9-BK 1 μM before 0.1 μM bradykinin; DesA^9: ischaemic hearts pretreated with 0.1 μM Des-Arg^9-BK; Leu^8 + DesA^9: pretreated with Lys [Leu^8] Des-Arg^9-BK 1 μM before 0.1 μM Des-Arg^9-BK. Results are expressed as means ± s.e.mean (vertical bars) in pmol mg^{-1} of heart protein content. $n = 5-8$. *$P<0.05$ compared with Isch.

Table 3 Concentrations of bradykinin (BK) measured in the Krebs-Henseleit (KH) buffer before the perfusion and in the perfusate buffer after their administration to the heart ($n = 5$)

BK concentration (μM) in KH buffer	*BK concentration (μM) in perfusate*	*Decrease (%)*
1	0.25 ± 0.03	− 75
0.1	0.05 ± 0.008	− 50
0.01	0.00375 ± 0.00095	− 62.5

Discussion

In 1973, Kimura *et al.* showed that during myocardial ischaemia induced by coronary ligation in anaesthetized dogs, bradykinin and bradykininase concentrations in coronary sinus blood were increased, while that of bradykininogen was decreased. In the dog model, levels of bradykinin varied considerably owing probably to differences in the development of collateral circulation and to the method of quantification. Recently, the beneficial effects of

bradykinin on myocardial ischaemia/reperfusion have been studied by various authors. Antiarrhythmic effects and improvement of cardiac performance after bradykinin infusion were observed in different animal species: pigs (Tio *et al.*, 1991), dogs (Linz & Schölkens, 1987) and rat isolated heart (Linz *et al.*, 1990). Carlsson & Abrahamsson (1989) have also reported that bradykinin (0.01–10 nM) reduced significantly noradrenaline efflux during reperfusion following total ischaemia in rat isolated hearts. Both of the latter studies used a constant rate of perfusion.

Catecholamines and their oxidation byproducts are known to have important deleterious effects accompanying reperfusion damages. In this context, Schömig *et al.* (1985) have shown that noradrenaline overflow during reperfusion represents a washout of the transmitter released from sympathetic nerve endings previously accumulated in the tissue during the ischaemic period. Noradrenaline is released via an inversion of the carrier system normally responsible for $uptake_1$ as a consequence of the metabolic imbalance that occurs within the nerve endings. It has also been shown that neuronal activity from a central origin does not lead to a substantial accumulation of noradrenaline within the ischaemic myocardium and that the intramyocardial noradrenaline stocks are expected to play the major role in modulating the transmitter release. Hence, the depletion of endogenous noradrenaline stores by α-methyl-*meta*-tyrosine reduces ventricular fibrillation and mortality in rats submitted to coronary artery ligation (Abrahamsson *et al.*, 1985).

It is important to mention that Rösen *et al.* (1983) have shown that in isolated perfused hearts bradykinin increased the rate of glucose uptake and oxidation as well as the formation of lactate, enhanced the nutritional flow across the capillary wall and indirectly glucose metabolism. Bradykinin caused also a concentration-dependent release of nitric oxide and of prostacyclin (Palmer *et al.*, 1987; Van Gilst *et al.*, 1987) which are known to block noradrenaline release and possess several pharmacological properties which render them potentially useful for the protection of the ischaemic myocardium. This could be an important contributing factor for myocardial tissue preservation.

In this study (using rat isolated hearts perfused at constant pressure) we have attempted to establish a link between arrhythmias and noradrenaline output at reperfusion. Because the amounts of noradrenaline released does not reflect those that are lost from the tissue (Lamontagne *et al.*, 1991), myocardial noradrenaline contents were also evaluated. Moreover, owing to the non-reproducibility of sampling in areas affected by coronary ligation (regional ischaemia), we used the model of acute total ischaemia to induce a global attack throughout the whole heart.

The results obtained showed that VF was prevented and VT significantly reduced only in hearts pretreated with bradykinin (1 μM). Under these conditions, noradrenaline release was completely abolished, while myocardial noradrenaline stocks were preserved. Thus bradykinin appears to reduce noradrenaline overflow after ischaemia and thereby affords a protection from arrhythmias.

It is conceivable that cells previously in anoxia react abnormally to a sudden oxygenation (Hess & Manson, 1984). Indeed, it has been shown that at reperfusion, there is enzyme leakage, increase of intracellular osmotic pressure, increased membrane permeability and an important Ca^{2+} entry resulting in an ionic alteration and a change of conduction velocity which, in the presence of catecholamines, may predispose the heart to arrhythmias.

Reperfusion-induced arrhythmias are resistant to most antiarrhythmic drugs (Naito *et al.*, 1981). Vasodilator drugs by decreasing cardiac afterload and by improving ventricular performance may prevent ventricular fibrillation following spasm or restoration of blood flow, a phenomenon that appears to be involved in sudden death.

One of the most important physiological effects of bradykinin is vasodilatation. However, as reported by Xiang *et al.* (1985), a great difference in cardiodynamics was observed between species when bradykinin was perfused in isolated hearts. Thus, bradykinin causes an increase of contractility in guinea-pigs, a decrease in rats and no change in rabbits. While coronary flow increased markedly in rabbits and guinea-pigs, the increase was moderate in rats. In the present study, the vasodilator effect of bradykinin was rather modest and not statistically significant: no change in heart rate was noted.

Bradykinin can be metabolized by various peptidases (Ward, 1991). Kininase I is well known to generate the active metabolite Des-Arg^9-BK, which has been shown to act via B_1 receptors. Using a specific carboxyterminal RIA for bradykinin quantification, we could not recover in the perfusate effluent the initial concentration added to the fluid perfusion. The low recovery of intact bradykinin pleads for an important metabolism of bradykinin by the myocardium. Indeed, Des-Arg^9-BK has been shown to be quite active in preventing ventricular fibrillation and noradrenaline overflow. This supports the hypothesis that B_1 receptors may be involved in the cardioprotective effect of bradykinin. This interpretation is supported by the results with antagonists. Thus, Hoe 140, the specific antagonist for B_2 receptor, which has been shown to antagonize the pharmacological effects of bradykinin in different *in vitro* models (Hock *et al.*, 1991) failed to demonstrate any significant effect on the bradykinin-mediated cardioprotection. On the contrary, Lys [Leu^8] Des-Arg^9-BK the specific antagonist of B_1 receptors abolished the effects of Des-Arg^9-BK and partially those of bradykinin. These observations, together with the low levels of bradykinin measured in the perfusate output versus those added initially to the perfusion medium suggest that the pharmacological effects of bradykinin may in part be due to its conversion to Des-Arg^9-BK and activation of B_1 receptors; this latter being expressed during the ischaemic period as suggested by Marceau & Regoli (1991) who noticed an induction of B_1 receptors in pathological conditions such as anoxia.

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Nitric oxide modulates vascular permeability in the rat coronary circulation

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1 The objective of the present study was to assess whether inhibition of nitric oxide (NO) production could modulate vascular permeability in the coronary circulation in conscious rats.

2 Intravenous injection of N^G-nitro-L-arginine methyl ester (L-NAME, 2 mg kg^{-1}) resulted in a slowly developing hypertension and evoked twofold increases in vascular permeability in the left ventricle and right atrium as measured by the extravasation of Evans blue dye. Maintenance of mean arterial blood pressure at the level observed following L-NAME injection by infusion of noradrenaline (620–820 ng kg^{-1} min^{-1}) did not induce significant protein extravasation in the coronary circulation.

3 L-NAME treatment markedly enhanced (up to 490%) protein extravasation both in the left ventricle and right atrium in response to platelet-activating factor (PAF, 1.9 nmol kg^{-1}, i.v.) and endothelin-1 (1 nmol kg^{-1}, i.v.). Noradrenaline infusion potentiated (up to 69%) endothelin-1-induced protein extravasation. The permeability effect of PAF was only slightly enhanced by noradrenaline.

4 The present findings indicate that inhibition of endogenous NO synthesis leads to an increase in protein extravasation and to potentiation of the permeability effects of PAF and endothelin-1 in the coronary circulation. These results also suggest that NO may be an important regulator of vascular permeability under physiological and pathological conditions.

Keywords: Nitric oxide; platelet-activating factor; endothelin-1; N^G-nitro-L-arginine methyl ester; protein extravasation; ischaemia-reperfusion; coronary circulation; inflammation

Introduction

Early myocardial oedema is a characteristic feature of the inflammatory response associated with acute myocardial ischaemia, especially with reperfusion of ischaemic tissues (Steenbergen *et al.*, 1985; Tranum-Jensen *et al.*, 1987). Recent studies have shown that ischaemia-reperfusion results in elevated plasma concentrations of platelet-activating factor (PAF) (Filep *et al.*, 1989; Montrucchio *et al.*, 1989) and endothelin-1 (Yasuda *et al.*, 1990; Watanabe *et al.*, 1991). Among other mediators, both PAF and endothelin-1 have been implicated as contributors to oedema formation during ischaemia-reperfusion (Braquet *et al.*, 1989; Filep *et al.*, 1991).

Nitric oxide (NO) synthesized by vascular endothelial cells (Ignarro *et al.*, 1987; Palmer *et al.*, 1988) or certain blood-borne cells (Ignarro, 1989) is a potent vasodilator substance. Endothelium-derived NO appears to play a role in the regulation of vascular tone and blood pressure (Ignarro, 1989; Rees *et al.*, 1989) and in the control of regional blood flow (Gardiner *et al.*, 1990). However, the actions of NO extend beyond its vasodilator potential, as inhibition of platelet aggregation (Radomski *et al.*, 1987a) and adhesion of platelets and neutrophil granulocytes to endothelial cells have also been reported (Radomski *et al.*, 1987b; Kubes & Granger, 1992). The objectives of the present experiments were to study whether or not inhibition of NO synthesis by N^G-nitro-L-arginine methyl ester (L-NAME) (Rees *et al.*, 1990) could affect microvascular protein extravasation and to examine whether or not L-NAME could modify the permeability effect of PAF and endothelin-1 in the coronary circulation in conscious rats.

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Methods

The experiments were performed on conscious, chronically catheterized male Wistar rats (235–315 g). The animals were kept in individual metabolic cages and were prepared as described previously (Filep *et al.*, 1987). Briefly, under anaesthesia (ketamine, 75 mg kg^{-1} and sodium pentobarbitone, 15 mg kg^{-1}, i.p.) catheters were implanted into the abdominal aorta and vena cava through the central tail artery and left femoral vein, respectively. The venous catheter was led subcutaneously to the root of the tail. The catheters emerging from the tail were protected by an acrylic cuff connected to a stainless steel spiral and were fed through the top of the metabolic cage. The cuff was glued to the tail. The animals were allowed to recover completely for at least 4 days following the surgical procedure. During the experiments the animals could move freely and had free access to food and water. Mean arterial blood pressure was monitored continuously with an electromanometer using a Statham P23 dB pressure transducer. To measure protein extravasation Evans blue dye was used as a marker of vascular permeability.

Experimental protocols

On the day of the experiments, following an equilibration period of 2 h, PAF (1.9 nmol kg^{-1}), endothelin-1 (1 nmol kg^{-1}) or their vehicle was injected i.v. together with Evans blue dye (20 mg kg^{-1}, 25 mg ml^{-1} in 0.9% NaCl). These doses of PAF and endothelin-1 have previously been shown to enhance protein extravasation in selected vascular beds in rats (Filep *et al.*, 1991). Some animals were pretreated with L-NAME (2 mg kg^{-1}), an inhibitor of NO synthesis (Rees *et al.*, 1990), for 10 min before injection of PAF or endothelin-1. Preliminary experiments showed that increasing the dose of L-NAME did not cause further increase in arterial blood pressure. In some rats, mean arterial blood pressure was maintained at the level observed following L-NAME injection by the continuous i.v. infusion of noradrenaline (620–820

ng kg^{-1} min^{-1}, 6.9 μl min^{-1}) for 10 min before injection of PAF or endothelin-1. Ten min after the injection of PAF or endothelin-1, the animals were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.v.) and the heart was perfused with 40 ml of 0.9% NaCl through a catheter inserted into the aorta. Portions of the anterior wall of the left ventricle and the right atrium were excised and weighed. Approximately half of each tissue was put in formamide (4 ml per g wet weight tissue at 20°C for 24 h), while the other half was dried at 60°C for 24 h. The amount of Evans blue extracted in formamide was determined by spectrophotometry. Evans blue content of each sample was expressed as μg dye per g dry weight of tissue to avoid underestimation of changes due to plasma fluid extravasation.

Materials

Endothelin-1 was a gift from Dr A. Fournier (INRS-Santé, Montreal, Canada). PAF (1-O-hexadecyl-2-O-acetyl-sn-glycero-3-phosphorylcholine) was purchased from Bachem, Bubendorf, Switzerland; L-NAME, noradrenaline hydrochloride and Evans blue dye were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All chemicals were dissolved in 0.9% NaCl immediately before the experiments.

Statistics

Results are expressed as means ± s.e.mean. Statistical analysis of the data were performed by Dunn's multiple contrast hypothesis test (Dunn, 1964) and by Mann-Whitney U test. A $P<0.05$ level was considered significant for all tests.

Results

Bolus i.v. injection of endothelin-1 (1 nmol kg^{-1}) increased myocardial Evans blue content on average by 84 and 123% in the left ventricle and right atrium, respectively, whereas PAF (1.9 nmol kg^{-1}) caused slight, statistically non-significant increases in both tissues (Figures 1, 2). Intravenous administration of L-NAME (2 mg kg^{-1}) resulted in a slowly developing sustained hypertension, with the maximal change occurring between 6 and 8 min after injection (Figure 3). Thereafter mean arterial blood pressure remained stable for the next 20 min. L-NAME produced an increase of 108 and 89% in Evans blue content in the left ventricle (Figure 1) and right atrium (Figure 2), respectively. Maintenance of blood pressure at the level observed following L-NAME injection by infusion of noradrenaline (620–820 ng kg^{-1} min^{-1}) did not affect protein extravasation significantly (Figures 1, 2). The time course of changes in mean arterial blood pressure in response to PAF and endothelin-1 was similar in animals receiving L-NAME or noradrenaline, except that L-NAME slightly attenuated the magnitude and duration of the depressor effect of endothelin-1 (36 ± 2 mmHg vs. 48 ± 4 mmHg decrease in L-NAME and noradrenaline-treated animals, respectively, $n=6$, $P<0.05$). The maximum depressor responses to PAF were 80 ± 3 mmHg and 78 ± 3 mmHg in L-NAME and noradrenaline-treated animals, respectively ($n=6$, $P>0.1$).

L-NAME treatment markedly potentiated the permeability effect of both PAF and endothelin-1, resulting in 198 and 490% increases in tissue Evans blue content in the left ventricle, respectively (Figure 1). Similarily, tissue Evans blue content in the right atrium increased on average by 160 and 373% in L-NAME-treated animals in response to PAF and endothelin-1, respectively (Figure 2). Noradrenaline infusion enhanced PAF-induced protein extravasation by 30 and 11% in the left ventricle and right atrium, respectively (Figures 1, 2). These changes were, however, statistically not significant. On the other hand, noradrenaline infusion significantly potentiated the permeability effects of endothelin-1 both in the ventricle and atrium, as evidenced by the 69 and 24%

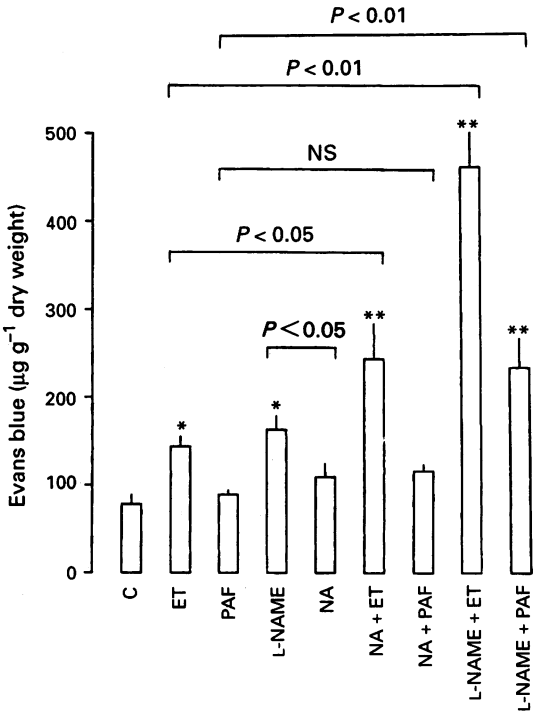

Figure 1 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) on protein extravasation in the left ventricle of conscious rats. The animals were pretreated with L-NAME (2 mg kg^{-1}), noradrenaline (NA, 620–820 ng kg^{-1} min^{-1}) or 0.9% NaCl (control, C) for 10 min before i.v. bolus injection of endothelin-1 (ET, 1 nmol kg^{-1}) or PAF (1.9 nmol kg^{-1}) plus Evans blue dye (20 mg kg^{-1}). The rats were killed 10 min after injection of endothelin-1 or PAF. Values are means of 6 experiments with s.e.mean shown by vertical lines. *$P<0.05$; **$P<0.01$ (compared to control by Dunn's multiple contrast hypothesis test).

increases in tissue Evans blue content, respectively (Figures 1, 2). Combined administration of L-NAME and PAF evoked 2 and 2.3 fold higher increases in protein extravasation in the left ventricle and right atrium, respectively, than those detected following PAF injection during noradrenaline infusion ($n=6$, $P<0.01$). Similarly, tissue Evans blue content in the left ventricle and right atrium were 1.9 and 1.7 times higher, respectively, in response to endothelin-1 in L-NAME-treated animals than those evoked by endothelin-1 in animals receiving noradrenaline infusion ($n=6$, $P<0.01$).

Discussion

The present results indicate that inhibition of NO synthesis increases microvascular protein extravasation and potentiates the permeability effect of PAF and endothelin-1 in the rat coronary circulation. Furthermore, L-NAME did not modify the depressor effect of PAF, whereas it slightly attenuated both the magnitude and duration of the depressor response to endothelin-1. Although we have not measured *in vivo* NO formation, it might be expected that with the supramaximal dose of L-NAME used, NO synthase would be completely blocked. Thus, it seems likely that the depressor effect of PAF and a major part of the depressor action of endothelin-1 may occur independently of NO formation.

Inhibition of NO formation with L-NAME resulted in about twofold increases in protein extravasation both in the left ventricle and right atrium. It seems unlikely that an increase in perfusion pressure (Humphries *et al.*, 1991), and consequently in capillary hydrostatic pressure could account for the increased protein extravasation, since elevation of mean arterial blood pressure by infusion of noradrenaline did

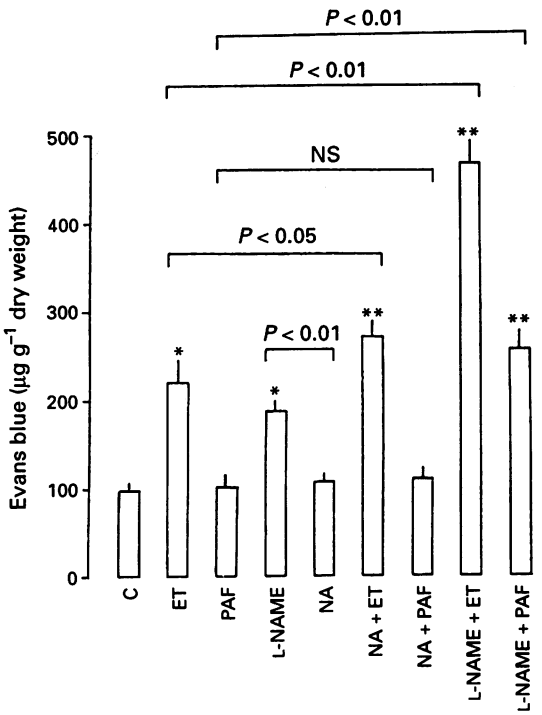

Figure 2 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) on protein extravasation in the right atrium of conscious rats. The animals were pretreated with L-NAME (2 mg kg^{-1}), noradrenaline (NA, 620–820 ng kg^{-1} min^{-1}) or 0.9% NaCl (control, C) for 10 min before i.v. bolus injection of endothelin-1 (ET, 1 nmol kg^{-1}) or PAF (1.9 nmol kg^{-1}) plus Evans blue dye (20 mg kg^{-1}). The rats were killed 10 min after injection of endothelin-1 or PAF. Values are means of 6 experiments with s.e.mean shown by vertical lines. $*P<0.05$; $**P<0.01$ (compared to control by Dunn's multiple contrast hypothesis test).

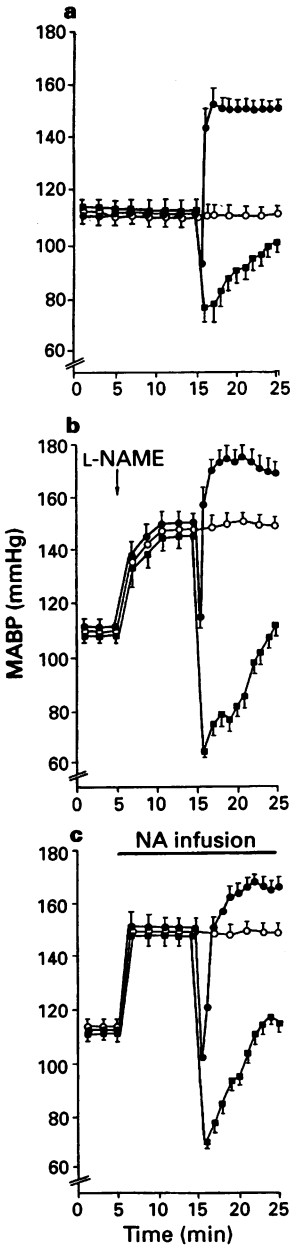

Figure 3 Blood pressure responses to endothelin-1 and PAF in untreated conscious rats (a), following N^G-nitro-L-arginine methyl ester (L-NAME) administration (b) and during infusion of noradrenaline (c). L-NAME (2 mg kg^{-1}) was injected i.v. or infusion of noradrenaline (NA, 620–820 ng kg^{-1} min^{-1}) was started at 5 min. Endothelin-1 (1 nmol kg^{-1}) (●), PAF (1.9 nmol kg^{-1}) (■) or 0.9% NaCl (○) were injected i.v. at 15 min in a volume of 4 μl per 100 g body weight. Values are means of 6 experiments with s.e.mean shown by vertical lines.

not mimic the effects of L-NAME on vascular permeability. Furthermore, L-NAME has recently been reported to decrease capillary hydrostatic pressure in the cat mesenteric circulation (Kubes & Granger, 1992). The enhanced protein extravasation by L-NAME cannot probably be attributed to an increase in capillary surface area for protein filtration since vasoconstrictors like L-NAME generally cause derecruitment of capillaries (Granger *et al.*, 1989). Another possibility might be that NO can preserve endothelial function. Indeed, inhibition of NO synthesis promotes adhesion of platelets and neutrophil granulocytes to the endothelium (Radomski *et al.*, 1987a,b; Kubes & Granger, 1992), an event that is known to induce endothelial dysfunction (Lefer *et al.*, 1991). Since the continuous release of NO may serve to scavange small amounts of superoxide released by endothelial cells, inhibition of NO synthesis might result in accumulation of superoxide radicals (Kubes & Granger, 1992), which, in turn, can enhance microvascular permeability (Del Maestro *et al.*, 1981).

Mediator-stimulated increase in protein extravasation is thought to be attributable primarily to induction of inter-endothelial cell gap formation exclusively in the venules (Bjork & Smedegard, 1983; Grega *et al.*, 1986). This leads to opening of the variable large-pore system, the dominant macromolecular transport pathway operant in inflammation (Grega *et al.*, 1986). Although activation of this transport pathway appears to be independent of haemodynamic changes, elevation of microvascular hydrostatic pressure can promote protein filtration if large pores are open (Grega *et al.*, 1986). The present findings with noradrenaline infusion are consistent with this hypothesis. By inducing capillary venoconstriction, noradrenaline is capable of increasing capillary hydrostatic pressure. However, this alone would not lead to enhanced protein filtration, as was confirmed in the present study. On the other hand, the increased capillary hydrostatic pressure might have potentiated endothelin-1-induced protein extravasation. A similar tendency was observed when PAF and noradrenaline administration was combined. However, these changes were not statistically significant probably due to the weak effect of PAF on protein extravasation in the coronary circulation and the relatively low number of observations.

The present study also showed that L-NAME markedly potentiated protein extravasation evoked by PAF and endothelin-1. Furthermore, Evans blue dye contents in the left ventricle and right atrium were approximately twofold higher when these mediators were injected in the presence of L-

NAME than in the presence of noradrenaline despite the fact that L-NAME and noradrenaline evoked similar changes in mean arterial blood pressure. Thus, it seems unlikely that the potentiating effect of L-NAME could be attributed to an increase in perfusion pressure. Since unlike noradrenaline, L-NAME has been reported to decrease rather than increase capillary hydrostatic pressure (Kubes & Granger, 1992), one may assume that L-NAME and noradrenaline do not enhance the permeability response to PAF and endothelin-1 through common mechanisms. Indeed, the potentiating effect of L-NAME could be explained by endothelial dysfunction secondary to inhibition of NO formation (see above). The findings that NO donors can decrease myocardial necrosis and attenuate endothelial dysfunction in the early phase of myocardial ischaemia-reperfusion (Siegfried *et al.*, 1992) lend further support to this hypothesis.

In conclusion, the present data demonstrate that inhibition of endogenous NO synthesis results in an increase in protein extravasation and potentiation of the permeability effects of PAF and endothelin-1 and suggest an important role for NO in regulating vascular permeability in the coronary circulation under physiological and pathological conditions.

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Dose-dependent effects of angiotensin converting enzyme (ACE) inhibitors on glomerular prostanoid production by normotensive rats

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1 This study was designed to investigate whether the angiotensin converting enzyme (ACE) inhibitors, captopril, enalapril and fosinopril have a dose-dependent effect on the production of prostaglandin E_2 (PGE_2), prostaglandin I_2 (prostacyclin, PGI_2) and thromboxane A_2 (TxA_2) by glomeruli isolated from normotensive Wistar-Kyoto rats.

2 Measurements of glomerular prostanoid production were made under basal conditions and in the presence of excess exogenous arachidonic acid.

3 All three ACE inhibitors demonstrated dose-dependent effects upon glomerular prostanoid production which varied with the individual ACE inhibitor.

4 Enalapril induced a dose-dependent increase in the ratio of $(PGE_2 + PGI_2)/TxA_2$, from 2.17 ± 0.20 to 5.35 ± 0.84 and to 10.0 ± 1.16 with the low and high doses of enalapril respectively. In contrast, the high dose of captopril tended to reduce the ratio when compared to the low dose.

5 The results obtained in this study suggest that although all three ACE inhibitors appear to induce prostacyclin synthetase and/or modulate phospholipase A_2 (PLA_2) activity, these effects differ with the ACE inhibitor studied and the dose employed.

6 This study has demonstrated dose-dependent effects of three ACE inhibitors on glomerular prostanoid production which may be significant in modulating glomerular haemodynamics and growth characteristics of glomerular cells.

Keywords: Angiotensin converting enzyme inhibitors; isolated glomeruli; prostanoids; angiotensin converting enzyme

Introduction

Recent studies in this laboratory have demonstrated that certain angiotensin converting enzyme (ACE) inhibitors which differed in their molecular structure could modulate prostanoid production by glomeruli from both normotensive (WKY) and spontaneously hypertensive rats (SHR) (Harding *et al.*, 1991). Their effects on prostaglandin E_2 (PGE_2), PGI_2 and thromboxane A_2 (TxA_2) differed with the nature of the ACE inhibitor, but were independent of any effect on blood pressure and unrelated to the presence of a sulphydryl group in the molecular structure. It was suggested that their differing effects on glomerular prostanoid synthesis might be the result of variations in the glomerular concentration or bioavailability of each compound and that this might also explain the many discrepancies recently reported relating to the ability of different ACE inhibitors to modify the severity of injury in certain forms of experimentally-induced models of glomerulonephritis (Anderson *et al.*, 1986; Ohishi *et al.*, 1989). It was also felt that a similar explanation could account for the findings of Ikoma *et al.* (1991), in that the protection offered by the ACE inhibitor, enalapril, in an experimental model of glomerulonephritis was dose-related/dependent.

Although the mechanisms responsible for the effects of ACE inhibitors on glomerular function and morphology are not clearly understood, it is thought that they may be mediated through their influence on glomerular angiotensin II (AII) generation (Lo *et al.*, 1990), kinin degradation and/or prostanoid production (Galler *et al.*, 1982); since these compounds play a central role in determining changes in the biological activities of glomerular cells and certain aspects of glomerular function, particularly those concerned with the maintenance of glomerular haemodynamics (Stork & Dunn, 1985; Nath *et al.*, 1987) and cell growth (Menè & Dunn, 1990; Paquet *et al.*, 1990). In this respect, it could be of relevance that the three ACE inhibitors previously studied all increased the ratio of $(PGE_2 + PGI_2)/TxA_2$, i.e. an increase in those prostanoids which are responsible for maintaining glomerular perfusion as opposed to those which compromise glomerular haemodynamics and also, those that inhibit cell growth compared with those prostanoids that have mitogenic properties.

Since initial experiments performed in this laboratory had demonstrated that doses of $60\ mg\ kg^{-1}\ day^{-1}$ captopril, $15\ mg\ kg^{-1}\ day^{-1}$ enalapril and $60\ mg\ kg^{-1}\ day^{-1}$ fosinopril gave equivalent reductions in the blood pressure of SHR but produced differing effects upon glomerular prostanoid production (Harding *et al.*, 1991); the present study was designed to establish whether the effects of ACE inhibitors on glomerular prostanoid production might be dose-dependent as this would have important therapeutic implications as suggested by Ikoma *et al.* (1991). The influence of both 15 and $60\ mg\ kg^{-1}\ day^{-1}$ doses of each ACE inhibitor on glomerular prostanoid production was therefore investigated.

Methods

Animals studied

Adult, 15 week-old, male WKY (Charles River, Margate, Kent) were studied. They were maintained on a diet containing 16.6% protein, 0.5% sodium and 0.7% potassium (Pilsbury, Birmingham), had free access to tap water and were housed in standard cages at a temperature of 20°C.

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Protocol

Rats were randomly allocated into 7 groups, each containing 15 animals. Groups 1 and 2 received 15 or 60 mg kg^{-1} day^{-1} captopril, groups 3 and 4, 15 or 60 mg kg^{-1} day^{-1} enalapril and groups 5 and 6, 15 or 60 mg kg^{-1} day^{-1} fosinopril respectively whereas Group 7 received water alone i.e. control animals. The ACE inhibitors were adminstered in the drinking water for 10 days and water consumption measured on a daily basis, with appropriate adjustments to ensure that the dosage of ACE inhibitor remained constant. Systolic blood pressure was measured by the indirect tail-cuff method on the day before the start of treatment with the ACE inhibitor and on day 10 of treatment (Pfeffer *et al.*, 1971).

Preparation of isolated glomeruli

Preparations of isolated glomeruli in Krebs-Henseleit buffer, pH 7.4 were obtained by a mechanical sieving technique with glomeruli collected from the surface of a 75 μm sieve (Fond & Drummond, 1968). The purity of the glomerular suspension was assessed by light microscopy, with suspensions of >85% purity being used for the experiments. Glomerular protein content was determined by the method of Lowry *et al.* (1951) after digestion of the glomeruli with NaOH 2 mol l^{-1}.

Glomerular prostanoid production

Glomerular prostanoid production was determined by modifications of previously described methods (Schambelan *et al.*, 1985; Lefkowith & Schreiner, 1987). Briefly, 0.9 ml portions of the glomerular suspension were incubated either alone or in the presence of 15 μmol l^{-1} arachidonic acid (Sigma) for 0 and 10 min at 37°C to provide basal production and production in the presence of excess exogenous arachidonic acid. Reactions were terminated by the addition of 50 μl of 2.8 mmol l^{-1} indomethacin followed by centrifugation at 1500 *g* for 3 min (4°C). The supernatants were then stored at −20°C before radioimmunoassay.

PGE_2, PGI_2 (assayed as the stable metabolite 6-keto $PGF_{1\alpha}$) and TxA_2 (assayed as the stable metabolite TxB_2) were determined in duplicate with radioimmunoassay kits (New England Nuclear, Boston, MA, U.S.A.). The cross-reactivities for the antisera are claimed by New England Nuclear to be as follows: PGE_2 antiserum to $PGF_{2\alpha}$, <0.01%; to 6-keto $PGF_{1\alpha}$, <0.01% and to TxB_2, 0.02%. The TxB_2 antiserum to $PGF_{2\alpha}$, 0.04%; to 6-keto $PGF_{1\alpha}$, 0.01% and to PGE_2, 0.003%. The 6-keto $PGF_{1\alpha}$ antiserum to $PGF_{2\alpha}$, 0.6%; to TxB_2, 0.4% to PGE_2, 0.4%. The results are expressed as ng of prostanoid produced mg^{-1} glomerular protein $10\ min^{-1}$.

Statistical analysis

Data are given as means ± s.e.mean. The results were subjected to statistical analysis to determine whether the data were normally distributed. The significance of differences between the untreated (control) and experimental groups was tested by a one-way analysis of variance followed by Dunnett's test for multiple comparisons. A P value of <0.05 was considered significant.

Results

Blood pressure (Table 1)

The ACE inhibitors had no influence on systolic blood pressure in either control animals (Group 7) or in Groups 1, 2, 3, 5 and 6. There was, however, a significant reduction in blood pressure from 137.8 ± 5.4 to 119.6 ± 5.1 mmHg, $P<0.05$ in Group 4, i.e. rats receiving a high dose of enalapril.

Glomerular prostanoid production (Figure 1 and Table 2)

In the following section, all P values refer to differences between untreated and treated animals.

Captopril Under basal conditions, neither high nor low doses of captopril had any effect upon either glomerular PGE_2 or TxA_2 production whereas PGI_2 production was increased by the low dose captopril ($P<0.005$).

Similarly, in the presence of arachidonic acid, only the low dose of captopril had any effect upon glomerular prostanoid production, affecting an increase in PGI_2 ($P<0.005$).

Enalapril Treatment with low dose enalapril decreased the basal production of all three prostanoids ($P<0.05$ for PGE_2, $P<0.01$ for PGI_2 and $P<0.005$ for TxA_2). In contrast the high dose affected an increase in the basal production of PGI_2 ($P<0.005$). A decrease in glomerular TxA_2 production was also observed following a high dose of enalapril ($P<0.05$).

In the presence of arachidonic acid, low dose enalapril reduced the production of TxA_2 ($P<0.05$) and a high dose of enalapril increased PGI_2 production ($P<0.005$).

Fosinopril Treatment with low dose fosinopril affected an increase in the basal production of PGI_2 ($P<0.01$) and a decrease in TxA_2 ($P<0.05$). The high dose had a similar effect upon PGI_2 production ($P<0.005$) and, in addition, affected an increase in PGE_2 production ($P<0.01$).

In the presence of arachidonic acid, neither the high nor the low dose of fosinopril had any effect upon glomerular TxA_2 production. The low dose increased the production of PGI_2 ($P<0.05$) whereas the high dose increased PGE_2 ($P<0.01$).

The ratio of $(PGE_2+PGI_2)/TxA_2$ (Figure 2)

Since a change in the ratio of vasodilator to vasoconstrictor prostanoids and those prostanoids with the potential to limit cell proliferation as opposed to those concerned with promoting mitogenesis may have more functional significance than a change in the absolute value of any individual prostanoid, the effect of the three ACE inhibitors, upon the ratio of

Table 1 The effect of low and high doses of captopril, enalapril and fosinopril on mean systolic blood pressure

	Mean systolic blood pressure (mmHg)			
	15 mg kg^{-1} day^{-1}		60 mg kg^{-1} day^{-1}	
	Before	*After*	*Before*	*After*
Control	129.0 ± 4.0	126.2 ± 4.1	129.0 ± 4.0	126.2 ± 4.1
Captopril	122.2 ± 5.2	116.6 ± 6.9	128.5 ± 2.7	121.6 ± 2.1
Enalapril	128.0 ± 3.0	124.1 ± 3.1	137.8 ± 5.4	119.6 ± 5.1*
Fosinopril	116.2 ± 4.8	123.9 ± 6.4	129.1 ± 2.7	127.1 ± 4.0

Values are means ± standard error of the mean ($n = 15$) for each group. Animals received either 15 or 60 mg kg^{-1} day^{-1} captopril, enalapril, or fosinopril for 10 days. Untreated animals (control) received water alone.
Statistical significance: *$P<0.05$ compared to values before treatment.

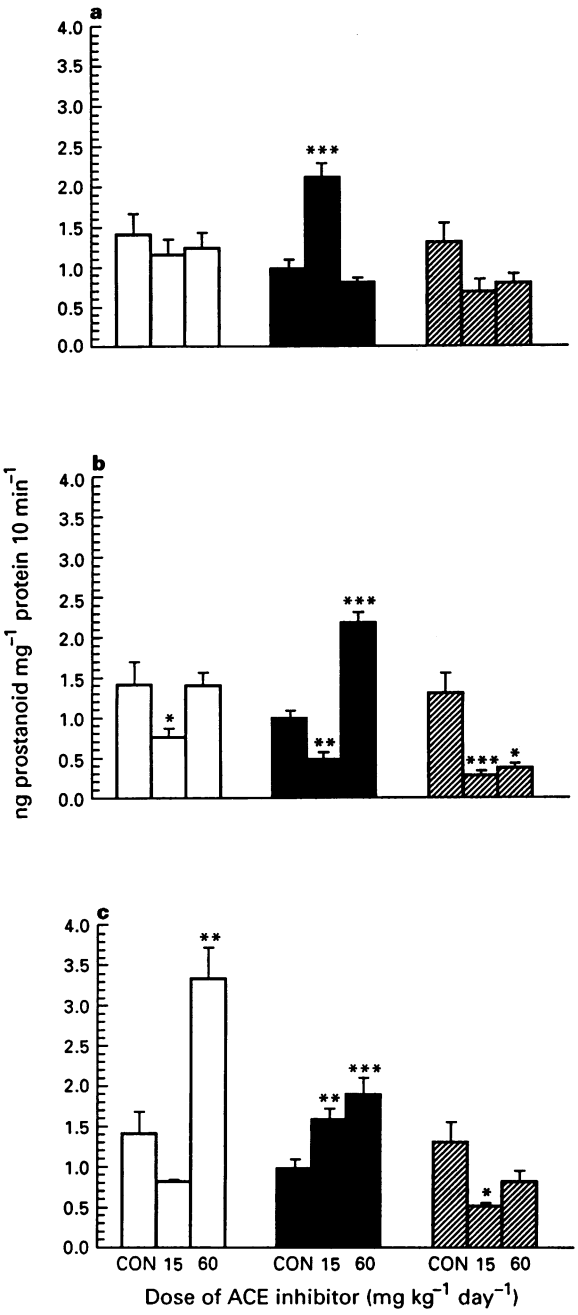

Figure 1 Prostaglandin E_2 (PGE_2, open columns), prostacyclin (PGI_2, solid columns; assayed as 6-keto $PGF_{1\alpha}$) and thromboxane A_2 (TxA_2, hatched columns; assayed as TxB_2) production by glomeruli from WKY rats after treatment with either 15 or 60 mg kg^{-1} day^{-1} captopril (a), enalapril (b) or fosinopril (c) for 10 days. Control (Con) animals received water alone. Results are means ± s.e.mean (vertical bars). Statistical significance: *$P<0.05$; **$P<0.01$; ***$P<0.005$ compared with respective values in control animals.

($PGE_2 + PGI_2$)/TxA_2 has been determined. Low doses of all three ACE inhibitors increased the ratio of ($PGE_2 + PGI_2$)/TxA_2 ($P<0.01$ for captopril, $P<0.005$ for enalapril and $P<0.001$ for fosinopril). Although further increases in this ratio were noted with high doses of enalapril and fosinopril, this change achieved statistical significance only for enalapril ($P<0.01$). However, there was no difference between the value of the ratio for high doses of enalapril and fosinopril.

Discussion

This study has demonstrated dose-dependent effects of three structurally dissimilar ACE inhibitors upon glomerular prostanoid production in normotensive Wistar Kyoto rats which vary for each ACE inhibitor. At low doses, captopril and fosinopril stimulated the basal production of PGI_2. All three prostanoids were reduced by low dose enalapril whereas fosinopril also reduced TxA_2. In contrast, the high dose of enalapril caused an increase in the basal production of PGI_2 and fosinopril caused an increase in both PGE_2 and PGI_2. The high dose of captopril had no effect on glomerular prostanoid production.

Although it is appreciated that the preparation of isolated glomeruli by the sieving technique employed in this study may stimulate some prostanoid production, this would be

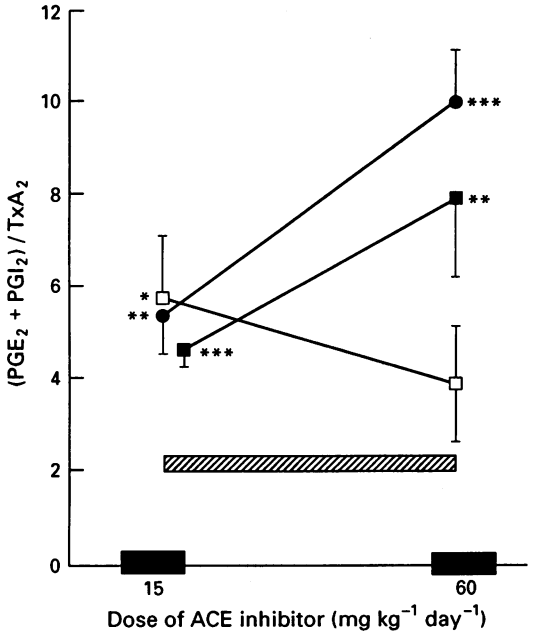

Figure 2 The effect of treatment with either 15 or 60 mg kg^{-1} day^{-1} captopril (□), enalapril (●) or fosinopril (■) for 10 days on the basal ratio of ($PGE_2 + PGI_2$)/TxA_2. Hatched bar represents the basal ratio in untreated animals. Results are means ± s.e.mean (vertical bars). Statistical significance: *$P<0.01$; **$P<0.005$ and ***$P<0.001$ compared to values in untreated animals.

Table 2 The effects of captopril, enalapril and fosinopril on glomerular prostanoid production in the presence of excess arachidonic acid

	PGE_2		*PGI_2*		*TxA_2*	
	15 mg kg^{-1}	60 mg kg^{-1}	15 mg kg^{-1}	60 mg kg^{-1}	15 mg kg^{-1}	60 mg kg^{-1}
Con	4.46 ± 1.02	4.46 ± 1.02	1.31 ± 0.19	1.31 ± 0.19	0.81 ± 0.18	0.81 ± 0.18
Cap	4.14 ± 0.31	5.16 ± 0.43	2.78 ± 0.20***	1.04 ± 0.15	1.15 ± 0.23	0.66 ± 0.12
Ena	4.23 ± 0.63	4.33 ± 0.58	1.36 ± 0.28	2.74 ± 0.34***	0.33 ± 0.09*	0.36 ± 0.06
Fos	3.28 ± 0.11	8.82 ± 0.82**	2.20 ± 0.23*	1.75 ± 0.18	0.81 ± 0.14	0.42 ± 0.15

Values represent mean prostanoid production ± s.e.mean (ng prostanoid mg^{-1} glomerular protein 10 min^{-1}) in the presence of 15 μmol l^{-1} arachidonic acid by glomeruli isolated from rats treated with either 15 or 60 mg kg^{-1} day^{-1} of captopril (Cap), enalapril (Ena) or fosinopril (Fos) for 10 days. Con are control animals which received only water.
Statistical significance: *$P<0.05$, **$P<0.01$ and ***$P<0.005$ compared to values in untreated animals.

likely to affect both untreated (control) and treated groups of rats and would therefore be taken into account in assessing any influence of an ACE inhibitor on glomerular prostanoid production. The alternative of measuring urinary prostanoid production is known to be a poor reflection of glomerular production because it relates to total renal production and to some prostanoid production from extra-renal sites. In addition, studies performed in this laboratory have shown that the *in vitro* incubation of isolated glomeruli with various concentrations of ACE inhibitors has no effect on glomerular prostanoid production.

Although there are no previous reports of studies designed to examine the influence of different doses of ACE inhibitors *in vivo* on prostanoid production, the present findings complement those reported by Nakagawa *et al.* (1987) in which a dose-dependent inhibitory effect of captopril on prostacyclin generation by cultured human vascular endothelial cells was demonstrated.

In the present study, untreated animals demonstrated a profile of basal glomerular prostanoid production consistent with that described by us and other investigators ($PGE_2 > TxA_2 = PGI_2$) (Folkert & Schlondorff, 1979; Schlondorff *et al.*, 1980). The addition of arachidonic acid to isolated glomeruli produced a greater increase in PGE_2 production than PGI_2, suggesting that endoperoxide E-isomerase has a greater affinity for PGH_2 than prostacyclin synthetase. In contrast, a decrease in thromboxane production was noted in the presence of excess arachidonic acid. Whilst an increase might have been anticipated, the reduced thromboxane suggests that thromboxane synthetase is substrate inhibited: a finding in agreement with the work described by Uehera *et al.* (1988) in studies using rat vascular smooth muscle cells.

The present findings suggest the induction of prostacyclin synthetase by all three ACE inhibitors under both basal conditions and in the presence of excess arachidonic acid, such that PGH_2 was preferentially metabolized to PGI_2 rather than PGE_2. The fact that low doses of captopril and fosinopril increased PGI_2 production under both assay conditions without any change in PGE_2 production also suggests that they increase prostacyclin synthetase activity rather than having an effect on phospholipase A_2 (PLA_2). In contrast, low dose enalapril reduced the synthesis of all three prostanoids under basal conditions, presumably by an inhibition of PLA_2. At the high dose, enalapril affected an increase in PGI_2 synthesis without any apparent effect on PLA_2 as the total sum of basal prostanoid production remained constant. This effect contrasts with the apparent stimulation of PLA_2 activity induced by high dose fosinopril. On the available evidence, one cannot exclude the possibility that some changes in glomerular prostanoid production might have resulted from an inhibition of one enzyme with the subsequent redirection of prostanoid synthesis.

All three ACE inhibitors produced increases in the ratio of $(PGE_2 + PGI_2)/TxA_2$. Although the maximum effects induced by enalapril and fosinopril were similar, only enalapril exhibited a dose-dependent effect. Whilst it is appreciated that the influence of ACE inhibitors on prostanoid production may differ between epithelial, mesangial and endothelial cells of the glomerulus, one could nevertheless speculate that an increase in the ratio of $(PGE_2 + PGI_2)/TxA_2$ may have beneficial influences on glomerular haemodynamics, cell growth and mesangial matrix expansion; all of which are considered to be important in the pathogenesis of progressive glomerular injury. Indeed, one might suggest that changes in glomerular prostanoid production induced by enalapril provide a possible explanation for the observations of Ikoma *et al.* (1991), that this ACE inhibitor limits experimentally-induced glomerular injury in a dose-dependent manner.

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Time-dependent blockade of neurogenic plasma extravasation in dura mater by 5-$HT_{1B/D}$ agonists and endopeptidase 24.11

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1 The delayed effects of stimulating the trigeminal ganglion unilaterally in rats and guinea-pigs were assessed by measuring the leakage of radiolabelled albumin from blood into the dura mater at intervals for up to 120 min after a 5 min stimulation period (5 Hz, 0.6 mA, 5 ms).

2 [^{125}I]-albumin (50 μCi kg^{-1}) was injected i.v. as a tracer 0, 20, 50, 80 or 110 min after stimulation and the animals were killed 10 min later. Extravasation of plasma protein developed for up to 90 min poststimulation.

3 To examine the mechanism underlying delayed plasma protein extravasation, CP-93,129 (5-HT_{1B} receptor agonist, 460 nmol kg^{-1}), sumatriptan (5-$HT_{1B/D}$ receptor agonist, 24 nmol kg^{-1}), or neutral endopeptidase 24.11 (1 nmol kg^{-1}) were administered 45 or 75 min after trigeminal stimulation and 5 min before radiolabelled albumin. The extravasation response was reduced at 45 min. CP-23,129 also blocked extravasation when injected 25 min after capsaicin administration (1 μmol kg^{-1}).

4 If the tracer was injected 5 min prior to electrical trigeminal stimulation, endopeptidase 24.11 (1 nmol kg^{-1}) given 10 min before stimulation blocked the leakage (as reported previously for CP-93,129 or sumatriptan). All three compounds blocked the leakage when administered 30 min (but not 60 min) poststimulation in this paradigm.

5 The data support the previously made contention that neuropeptide release from sensory fibres mediates the plasma extravasation response following trigeminal ganglion stimulation, and that release and plasma leakage continue many minutes beyond the stimulation period. Hence, drugs that inhibit neuropeptide release (CP-93,129, sumatriptan), or enhance breakdown of neuropeptide mediators (endopeptidase 24.11) block the delayed extravasation response. Extravasation developing later than 45 min poststimulation was not neurogenically mediated.

Keywords: CP-93,129; sumatriptan; endopeptidase 24.11; 5-HT_{1B} receptors; 5-HT_{1D} receptors; migraine; dura mater; neurogenic inflammation.

Introduction

High intensity electrical trigeminal ganglion stimulation is accompanied by leakage of albumin (Saito *et al.*, 1988; Buzzi & Moskowitz, 1990) and horseradish peroxidase from postcapillary venules within the dura mater (Dimitriadou *et al.*, 1991), and these changes are mediated by neuropeptide release from perivascular sensory fibres (Buzzi *et al.*, 1991a). Leakage is evident immediately after stimulation and is accompanied by the formation of endothelial pinocytotic vesicles and vacuoles, and platelet aggregates. Mast cells in the vicinity of vessels exhibit morphological changes consistent with activation and secretion. Fifteen minutes after stimulation, mast cells demonstrate frank degranulation. Overall, the histological appearance suggests an evolving sterile inflammatory response (Dimitriadou *et al.*, 1991; 1992).

Neurogenic inflammation (NI) within the meninges has been suggested as a model to explain the pharmacological mechanisms in migraine and vascular headaches (Moskowitz, 1992). It was postulated that NI contributes to the sensitization of polymodal nociceptors, the development of hyperalgesia and the prolongation of pain in the clinical condition (Moskowitz, 1991). Sumatriptan, a 5-$HT_{1B/D}$ receptor agonist useful for treating acute migraine headaches (Buzzi & Moskowitz, 1990), and CP-93,129, a selective agonist at the 5-HT_{1B} receptor, respectively blocks neurogenic plasma leakage selectively in dura mater (Matsubara *et al.*, 1991). As a possible drug mechanism, blockade of neuropeptide release was proposed (Saito *et al.*, 1988; Buzzi *et al.*, 1991b) which is consistent with the ability of sumatriptan and dihydroergotamine to inhibit the accompanying ultrastructural changes within postcapillary venules and mast cells (Buzzi *et al.*, 1992). Because sumatriptan is effective clinically when given before as well as after the onset of headache, and tissue ultrastructure suggests an evolving histopathological process, we examined the time-dependent changes in plasma leakage that develops following electrical trigeminal stimulation and the effects of drugs which either block neuropeptide release or promote degradation of released neuropeptides. Hence, sumatriptan, CP-93,129 (inhibitors of neuropeptide release), and the recombinant form of the ectoenzyme, endopeptidase 24.11 (which cleaves peptides at the amino group of hydrophobobic residues) were examined in this model.

Methods

Electrical trigeminal ganglion stimulation (see Markowitz *et al.*, 1987; Matsubara *et al.*, 1991)

Male Sprague-Dawley rats and male Hartley guinea-pigs (200–250 g) (Charles River Laboratories, Wilmington, MA, U.S.A.) were kept under diurnal lighting conditions and allowed food and water *ad libitum*. Anaesthetized animals [pentobarbitone; 50 or 40 mg kg^{-1}, i.p., in rats or guinea-pigs, respectively], were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) with the incisor bar set at −1.5 mm (rats) or −4.0 mm (guinea-pigs). Symmetrical burr holes were drilled 3.1 mm laterally and 3.7 mm posteriorly from bregma in rats or 4.2 mm laterally and 4.0 mm poster-

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iorly in guinea-pigs. Bipolar electrodes (5 mm shaft, Rhodes Medical Instruments, Woodland Hills, CA) were lowered 9.5 mm (rats) or 10.5 mm (guinea-pigs) from the dura mater. The right ganglion was stimulated (5 min, 0.6 mA, 5 Hz, 5 ms duration) (Pulsemaster A300, Stimulus Isolator A365, World Precision Instruments, San Carlos, CA; Hitachi Densi Oscilloscope, Tokyo, Japan). Animals were perfused with saline via the left cardiac ventricle for 2 (rats) or 3 min (guinea-pigs) at constant pressure (100 mmHg). The dura mater was then dissected bilaterally as previously described and radioactivity determined and compared on the two sides (Micromedic Systems Inc., Huntsville, AL, U.S.A.).

Rats (200–250g) were used for all experiments except that guinea-pigs were used for the sumatriptan experiments. Experimental protocols were as follows.

(1) Time course of plasma protein extravasation following electrical trigeminal stimulation

To examine whether plasma proteins leak during the post stimulation period, [^{125}I]-BSA (50 μCi kg^{-1}) was injected via the left femoral vein 0, 20, 50, 80 or 110 min after electrical stimulation in anaesthetized animals. Supplementary pentobarbitone (20 mg kg^{-1}, i.p.) was injected every 60 min to maintain anaesthesia. Ten minutes after [^{125}I]-BSA injection, animals were perfused, the dura mater was dissected and radioactivity counted and compared on the two sides.

To examine the leakage of plasma proteins during trigeminal ganglion stimulation, [^{125}I]-BSA was injected 5 min before electrical stimulation via the left femoral vein. Rats were perfused immediately after stimulation or 30, 60, 90, 120 or 180 min later. Supplementary anaesthesia (pentobarbitone, 20 mg kg^{-1}, i.p.) was administered every 60 min. The dura mater was dissected and radioactivity counted and compared on the two sides.

(2) Drug effects on the delayed plasma extravasation

Tracer and drug administered after trigeminal ganglion stimulation:

CP-93,129 (460 nmol kg^{-1}), sumatriptan (24 nmol kg^{-1}), or endopeptidase 24.11 (1 nmol kg^{-1}) were administered 45 or 75 min after electrical stimulation and 5 min before [^{125}I]-BSA injection to anesthetized animals. Ten min after [^{125}I]-BSA injection, animals were perfused and killed.

(3) Drug effects on plasma extravasation during and after stimulation

Tracer and drug administered prior to trigeminal ganglion stimulation:

CP-93,129 and sumatriptan The dose-responses for both drugs have been published in this model (Matsubara *et al.*, 1991; Buzzi & Moskowitz, 1990). When administered 10 min prior to trigeminal stimulation and 5 min before radiolabelled albumin, threshold dosages of 140 nmol kg^{-1} and 7 nmol kg^{-1} were obtained in rat and guinea-pig for CP-93,129 and sumatriptan, respectively. Matsubara *et al.* (1991) and Buzzi & Moskowitz (1990) determined that the dosages used blocked plasma protein extravasation by more than 75%.

Endopeptidase 24.11 [^{125}I]-BSA was given intravenously 5 min before unilateral trigeminal ganglion stimulation. Endopeptidase 24.11 (0.1, 0.3, 1 or 3 nmol kg^{-1}) was injected i.v. 3 min before electrical stimulation. Rats were perfused immediately after stimulation.

Tracer administered before, and drug after trigeminal stimulation:

[^{125}I]-BSA was injected 5 min before electrical stimulation. CP-93,129 (460 nmol kg^{-1}, rats), sumatriptan (24 nmol kg^{-1}, guinea-pigs), or endopeptidase 24.11 (1 nmol kg^{-1}, rat) were administered as a bolus 0, 30 or 60 min after electrical stimulation. Animals were perfused 10 min after each drug treatment.

(4) Capsaicin administration

Tracer and drug were administered after capsaicin.

The left femoral vein was exposed in pentobarbitone anaesthesized rats and capsaicin (1 μmol kg^{-1}) was infused over 30 s. Either CP-93,129 (460 nmol kg^{-1}) or vehicle were administered 25 min later followed in 5 min by [^{125}I]-BSA injection. Rats were perfused 10 min after [^{125}I]-BSA injection. The dura mater was then dissected and radioactivity compared between drug and vehicle-treated group.

Data analysis

[^{125}I]-BSA extravasation is expressed as the ratio: c.p.m. mg^{-1} wet weight (wet wt.) [stimulated side]/c.p.m. mg^{-1} wet wt. [unstimulated side]. In capsaicin experiments, data are expressed as percentage of c.p.m. mg^{-1} wet wt. of vehicle-treated animals. Data are expressed as mean ± s.e.mean. Paired Student's t test with the Bonferroni correction was used to compare time-dependent differences in c.p.m. mg^{-1} wet wt. on each side. Unpaired Student's t test was used for statistical comparisons between vehicle- and drug-treated groups. Probability values (P) of less than 0.05 were considered significant. Each data point was calculated from at least two separate experiments.

Drugs

^{125}I-labelled bovine serum albumin (BSA; New England Nuclear, Boston, MA, U.S.A.) was diluted in saline; sumatriptan (Glaxo Ltd., Hertfordshire, U.K.) and endopeptidase 24.11 (Genentech Inc., South San Francisco, CA, U.S.A.) were dissolved in saline; CP-93,129 (5-hydroxy-3 (4-1,2,5,6-tetrahydropyridyl-4-azaindole; Pfizer, Inc., Groton, CT, U.S.A.) was solubilized in dimethyl sulphoxide:saline 1:19; capsaicin (Polyscience Inc., Wilmington, Pennsylvania, U.S.A.) was dissolved in saline:ethanol:Tween 80, 8:1:1 (200 μl) and then diluted (1:200) with saline. Capsaicin was made up from powder for each experiment. All drugs or an equivalent volume of vehicle were injected as a bolus intravenously (1 ml kg^{-1}).

Results

Tracer administered before electrical stimulation

The leakage of albumin was greater ipsilateral to the stimulation and the differences remained highly significant up to and including 120 min but not later. The ratio between the two sides was >1.50 at intervals up to 90 min ($P<0.01$), decreased to 1.30 ($P<0.05$) at 120 min and reached baseline (1.05) at 180 min (Figure 1), when plasma isotope levels had only decreased to approximately 50% (data not shown) post-stimulation. C.p.m. mg^{-1} wet wt. did not change on the side contralateral to the stimulation over 180 min and ranged from 18.9 ± 1.9–20.9 ± 1.6.

Endopeptidase 24.11, when administered 3 min before stimulation, attenuated the albumin leakage at 1 nmol kg^{-1} [1.75 ± 0.08 ($n=7$) versus 1.20 ± 0.06 ($n=8$) in vehicle and drug-treated group, respectively, $P<0.001$], and 3 nmol kg^{-1} [1.79 ± 0.07 ($n=7$) versus 1.01 ± 0.06 ($n=8$), respectively, $P<0.001$], but not at 0.3 nmol kg^{-1} [1.80 ± 0.11 ($n=7$) versus 1.93 ± 0.11 ($n=8$), respectively] (Figure 2). Endopeptidase 24.11 [1 nmol kg^{-1} ($n=4$)] did not affect mean arterial blood pressure when measured over 20 min (Data not shown).

CP-93,129 (460 nmol kg^{-1}), sumatriptan (24 nmol kg^{-1}), or endopeptidase 24.11 (1 nmol kg^{-1}) attenuated albumin leakage in the dura mater when given immediately (data not

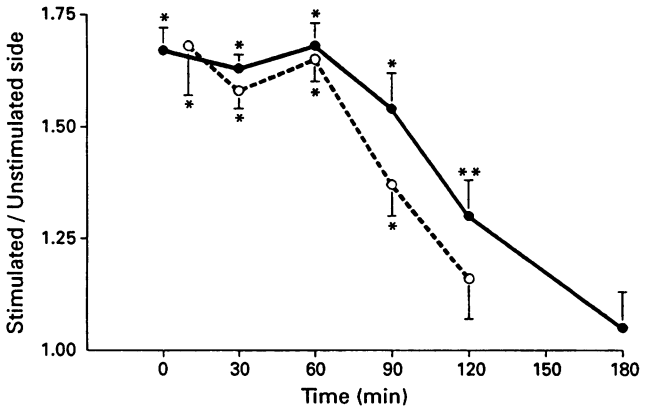

Figure 1 Time-dependent changes in plasma protein (^{125}I-labelled bovine serum albumin, [^{125}I]-BSA) extravasation within rat dura mater during and after electrical trigeminal ganglion stimulation. Tracer administered before stimulation (●): [^{125}I]-BSA (50 μCi kg^{-1}, i.v.) was given 5 min before electrical stimulation. Animals were then perfused with saline immediately ($n = 7$) or 30 ($n = 5$), 60 ($n = 7$), 90 ($n = 8$), 120 ($n = 7$), or 180 ($n = 5$) min after stimulation. Tracer administered at varying times after stimulation (○): [^{125}I]-BSA was injected immediately ($n = 6$) or 20 ($n = 5$), 50 ($n = 11$), 80 ($n = 10$) or 110 ($n = 6$) min after stimulation. Ten min later, animals were perfused with saline. Dura was then dissected and counted for radioactivity as described in Methods. Extravasation newly developing after stimulation ceased by 120 min whereas the amount accumulated both during and after stimulation remained for at least 120 min. Data are expressed as the mean ± s.e.mean (vertical bars) of c.p.m. mg^{-1} wet wt. on the stimulated divided by the unstimulated sides. *$P < 0.01$; **$P < 0.05$ when the differences in c.p.m. mg^{-1} wet wt. were compared between the two sides.

shown) and 30 min after electrical stimulation, the animals being killed 10 min later (Figure 3).

Tracer administered after stimulation

The c.p.m. mg^{-1} ipsilaterally was greater compared to that contralaterally at each interval up to 90 min ($P < 0.01$), but not at 120 min following electrical stimulation. The ratios were lower than those when tracer was injected before stimulation. The ratio of albumin leakage, >1.35 up to 90 min ($P < 0.01$), decreased to 1.15 at 120 min ($P > 0.05$) after electrical stimulation (Figure 1). The c.p.m. mg^{-1} on the side contralateral to stimulation was similar when assessed 10 min

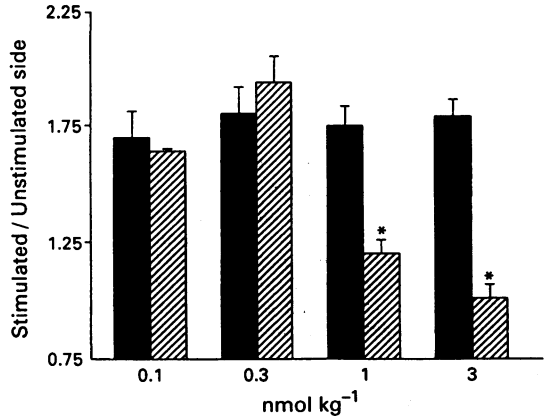

Figure 2 Dose-dependent blockade of plasma extravasation within rat dura mater by endopeptidase 24.11 when ^{125}I-labelled bovine serum albumin ([^{125}I]-BSA) was injected 5 min before electrical stimulation. Endopeptidase 24.11 was administered 3 min before electrical stimulation. Immediately after stimulation, rats were perfused with saline. Data are expressed as in Figure 1. Solid columns represent vehicle-treated group and hatched drug-treated group. *$P < 0.001$ as compared to vehicle-treated group.

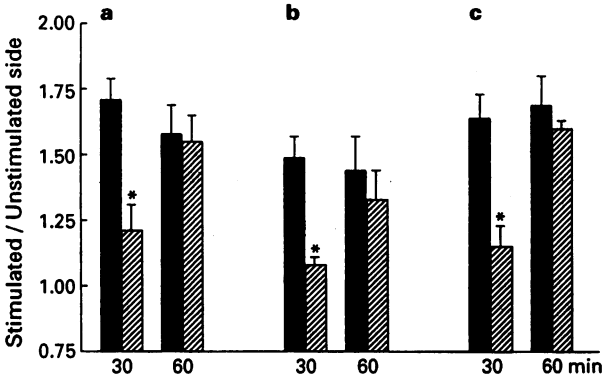

Figure 3 CP-93,129, sumatriptan, or endopeptidase 24.11 blocks plasma leakage when administered 30 but not 60 min after nerve stimulation; isotopic tracer was administered before stimulation in rats or guinea-pigs (sumatriptan only). (a) CP-93,129 [460 nmol kg^{-1}, 30 ($n = 10$) and 60 ($n = 6$) min], (b) sumatriptan (24 nmol kg^{-1}, 30 ($n = 16$) and 60 ($n = 5$) min], (c) endopeptidase 24.11 [1 nmol kg^{-1}, 30 ($n = 9$) and 60 ($n = 5$) min]. ^{125}I-labelled bovine serum albumin was injected 5 min before stimulation and animals perfused with saline 10 min after each drug injection. Data are expressed as in Figure 1. Solid and hatched columns represent vehicle-treated group and drug-treated group, respectively. *$P < 0.005$ as compared to vehicle-treated group.

after tracer injection and ranged from 15.1 ± 1.7 to 20.4 ± 0.8 over 120 min.

CP-93,129 (460 nmol kg^{-1}), sumatriptan (24 nmol kg^{-1}), or endopeptidase 24.11 (1 nmol kg^{-1}) attenuated the plasma extravasation in dura mater when administered 45 min, but not 75 min, after electrical stimulation (Figure 4).

Tracer injected after capsaicin administration

Albumin extravasation was measured within rat dura mater when tested as late as 40 min after capsaicin infusion (1 μmol kg^{-1}), and increased to 142 ± 3% ($n = 7$) as compared to the vehicle-treated group. When CP-93,129 (460 nmol kg^{-1}) was administered 25 min after capsaicin infusion, the response was attenuated to 96 ± 4% ($n = 7$, $P < 0.05$ as compared to capsaicin-treated group).

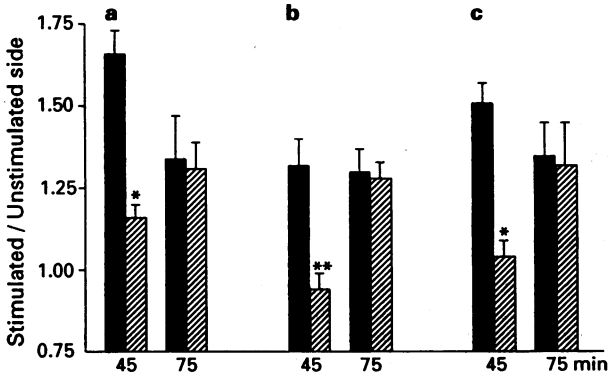

Figure 4 CP-93,129 (a), sumatriptan (b), or endopeptidase 24.11 (c) blocks plasma leakage when administered 45 min after trigeminal ganglion stimulation in rats or guinea-pigs (sumatriptan only); [^{125}I]-albumin (50 μCi kg^{-1}, i.v.) was injected 5 min after each drug administration. CP-93,129 [460 nmol kg^{-1}; $n = 7$ (45 min) or 5 (75 min)], sumatriptan [24 nmol kg^{-1}; $n = 4$ (45 min) or 6 (75 min)], or endopeptidase 24.11 [1 nmol kg^{-1}; $n = 5$ (45 min) or 5 (75 min)] was administered 45 or 75 min after electrical stimulation, respectively. Fifteen min after drug administration, animals were perfused with saline. Data are expressed as described in Figure 1. Solid columns represent vehicle-treated group and hatched columns drug-treated group. Note that the differences in the extravasation response between 45 and 75 min in the vehicle groups reflect the expected reduction in leakage with time (see Figure 1). *$P < 0.001$, **$P < 0.01$ as compared to vehicle-treated group.

Discussion

The experiments described here demonstrate that blood vessels of the dura mater continue to leak plasma proteins for many minutes following a relatively brief (5 min) period of electrical trigeminal ganglion stimulation and that this delayed leakage may depend upon impulse generation (see below) and neuropeptide release from sensory fibres. As evidence, endopeptidase 24.11, a Zn^{2+}-metallopeptidase which degrades extracellular peptides including tachykinins and probably calcitonin gene-related peptide (CGRP) (see Roques & Beaumont, 1990), inhibited plasma leakage when administered before stimulation as well as at varying intervals after trigeminal nerve stimulation (Figures 2, 3 and 4). Sumatriptan and CP-93,129, drugs previously shown to block plasma extravasation by prejunctional mechanisms, inhibited the response at similar time points as well (Figures 3 and 4).

The capsaicin experiments also suggest that neurogenically mediated mechanisms may persist after chemical stimulation of trigeminovascular fibres. However, in this instance, it is difficult to exclude effects due to residual and/or recirculating drug. Nevertheless, the data are consistent with more recent findings suggesting that the mechanism by which capsaicin promotes neuropeptide release (blockade by tetrodotoxin- and ω-contoxin-sensitive mechanisms; Lou *et al.*, 1991) may require the presence of membrane depolarization and action potentials.

The present paper is the first to our knowledge to show that admininstered endopeptidase 24.11 can block the development of neurogenic plasma protein extravasation. The ability of endopeptidase 24.11 to block neurogenic plasma protein extravasation was anticipated based on previous reports showing that peptidase inhibitors potentiate NI in other models (Sekizawa *et al.*, 1987a,b; Iwamoto *et al.*, 1990), and that the tachykinins mediate the plasma protein extravasation response in dura mater. In previous reports, inhibitors of neutral endopeptidase selectively potentiated substance P-induced effects on airway submucosal gland secretion (Borson *et al.*, 1987), vascular permeability in skin (Iwamoto *et al.*, 1989), smooth muscle contraction (Sekizawa *et al.*, 1987a,b), and potentiated NI in the rat trachea induced by capsaicin or electrical stimulation (Umeno *et al.*, 1989). Of course, our studies have not ruled out the potential importance of other peptide mediators such as bradykinin which serve as substrate for endopeptidase 24.11.

We infer that the extravasated proteins existed in a pool which was rapidly turning over since leakage was at least partially reversible within 10 min after drug administration. At time points beyond 45 min however, non-neurogenic mechanisms predominated. Hence, new leakage was not blocked by sumatriptan, CP-93,129 or endopeptidase 24.11 (Figures 3 and 4). We postulate that damage to endothelial membranes, perhaps mediated by prolonged contact with peptides and mast cell products, or other mediators such as those released from white cells may account for leakage at this time.

We interpret our present findings as further evidence that 5-$HT_{1B/D}$ receptor agonists inhibit neuropeptide release from trigeminovascular fibres. As noted above, CP-93,129 and sumatriptan exhibited time-dependent actions similar to endopeptidase 24.11 (Figures 3 and 4). The findings are consistent with previously published data showing that: (a) sumatriptan and another 5-HT_1 agonist, dihydroergotamine (DHE), attenuate plasma levels of CGRP within venous effluent during and following electrical trigeminal stimulation (Buzzi *et al.*, 1991b); (b) sumatriptan, CP-93,129 and DHE block plasma protein extravasation that is neurogenically-mediated and do not block plasma extravasation caused by intravenously administered substance P, neurokinin A (Saito *et al.*, 1988; Buzzi & Moskowitz, 1990; Matsubara *et al.*, 1991) or α-methyl-5-hydroxytryptamine (α-methyl-5-HT), a 5-HT_2 receptor agonist. In the latter experiments, 242 nmol kg^{-1} sumatriptan administered 10 min prior to α-methyl-5-HT (0.33 μmol kg^{-1}, $n = 8$) did not block extravasation whereas pizotifen (1 μmol kg^{-1}, $n = 9$) did (unpublished); (c) sumatriptan or dihydroergotamine attenuate nerve stimulation-induced ultrastructural responses in mast cells and endothelium (Buzzi *et al.*, 1992); (d) 5-$HT_{1B/D}$ receptors function as presynaptic autoreceptors inhibiting neurotransmitter release in other nerve cells (see Maura & Raiteri, 1986; Sharp *et al.*, 1989).

We previously suggested the importance of NI in the dura mater to the pathogenesis and treatment of migraine headaches based on the acknowledged importance of the dura mater as a source of head pain, and the ability of antimigraine drugs such as sumatriptan and ergot alkaloids to block NI when administered as a pretreatment. However, the relevance of the model to the clinical situation was challenged because the original model involved the administration of drug prior to electrical stimulation. The protocol as described here, may be more relevant in this regard, particularly within the first 45 min after stimulation. At these times, sumatriptan blocked plasma protein extravasation in dosages which are similar to those required to treat headaches in man (Buzzi & Moskowitz, 1990).

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Chloroethylclonidine: an irreversible agonist at prejunctional α_2-adrenoceptors in rat vas deferens

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1 The possibility that chloroethylclonidine (CEC) activates prejunctional α_2-adrenoceptors was studied in the isolated vas deferens of the rat. Tissues were stimulated electrically and both the stimulation-evoked overflow of tritium (after preincubation with [^{3}H]-noradrenaline) and the purinergic contraction component (isolated by prazosin 0.3 μM) were measured.

2 CEC (0.1–3 μM) concentration-dependently reduced the overflow of tritium evoked by trains of 6 pulses/100 Hz. The inhibition by CEC was not altered by prazosin (0.3 μM) but was prevented by pre-exposure to rauwolscine (0.3 μM). The inhibition, once established, did not fade upon washout of CEC, even when the washout fluid contained rauwolscine (0.3 μM).

3 CEC (0.1–3 μM) concentration-dependently reduced the purinergic component of contractions elicited by single pulses. The inhibition, again, was prevented by pre-exposure to rauwolscine (0.3 μM) and once established, did not fade upon washout of CEC, even when the washout fluid contained rauwolscine (0.3 μM).

4 CEC (3 μM) reduced the overflow of tritium evoked by 20 pulses/10 Hz, did not alter the overflow evoked by 100 pulses/10 Hz and increased the overflow evoked by 500 pulses/10 Hz.

5 CEC (3 μM) reduced the early peak, but increased the late plateau phase, of purinergic contractions elicited by 100 pulses/10 Hz.

6 It is concluded that CEC reduces the release of noradrenaline and a purinergic co-transmitter by irreversible activation of prejunctional α_2-adrenoceptors. CEC seems to be a partial α_2-agonist with an efficacy lower than that of noradrenaline. The prejunctional inhibitory effect limits the suitability of CEC for the characterization of postjunctional α_1-adrenoceptors mediating responses to sympathetic nerve stimulation.

Keywords: Chloroethylclonidine; rauwolscine; prejunctional α_2-adrenoceptors; α_1-adrenoceptor subtypes; co-transmission; purinergic transmission; irreversible agonism; rat vas deferens

Introduction

The clonidine derivative *N*-β-chloroethyl-*N*-methylaminomethylclonidine (chloroethylclonidine; CEC) was first described by Leclerc *et al.* (1980). The authors showed that CEC (i) inhibited brain synaptosomal binding of [^{3}H]-clonidine, (ii) caused a phentolamine-sensitive contraction of rat aorta that persisted during washout, (iii) caused a long-lasting blood pressure increase in pithed rats and (iv) lowered the blood pressure of anaesthetized rats upon intracerebroventricular injection. They concluded that CEC alkylates and thereby persistently activates α-adrenoceptors, 'the first example of an α-agonist with an irreversible effect'. Retrospectively, a long-lasting activation of α_1-adrenoceptors seems a likely interpretation, since only α_1-adrenoceptors mediate contraction in rat aorta (see Flavahan & Vanhoutte, 1986; Docherty, 1989). Activation of α_2-adrenoceptors also seems possible since this subtype mediates (much of) the central hypotensive effect of clonidine-like drugs (see Kobinger, 1986).

CEC was not studied further until several years later, when it was introduced as an irreversible α_1-adrenoceptor antagonist, and one that blocked the α_{1B} subtype selectively (Johnson & Minneman, 1987; Minneman *et al.*, 1988). Since then it has become a key compound to distinguish α_1 subtypes, including subtypes of postjunctional receptors mediating adrenergic responses to sympathetic nerve stimulation (Muramatsu, 1991; Sulpizio & Hieble, 1991; Mallard *et al.*, 1992). The possibility that the blockade by CEC of responses to nerve stimulation might in part be due to activation of prejunctional α_2-adrenoceptors has not so far been taken into account.

We investigated the effect of CEC on prejunctional α_2-adrenoceptors in the rat vas deferens. Both effects on tritium overflow from tissues prelabelled with [^{3}H]-noradrenaline and effects on purinergic neurogenic contractions were examined.

Methods

Male Wistar rats (240–340 g) were decapitated. The vasa deferentia were removed and cleaned of adherent tissue. Unless stated otherwise, the medium used for incubation and superfusion contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 0.9, $NaHCO_3$ 25, glucose 11, ascorbic acid 0.3 and disodium EDTA 0.03. It was saturated with 95% O_2/5% CO_2 and kept at 37°C.

Tritium overflow

Vasa deferentia were split open longitudinally. Four pieces of about 10 mg each were cut from the prostatic portions. The eight pieces were incubated for 30 min in medium with reduced Ca^{2+} ($CaCl_2$ 0.2 mM; see Limberger *et al.*, 1992) containing 0.2 μM (−)-[^{3}H]-noradrenaline, specific activity 56.9 Ci mmol^{-1}. They were then rinsed, and one piece was transferred to each of six 0.16 ml superfusion chambers. The tissue was held by a polypropylene mesh between platinum wire electrodes 6 mm apart and was superfused with [^{3}H]-noradrenaline-free medium at 2 ml min^{-1} ($CaCl_2$ 2.5 mM). A Stimulator I (Hugo Sachs Elektronik, Hugstetten, Germany) operating in the constant voltage mode was used for electrical stimulation (1 ms pulse width, 36 V cm^{-1} voltage drop between the electrodes of each chamber, yielding a current of 60 mA). An initial stimulation period (180 pulses, 1 Hz) was applied after 30 min of superfusion; it was not used for

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determination of tritium overflow. The superfusate was collected in 2 or 10 min periods from 116 min of superfusion onwards. Two different stimulation protocols were then used. In the first protocol, tissues were stimulated five times (S_1 to S_5), after 120, 146, 172, 198 and 224 min of superfusion; each of the five stimulation periods consisted of a train of 6 pulses/100 Hz. In the second protocol, tissues were stimulated twice (S_1 and S_2), after 120 and 150 min of superfusion; each of the two stimulation periods consisted of a train of either 20, 100 or 500 pulses at a frequency of 10 Hz; S_1 and S_2 had the same pulse number in a single piece of tissue. At the end of an experiment, tissues were solubilized in 1 ml Soluene-350 (Packard Instrument, Frankfurt am Main, Germany). Tritium was measured in superfusate samples and solubilized tissues by liquid scintillation spectrometry.

The outflow of tritium was calculated as a fraction of the amount of tritium in the tissue at the start of the respective collection period (fractional rate of outflow, min^{-1}). The stimulation-evoked overflow was calculated as the difference between the total outflow of tritium in the 4 min (first protocol) or 6 min (second protocol) following the start of a stimulation period, and the estimated basal outflow during this time; the basal outflow was assumed to decline linearly from the 2 min interval before, to the interval 4–6 min (first protocol) or 6–8 min (second protocol) after, the start of stimulation; the difference (nCi) was expressed as a percentage of the amount of tritium in the tissue (nCi) at the start of stimulation. For further evaluation of basal efflux, ratios were calculated of the fractional rate of outflow in the 2 min before each stimulation period S_n (b_n) over that in the 2 min before S_1 (b_1). Ratios S_n/S_1 were calculated for further evaluation of the stimulation-evoked overflow.

Contraction

The vasa deferentia were suspended vertically in an organ bath with 5.7 ml medium. The medium contained prazosin (0.3 μM) in order to isolate the purinergic contraction component. The bath fluid was exchanged every 13 to 26 min. The lower end of the vas deferens was fixed and the upper end attached to an isometric force transducer (K30; Hugo Sachs Elektronik) under an initial tension of 9.8 mN. The tissues were allowed to relax to a resting tension of approximately 3 mN during a 60 min equilibration period. This final resting tension remained constant during the course of the experiment. The tension was recorded on a Graphtec thermal pen recorder (Ettlingen, Germany). A Stimulator II (Hugo Sachs Elektronik) operating in the constant current mode was used for electrical stimulation (0.3 ms pulse width, current strength 100 mA). The platinum electrodes were located at the top and the bottom of the organ bath. Two protocols were used. In the first protocol, tissues were stimulated five times (S_1 to S_5), 60, 86, 112, 138 and 164 min after the preparation had been set up; each of the five stimulations consisted of a single pulse. In the second protocol, tissues were stimulated three times (S_1 to S_3), 60, 90 and 120 min after the preparation had been set up; each of the three stimulation periods consisted of a train of 100 pulses/10 Hz. In the case of biphasic contractions (100 pulses/10 Hz), amplitudes of both the initial and the secondary phase were measured. Amplitude ratios S_n/S_1 were calculated for further evaluation.

Some additional experimental details are described in the Results section.

Materials

The following drugs were used: chloroethylclonidine dihydrochloride (purity > 99.9%; Biotrend, Köln, Germany); clonidine hydrochloride (Boehringer, Ingelheim, Germany); (−)-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 56.9 Ci $mmol^{-1}$ (Du Pont, Dreieich, Germany); prazosin hydrochloride (Pfizer, Karlsruhe, Germany); rauwolscine hydrochloride (Roth, Karlsruhe, Germany); suramin hexasodium salt (Bayer, Wuppertal, Germany); tetrodotoxin (Sigma, Deisenhofen, Germany). Tetrodotoxin was dissolved in 0.1 M sodium acetate buffer pH 4.85. Other drugs were dissolved in distilled water. In tritium overflow experiments, drug solutions were added to the superfusion medium reservoir; in contraction experiments, they were added to the organ bath in volumes not exceeding 30 μl.

Statistics

The effect of a drug, added after S_1, on an S_n/S_1 ratio is expressed as a percentage of the average S_n/S_1 value obtained in a control group without addition of this drug but otherwise treated identically. For example, the effect of CEC (1 μM) added before S_4 in the continuous presence of rauwolscine (0.3 μM), on S_4/S_1 is expressed as a percentage of the average S_4/S_1 value obtained in experiments with rauwolscine alone.

Arithmetic means ± s.e.mean are given throughout, except for IC_{50} values which are geometric means with 95% confidence intervals (c.i.). IC_{50} values of CEC were interpolated as the concentrations producing 50% inhibition. Differences between means were tested by the Mann-Whitney test. They were taken to be statistically significant when the error probability was $P < 0.05$. For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni.

Results

Tritium overflow

In the first series of experiments, vasa deferentia prelabelled with [^{3}H]-noradrenaline were stimulated electrically five times, each stimulation period (S_1 to S_5) consisting of a train of 6 pulses/100 Hz. In the absence of drugs, the fractional rate of tritium outflow immediately before S_1 (b_1) was $0.00133 \pm 0.00002\ min^{-1}$, and the overflow evoked by S_1 amonted to $0.043 \pm 0.002\%$ of the tritium content of the tissue ($n = 50$). The evoked overflow remained approximately constant from S_1 to S_5 in control experiments without any drug (for example, ratio $S_5/S_1 = 1.10 \pm 0.13$; $n = 7$). Tetrodotoxin (0.3 μM), when added 20 min before S_5, almost abolished the evoked overflow ($S_5/S_1 = 0.10 \pm 0.02$; $n = 6$).

Cumulative addition of increasing concentrations of CEC (0.1–3 μM) after S_1 produced increasing inhibition (Figure 1a), with an IC_{50} of 0.4 (95% c.i. 0.3–0.6) μM. The basal efflux of tritium was not changed (based on b_n/b_1 ratios). The same concentrations of CEC were used in the same cumulative protocol in order to examine its interaction with prazosin or rauwolscine. Prazosin (0.3 μM), when added 60 min before S_1 and kept for the remainder of the experiment, increased the basal efflux of tritium ($b_1 = 0.00221 \pm 0.00004\ min^{-1}$; $P < 0.01$) but did not by itself change the evoked overflow ($S_1 = 0.042 \pm 0.003\%$; $n = 14$). As in the absence of any drug, the evoked overflow remained approximately constant when prazosin alone was present (for example, $S_5/S_1 = 0.91 \pm 0.11$; $n = 7$). Prazosin (0.3 μM) did not affect the concentration-dependent inhibition produced by CEC (for example, $S_5/S_1 = 0.10 \pm 0.03$ when CEC, 3 μM, was administered at S_5 in the presence of prazosin as the highest concentration of the concentration-response curve; $n = 7$). Rauwolscine (0.3 μM), when added 60 min before S_1, did not change basal tritium efflux (b_1) but slightly increased the evoked overflow ($S_1 = 0.050 \pm 0.002\%$ of tissue tritium; $n = 16$; $P < 0.01$). In the presence of rauwolscine (0.3 μM) alone, the evoked overflow again remained stable (for example, $S_5/S_1 = 0.94 \pm 0.10$; $n = 8$). Rauwolscine completely prevented the inhibitory effect of CEC (Figure 1a).

The reversibility of the effect of CEC was tested by addi-

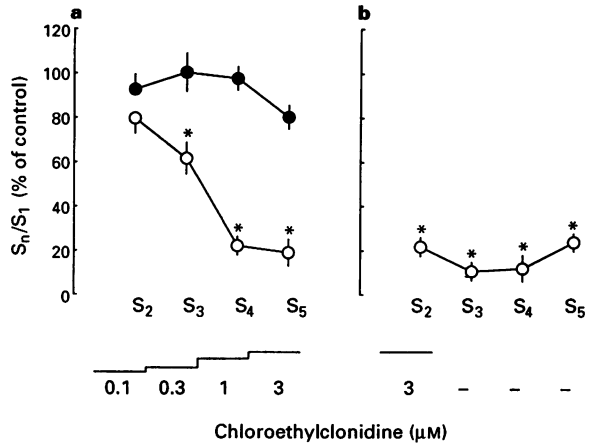

Figure 1 Effect of chloroethylclonidine (CEC) on tritium overflow evoked by brief pulse trains, and interaction with rauwolscine. Tissues were stimulated five times (S_1 to S_5). Each stimulation period consisted of 6 pulses/100 Hz. In (a), CEC was administered at increasing concentrations (0.1–3 μM), in a cumulative manner, 20 min before S_2 to S_5, either in the absence (○) or in the presence of rauwolscine 0.3 μM (●); rauwolscine was added 60 min before S_1 and was present for the remainder of the experiment. In (b), CEC (3 μM) was added from 20 min before until 6 min after S_2 and then washed out. Ordinate scale shows S_n/S_1 ratios obtained in the presence of CEC, expressed as a percentage of the average S_n/S_1 ratio obtained in corresponding controls. Means and s.e.mean (vertical lines) from 7–9 experiments. *denotes a significant difference from corresponding control ($P<0.01$).

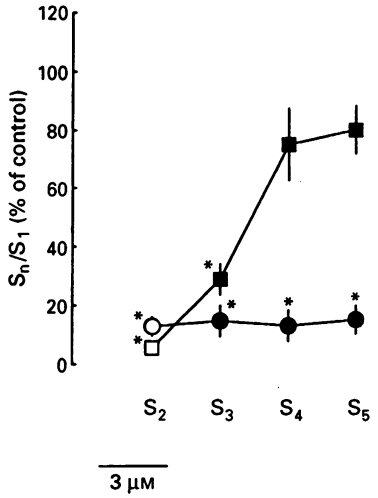

Figure 2 Reversibility of the effects of chloroethylclonidine (CEC) and clonidine on evoked tritium overflow. Tissues were stimulated five times (S_1 to S_5). Each stimulation period consisted of 6 pulses/100 Hz. CEC (circles) or clonidine (squares) was added at a single concentration (3 μM) from 20 min before until 6 min after S_2 and then washed out. Throughout the washout phase (i.e., from 6 min after S_2 to the end of the experiment), rauwolscine (0.3 μM) was added to the superfusion medium (indicated by solid symbols). Ordinate scale shows S_n/S_1 ratios obtained in experiments with CEC and clonidine, expressed as a percentage of the average S_n/S_1 ratio obtained in corresponding controls (rauwolscine only from S_3 to S_5). Means and s.e.mean (vertical lines) from 6–8 experiments. *denotes a significant difference from corresponding control ($P<0.01$).

tion of CEC (3 μM) at S_2 only. The ensuing reduction of evoked tritium overflow was similar to that obtained in the cumulative concentration-response curve (Figure 1b). The inhibition persisted, despite superfusion with CEC-free medium, throughout the stimulation periods S_3 to S_5, i.e., even after 72 min of washout (S_5; Figure 1b). To examine further the reversibility, rauwolscine, at the concentration (0.3 μM) that had prevented the effect of CEC when given before the latter (Figure 1a), was added during the phase after S_2 when CEC was washed out. The inhibition by CEC persisted throughout S_3 to S_5 even during exposure to the antagonist (Figure 2). Rauwolscine (0.3 μM) alone, added after S_2, did not change the evoked overflow of tritium (for example, $S_5/S_1 = 1.09 \pm 0.14$; $n = 7$).

The reversibility of the effect of an equimolar concentration of clonidine (3 μM) was tested for comparison. When added 20 min before S_2, clonidine (3 μM) virtually abolished the evoked overflow of tritium (Figure 2). However, this inhibition disappeared upon superfusion with clonidine-free medium containing rauwolscine (0.3 μM; Figure 2).

In the second set of experiments, two trains of 20, 100 or 500 pulses and a frequency of 10 Hz were applied to each vas deferens (S_1, S_2). The overflow of tritium evoked by S_1 was 0.070 ± 0.005% (20 pulses; $n = 13$), 0.366 ± 0.022% (100 pulses; $n = 14$) and 1.126 ± 0.064% (500 pulses; $n = 13$) of tissue tritium, respectively. In the absence of drugs, the overflow evoked by S_2 was similar to S_1 (Figure 3). Tetrodotoxin (0.3 μM), when added 20 min before S_2, abolished the overflow of tritium evoked by 500 pulses/10 Hz ($S_2/S_1 = 0.00$ and 0.01 in 2 experiments). CEC (3 μM), also added 20 min before S_2, reduced by about 46% the overflow evoked by 20 pulses/10 Hz (train length 2 s), did not change the overflow evoked by 100 pulses/10 Hz (train length 10 s), and increased by about 25% the overflow evoked by 500 pulses/10 Hz (train length 50 s; Figure 3).

Contractions

These experiments were devised to mirror the tritium overflow experiments (pulse pattern, drug administration, reversibility). The purinergic component of neurogenic contractions was isolated by prazosin (0.3 μM; Bourreau *et al.*, 1991; Mallard *et al.*, 1992).

In the first series of experiments, each of five stimulation periods (S_1 to S_5) consisted of a single pulse. The pulses elicited rapid monophasic contractions in the presence of prazosin, the average force developed at S_1 amounting to 17.4 ± 0.7 mN ($n = 31$). The twitches remained constant in control experiments without other drugs (for example, $S_5/S_1 = 1.01 \pm 0.05$; $n = 6$). Tetrodotoxin (0.3 μM; $n = 2$) or suramin (300 μM; Dunn & Blakeley, 1988; $n = 3$), when added after S_5 of control experiments in which further

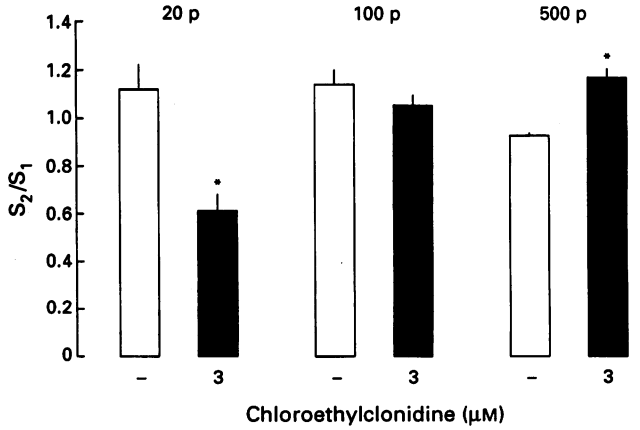

Figure 3 Effect of chloroethylclonidine (CEC) on tritium overflow evoked by long pulse trains. Tissues were stimulated twice (S_1 and S_2). Each stimulation period consisted of either 20, 100 or 500 pulses (p) as indicated and a frequency of 10 Hz. CEC (3 μM) was added 20 min before S_2 and was present for the remainder of the experiment. Ordinate scale shows S_2/S_1 ratios obtained in control experiments (open columns) and in experiments with CEC (solid columns). Means and s.e.mean (vertical lines) from 5–8 experiments. *denotes a significant difference from corresponding control ($P<0.01$).

stimulation periods were applied, abolished the twitches, thus confirming their neurogenic and purinergic character.

Cumulative addition of increasing concentrations of CEC (0.1–3 μM) after S_1 did not change the basal tension of the tissue but produced increasing inhibition of electrically evoked twitches (Figure 4a), with an IC_{50} value of 1.6 (0.7–3.5) μM. Rauwolscine (0.3 μM), when present in the medium in addition to prazosin, did not by itself alter the twitches ($S_1 = 18.5 \pm 1.1$ mN; $n = 8$), and as in the absence of rauwolscine, twitches remained constant from S_1 to S_5 (for example, $S_5/S_1 = 1.00 \pm 0.05$; $n = 4$). Rauwolscine prevented the inhibition by CEC (Figure 4a).

As in the tritium overflow experiments, the reversibility of the effect of CEC was tested by exposure to CEC (3 μM) at S_2 only. The ensuing inhibition was similar to that obtained in the cumulative concentration-response curve (Figure 4b). The inhibition persisted, despite repeated washing with CEC-free medium, throughout the stimulation periods S_3 to S_5, i.e., even after 72 min of washout (S_5; Figure 4b). The inhibition by CEC persisted even when rauwolscine (0.3 μM) was added to the CEC-free medium during washout (Figure 5). Rauwolscine alone, added after S_2, did not change the twitch contraction (for example, $S_5/S_1 = 0.99 \pm 0.04$; $n = 5$).

The reversibility of the effect of an equimolar concentration of clonidine (3 μM) was again tested for comparison. When added 20 min before S_2, clonidine (3 μM) abolished the twitch responses (Figure 5). This inhibition was markedly attenuated upon washout with clonidine-free medium containing rauwolscine (0.3 μM; Figure 5).

The three trains of 100 pulses/10 Hz applied in the second series of experiments elicited biphasic tetanic contractions: an initial peak within the first 2 s was followed by a secondary plateau with a maximum beyond 4 s of stimulation (Figure 6). The responses remained approximately constant from S_1 to S_3 in control experiments with no drug added except prazosin (for example, S_2/S_1 for initial peak = 1.05 ± 0.01; for plateau = 1.12 ± 0.02; Figure 6a). Tetrodotoxin (0.3 μM; $n = 2$) and suramin (300 μM; $n = 3$), when added after S_3 of control experiments in which further stimulation periods were applied, abolished or almost abolished the contractions. CEC (3 μM), when added before and during S_2 and then washed out, reduced the initial peak ($S_2/S_1 = 0.80 \pm 0.04$; $P<0.01$) but enhanced the plateau ($S_2/S_1 = 1.32 \pm 0.04$; $P<0.01$; $n = 5$; Figure 6b). These changes were not attenuated after 30 min of washout (Figure 6b).

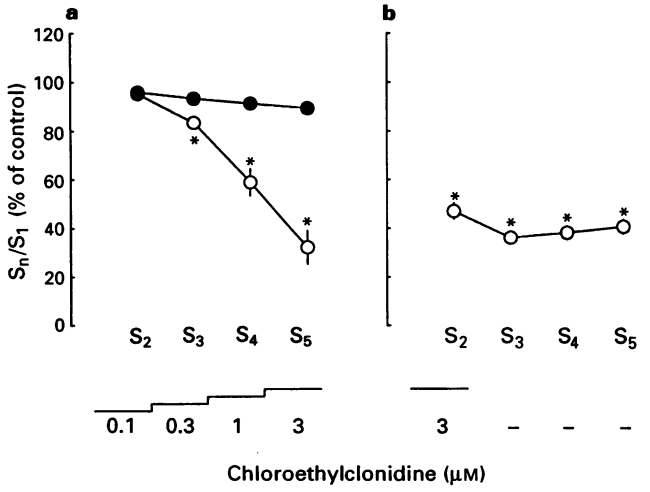

Figure 4 Effect of chloroethylclonidine (CEC) on purinergic contractions evoked by single pulses, and interaction with rauwolscine. Prazosin (0.3 μM) was present in the medium from the beginning. Tissues were stimulated five times (S_1 to S_5). Each stimulation consisted of a single pulse. In (a), CEC was administered at increasing concentrations (0.1–3 μM), in a cumulative manner, 20 min before S_2 to S_5, either in the absence (○) or in the presence of rauwolscine 0.3 μM (●); rauwolscine was present in the medium from the beginning, i.e., 60 min before S_1. In (b), CEC (3 μM) was added from 20 min before until 6 min after S_2 and then washed out by replacement of the bath fluid every 13 min. Ordinate scale shows S_n/S_1 ratios obtained in the presence of CEC, expressed as a percentage of the average S_n/S_1 ratio obtained in corresponding controls. Means and s.e.mean (vertical lines) from 4 or 5 experiments. *denotes a significant difference from corresponding control ($P<0.01$).

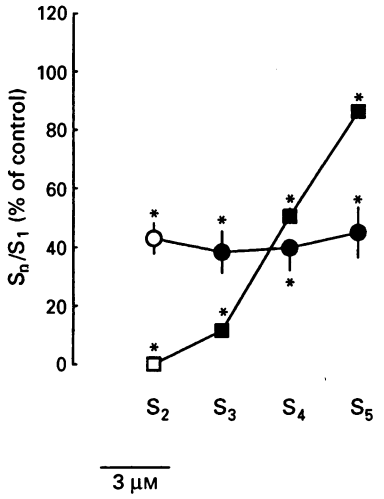

Figure 5 Reversibility of the effects of chloroethylclonidine (CEC) and clonidine on purinergic contractions. Prazosin (0.3 μM) was present in the medium from the beginning. Tissues were stimulated five times (S_1 to S_5). Each stimulation consisted of a single pulse. CEC (circles) or clonidine (squares) was added at a single concentration (3 μM) from 20 min before until 6 min after S_2 and then washed out by replacement of the bath fluid every 13 min. Throughout the washout phase (i.e., from 6 min after S_2 to the end of the experiment), rauwolscine (0.3 μM) was added to the medium (indicated by solid symbols). Ordinate scale shows S_n/S_1 ratios obtained in experiments with CEC and clonidine, expressed as a percentage of the average S_n/S_1 ratio obtained in corresponding controls (rauwolscine only from S_3 to S_5). Means and s.e.mean (vertical lines) from 5 or 6 experiments. *denotes a significant difference from corresponding controls ($P<0.01$).

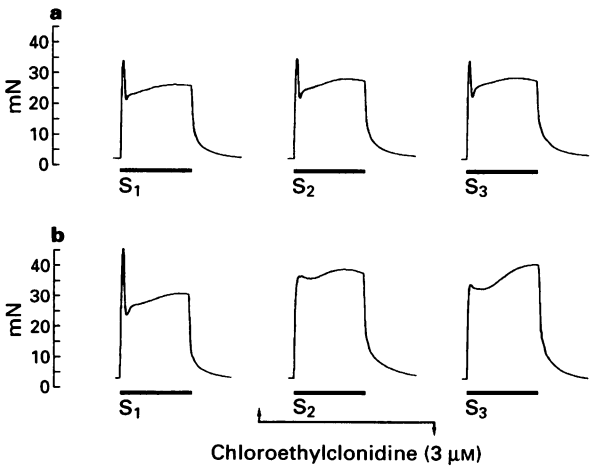

Figure 6 Effect of chloroethylclonidine (CEC) on purinergic contractions evoked by long pulse trains. Prazosin (0.3 μM) was present in the medium from the beginning. Tissues were stimulated three times (S_1 to S_3). Each stimulation period consisted of 100 pulses/10 Hz (10 s; horizontal bars). (a) Shows a control experiment without CEC; (b) shows an experiment in which CEC (3 μM) was added 20 min before S_2 and was washed out immediately afterwards. Representative tracings from 5 experiments each.

Discussion

CEC concentration-dependently reduced the overflow of tritium (and presumably the release of [^{3}H]-noradrenaline; Starke, 1977) elicited by brief (50 ms) trains of high frequency. Release of transmitter evoked by such brief trains resembles release by a single pulse and is free, or almost free, from prejunctional autoinhibition (Singer, 1988; Limberger *et al.*, 1992), as confirmed here by the failure of rauwolscine to cause any major increase. The IC_{50} of CEC (0.4 μM) was close to its K_i value for inhibition of the binding of [^{3}H]-clonidine to brain synaptosomes (1.7 μM; Leclerc *et al.*, 1980). Moreover, its effect was not changed by prazosin (0.3 μM) but was prevented by pre-exposure to rauwolscine (0.3 μM). The effect, hence, was mediated by α_2-adrenoceptors, specifically, the α_2-autoreceptor recently characterized as α_{2D} in rat vas deferens (Limberger *et al.*, 1992; Smith & Docherty, 1992). CEC, hence, is an agonist at α_2-adrenoceptors.

Noradrenaline and adenosine 5'-triphosphate (ATP) (or a related nucleotide) are postganglionic sympathetic co-transmitters in rat vas deferens (French & Scott, 1983; Bourreau *et al.*, 1991; Mallard *et al.*, 1992; see Burnstock, 1990; von Kügelgen & Starke, 1991). If CEC is an α_2-agonist, it should also inhibit the release of ATP. In order to examine this possibility, we isolated the rapid purinergic component of single pulse-evoked contractions by prazosin. In fact, CEC concentration-dependently decreased the purinergic twitches, and again the effect was prevented by pre-exposure to rauwolscine. CEC was less potent at inhibiting the purinergic twitches (IC_{50} 1.6 μM) than the release of noradrenaline (0.4 μM). More or less marked differences in the sensitivity of purinergic postjunctional effects (or ATP overflow) on the one hand, and adrenergic postjunctional effects (or noradrenaline overflow) on the other hand, to α_2-adrenoceptor agonists have been noticed previously (Brown *et al.*, 1983; Hammond *et al.*, 1988; Bültmann *et al.*, 1991; von Kügelgen & Starke, 1991; but see Msghina *et al.*, 1992); the reason is not known.

The effects of CEC on tritium overflow and single pulse-evoked purinergic twitches persisted, not only during washout with normal buffer but even upon washout with buffer containing rauwolscine at a concentration that, when given before CEC, had completely prevented the effect of the latter. In this respect, CEC contrasted sharply with clonidine, despite the fact that, in contrast to CEC, clonidine was given at a greatly supramaximal concentration (as little clonidine as 10 nM inhibited the evoked overflow of tritium to the same extent as did CEC, 3 μM, under the conditions of Figure 1; R. Bültmann, unpublished observation). An irreversible activation of α_1-adrenoceptors by CEC has been observed repeatedly (Leclerc *et al.*, 1980, in rat aorta as explained in the Introduction; Muramatsu *et al.*, 1991, in rat aorta; Schwietert *et al.*, 1991, in rat portal vein). Our results demonstrate that CEC is an irreversible α_2-adrenoceptor agonist as well, presumably because it alkylates the α_2-adrenoceptor (Leclerc *et al.*, 1980). CEC may irreversibly activate both α_1- and α_2-adrenoceptors in dog saphenous vein (Nunes & Guimarães, 1992). The mechanism of irreversible activation by covalent binding is not known. It has been suggested that the phenomenon argues against the rate theory of receptor activation (Lohse *et al.*, 1986). In the case of CEC, however, the alkylating β-chloroethylamino group may be sufficiently remote from the region of the molecule responsible for agonism (see Tian *et al.*, 1990) to permit successive attachment and detachment of that region to and from the receptor, as the rate theory postulates.

Clonidine acts as a partial α_2-agonist on many cells including noradrenergic neurones (Medgett *et al.*, 1978; Cichini *et al.*, 1986; see Starke, 1987). The same seems to be true for CEC in rat vas deferens. It reduced the release of noradrenaline elicited by 6 pulses/100 Hz and also 20 pulses/10 Hz, caused no change for 100 pulses/10 Hz, and increased the release elicited by 500 pulses/10 Hz. This is a pattern predicted for a prejunctional α_2-agonist with lower efficacy than noradrenaline (Starke *et al.*, 1974): the agonist effect prevailed early in trains of pulses, up to 2 s in our experiments; later in the 10 Hz trains, sufficient noradrenaline presumably accumulated in the autoreceptor biophase to reveal antagonism against noradrenaline; early inhibition and later facilitation of release cancelled each other in the 10 s trains, but facilitation prevailed in the 50 s train. Results obtained on purinergic contractions, in this case those elicited by 100 pulses/10 Hz, again confirm the observations on tritium overflow, and in fact show the change from agonism to antagonism directly. Just as CEC diminished the release of noradrenaline elicited by 2 s stimulation periods (Figure 3), it markedly reduced the initial purinergic contraction peak (Figure 6); just as CEC increased the release of noradrenaline later in the trains (Figure 3), it increased the secondary plateau with its maximum beyond 4 s.

The fact that CEC is an irreversible agonist at α_2-adrenoceptors, specifically at α_2-autoreceptors, limits its suitability for the distinction of postjunctional α_1-adrenoceptor subtypes mediating responses to nerve stimulation (see Introduction). Muramatsu (1991) administered CEC in the presence of an α_2-antagonist and, hence, CEC possibly did not act at α_2-adrenoceptors in his study. However, CEC may have activated prejunctional α_2-autoreceptors in other investigations (Sulpizio & Hieble, 1990; Mallard *et al.*, 1992).

Mallard *et al.* (1992) showed, in the rat vas deferens, that CEC (0.1 to 3 μM) selectively blocked the slow, adrenergic but not the rapid, purinergic component of single pulse-evoked twitches; they also reported that CEC (3 μM) blocked the early peak (<2 s) but not the late plateau (>4 s) of the adrenergic component of the tetanus elicited by 100 pulses/10 Hz; they concluded that the adrenergic component of the single pulse twitches and of the early tetanic peak is mediated by α_{1B}-adrenoceptors whereas the adrenergic component of the secondary tetanic plateau is mediated by α_{1A}-adrenoceptors. The presynaptic α_2-agonist effect of CEC does not disprove, but questions, this conclusion. In conditions when CEC depresses adrenergic contractions (Mallard *et al.*, 1992), it also reduces the release of noradrenaline (Figures 1 and 3). Late in a 10 Hz train, when CEC fails to attenuate the adrenergic contraction plateau (Mallard *et al.*, 1992), it also fails to reduce the release of noradrenaline (Figure 3). Strikingly, just as CEC (3 μM) abolishes the early peak of the adrenergic response to 100 pulses/10 Hz (Mallard *et al.*, 1992), it also abolishes the early peak of the purinergic response to 100 pulses/10 Hz (Figure 6), a clear indication of prejunctional inhibition. In unpublished experiments (R. Bültmann), direct evidence has been obtained for at least a contribution of prejunctional α_2-adrenergic inhibition to the blockade of postjunctional adrenergic responses by CEC: CEC (1 and 3 μM) abolished the adrenergic component of single pulse twitches, isolated by nifedipine (10 μM) in the absence of rauwolscine, but reduced them only by 70% when prejunctional α_2-adrenoceptors were blocked by rauwolscine (0.3 μM). One finding might argue against prejunctional inhibition in the study of Mallard *et al.* (1992): CEC did not reduce the purinergic component of single pulse-evoked twitches in their experiments, in contrast to the present work. Mallard *et al.* (1992) did not pharmacologically isolate the purinergic component, and the ensuing purinergic-adrenergic overlap may have obscured the inhibitory effect of CEC. Moreover, we also find that the purinergic twitch is slightly less sensitive to CEC than the release of noradrenaline. However when an antagonist is used to identify postjunctional adrenoceptor types mediating neurogenic responses, it is effects on the release of noradrenaline that impede interpretation.

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Allopurinol and amlodipine improve coronary vasodilatation after myocardial ischaemia and reperfusion in anaesthetized dogs

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1 We have assessed the effect of allopurinol, amlodipine and propranolol pretreatment on both endothelium-dependent and endothelium-independent coronary vasodilatation *in vivo*, by comparing pre-ischaemic responses with those measured after 60 min of coronary artery occlusion and 30 min of reperfusion in anaesthetized dogs.

2 In 15 untreated dogs ischaemia and reperfusion attenuated the increases in coronary blood flow produced by either acetylcholine (0.01–0.05 $\mu g\ kg^{-1}$, i.a.) or glyceryl trinitrate (0.05–0.2 $\mu g\ kg^{-1}$, i.a.), to an average of $39 \pm 4\%$ and $42 \pm 5\%$ of the pre-ischaemic control response, respectively (both $P < 0.05$).

3 In 5 dogs treated with allopurinol (25 $mg\ kg^{-1}$, orally, 24 h previously, plus 50 $mg\ kg^{-1}$, i.v., 5 min before occlusion), the increases in coronary blood flow after ischaemia and reperfusion (acetylcholine: $78 \pm 12\%$, glyceryl trinitrate: $60 \pm 3\%$ of pre-ischaemic response) were significantly larger than post-ischaemic responses in untreated dogs (both $P < 0.05$).

4 Similarly, amlodipine treatment (3 $\mu g\ kg^{-1}\ min^{-1}$, i.v., starting 90 min before occlusion) in 5 dogs improved post-ischaemic increases in blood flow (acetylcholine: 58.5%, glyceryl trinitrate: $66 \pm 6\%$ of pre-ischaemic response, significantly greater than post-ischaemic responses in untreated dogs, $P < 0.05$).

5 In contrast, in a further 6 dogs pretreated with propranolol (1 $mg\ kg^{-1}$, i.v., 30 min before occlusion, plus 0.5 $mg\ kg^{-1}\ h^{-1}$, i.v.), blood flow responses after ischaemia and reperfusion were not different from post-ischaemic responses in untreated dogs (acetylcholine: $46 \pm 6\%$, glyceryl trinitrate: $46 \pm 6\%$ of pre-ischaemic response).

6 These results suggest that allopurinol and amlodipine protect against the post-ischaemic impairment of endothelium-dependent and endothelium-independent coronary vasodilatation *in vivo* by mechanisms additional to endothelial protection.

Keywords: Acetylcholine; allopurinol; amlodipine; coronary artery; endothelium; ischaemia; glyceryl trinitrate; propranolol; reperfusion; vasodilatation

Introduction

Besides regional myocardial infarction and reduced cardiac function, an acute period of coronary artery occlusion and reperfusion causes non-specific attenuation of dilator responses of the large coronary artery (Sobey *et al.*, 1990) and coronary resistance vessels *in vivo* (Nichols *et al.*, 1988; Mehta *et al.*, 1989b,c; Sobey *et al.*, 1990; Vanhaecke *et al.*, 1990). Endothelium-dependent relaxation of the isolated large coronary artery is also impaired, but in contrast to the *in vivo* findings, endothelium-independent relaxation *in vitro* is normal (Ku, 1982; VanBenthuysen *et al.*, 1987; Mehta *et al.*, 1989a; Sobey *et al.*, 1990; Lefer *et al.*, 1991). These observations indicate that whilst coronary artery endothelial dysfunction is one consequence of ischaemia and reperfusion, other mechanisms besides vascular smooth muscle damage are involved in the non-specific attenuation of coronary dilatation *in vivo* after ischaemia and reperfusion. We have recently reported that administration of amlodipine (a dihydropyridine-type calcium entry inhibitor), allopurinol (a xanthine oxidase inhibitor) or propranolol (a β-adrenoceptor antagonist) *in vivo* prevents the attenuation by ischaemia and reperfusion of endothelium-dependent relaxation *in vitro* (Sobey *et al.*, 1992). These data suggested that processes previously considered to contribute to the post-ischaemic myocardial damage such as excessive levels of intracellular calcium (Kloner & Prsyklenk, 1990), generation of cytotoxic oxygen-derived free radicals (Jolly *et al.*, 1984; Ambrosio *et al.*, 1986; Werns *et al.*, 1986; 1988; Chambers *et al.*, 1987; Tamura *et al.*, 1988) and accelerated myocardial energy consumption (Nayler *et al.*, 1980), may also be involved in the impaired endothelial function caused by ischaemia and reperfusion. In the present study we have investigated the protective effects of these agents on both endothelium-dependent and endothelium-independent coronary dilator responses *in vivo* following 60 min of coronary artery occlusion and 30 min of reperfusion.

Methods

Experimental preparation

Thirty-one mongrel dogs of either sex (10–33 kg) were sedated with an intramuscular injection of xylazine (0.1–0.5 mg kg^{-1}; Bayer, Australia), and anaesthetized by intravenous injection of sodium thiopentone (25–30 $mg\ kg^{-1}$; May & Baker, Australia), followed by α-chloralose (70 $mg\ kg^{-1}$; BDH Chemicals, England) which was supplemented as necessary. The dogs were ventilated artificially with room air plus additional oxygen as necessary to maintain arterial blood gas and pH levels within the physiological range (P_{O_2}: 85–110 mmHg; P_{CO_2}: 30–35 mmHg; pH: 7.35–7.45). Systemic arterial blood pressure was measured by inserting a catheter into the right femoral artery, and connecting it to a Druck pressure transducer. Heart rate was monitored with a cardiotachometer coupler which was triggered by the electrocardiogram (ECG). A left thoracotomy was performed and the heart was suspended in a pericardial cradle. A proximal segment

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(approximately 1 cm in length) of the left circumflex coronary artery was dissected clear of connective tissue and inserted securely into an electromagnetic flow probe (2.0–2.5 mm internal diameter) connected to a flowmeter (Gould Statham, U.S.A.) for coronary blood flow measurement. The 5-mm tip of a coronary arterial catheter, consisting of a 23-guage hypodermic needle bent at 120° and joined to 30 cm of polyethylene tubing, was inserted into the artery distal to the flow probe. Blood flow in the left circumflex coronary artery was therefore maintained at all times. Intracoronary injections were administered as a bolus of 0.5 ml or less.

Experimental protocol

The effects of ischaemia and reperfusion on coronary dilatation were studied in four groups of dogs: (1) Dogs receiving no pretreatment (untreated, $n = 15$); (2) Dogs pretreated with amlodipine ($3\,\mu g\,kg^{-1}\,min^{-1}$, i.v., starting 90 min before arterial occlusion and continuing throughout the period of ischaemia and reperfusion; $n = 5$); (3) Dogs pretreated with allopurinol ($25\,mg\,kg^{-1}$, orally 24 h previously, plus $50\,mg\,kg^{-1}$, i.v., administered 5 min prior to arterial occlusion; $n = 5$); and (4) Dogs pretreated with propranolol ($1\,mg\,kg^{-1}$, i.v., 30 min prior to arterial occlusion, and supplemented with $0.5\,mg\,kg^{-1}\,h^{-1}$ i.v.; $n = 6$). Each dog was included in only one experimental group. In all dogs, left circumflex coronary artery blood flow, systemic arterial blood pressure and heart rate were recorded simultaneously on a Grass model 7D Polygraph. Total coronary vascular resistance was calculated as the quotient of arterial pressure and coronary blood flow. In Groups 1 (in 7 of 15 dogs), 2 and 3 left ventricular pressure was measured with a pressure transducer-tipped catheter (Miller) passed through the left atrium into the left ventricle. Peak positive *LV dP/dt*, an index of ventricular contractility, was obtained by electronic differentiation of the left ventricular pressure pulse.

A stablization period of approximately 20 min was allowed following surgery. In all dogs, control coronary vasodilator responses to acetylcholine (0.01, 0.02 and $0.05\,\mu g\,kg^{-1}$) and glyceryl trinitrate (0.05, 0.1 and $0.2\,\mu g\,kg^{-1}$) were obtained by injection directly into the left circumflex coronary artery. In pretreated dogs these control dilator responses were obtained in the presence of amlodipine (Group 2) or propranolol (Group 4), or in dogs that had received allopurinol 24 h previously (Group 3). Group 3 dogs were then given the second dose of allopurinol 5 min before coronary occlusion. All variables were allowed to return to baseline levels for at least 5 min between injections.

Five min before occlusion of the left circumflex coronary artery, lignocaine hydrochloride (50 mg, i.v.; Astra Pharmaceuticals, Australia) was administered to reduce arrhythmias. Total occlusion of the left circumflex coronary artery, proximal to the flow probe and distal to the first main branch, was performed with a small bulldog clip. After 60 min of arterial occlusion the clip was removed, allowing reperfusion of the ischaemic myocardium. Left circumflex coronary artery blood flow was restricted to the pre-occlusion level for approximately 10 min by partially occluding the artery with a 10 cm length of cotton tape placed around the artery. This procedure limits reperfusion hyperaemia, thus reducing the severity of reperfusion arrhythmias and the incidence of haemorrhagic myocardial infarction (Lucchesi *et al.*, 1976). Recordings of the ECG (leads I, II and III) were made periodically in each experiment. After 30 min of reperfusion, vasodilator injections were repeated. The effect of coronary artery occlusion and reperfusion on baseline levels of all variables was studied by comparing resting levels recorded: (i) 10 min before coronary artery occlusion, (ii) at the end of the 60 min occlusion period, (iii) after 30 min of reperfusion, and (iv) at the end of the experiment (106 ± 7 min of reperfusion in Group 1; 75 ± 8 min in Group 2; 103 ± 6 min in Group 3; and 111 ± 14 min in Group 4).

Drugs

Acetylcholine perchlorate (BDH Chemicals, Poole, Dorset) and (±)-propranolol hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) were dissolved and diluted in 0.9% saline. Glyceryl trinitrate (GTN, Tridil, Boots Co., Australia) was diluted in 0.9% saline. Amlodipine maleate (Pfizer, Sandwich, Kent) was dissolved and diluted in water for injection. Allopurinol (Sigma) was dissolved in 50 ml 0.9% saline (high pH with 2 M NaOH).

Calculations and statistics

All data presented are the mean ± s.e.mean of n experiments. A one-way analysis of variance and Neuman-Keul's test for multiple comparisons were used to analyse the effect of drug treatment and ischaemia/reperfusion on baseline levels. Dose-response curves generated by acetylcholine and glyceryl trinitrate before and after ischaemia/reperfusion were compared using a two-way analysis of variance and Neuman-Keul's test for multiple comparisons.

To evaluate the protection against ischaemia-induced impairment of coronary vasodilatation by each pharmacological agent, post-ischaemic dilator responses in Groups 2–4 were compared with those in Group 1. For this analysis, post-ischaemic increases in coronary blood flow produced by acetylcholine and glyceryl trinitrate were expressed as a percentage of the pre-ischaemic response, and an average value for each dilator was then calculated in each experiment. The mean of these post-ischaemic values in each of Groups 2–4 was then compared with the Group 1 value by a one-way analysis of variance and a planned comparison test. A P value of less than 0.05 was considered to be statistically significant in each analysis.

Results

Effects of vasodilators on cardiovascular variables before coronary occlusion

Intra-coronary injection of either acetylcholine ($0.01-0.05\,\mu g\,kg^{-1}$) or glyceryl trinitrate ($0.05-0.2\,\mu g\,kg^{-1}$) produced a dose-dependent increase in coronary blood flow in all groups (Figures 1–4). There was no change in arterial pressure, heart rate or *LV dP/dt* produced by these doses of the dilators (data not shown).

Effects of pharmacological pretreatments on baseline cardiovascular variables before coronary occlusion

Amlodipine treatment resulted in a reduced systemic arterial pressure compared with untreated dogs (Table 1, $P<0.05$, one-way analysis of variance). Allopurinol administration caused an elevation of heart rate for approximately 30 min (Table 1, $P<0.05$, one-way analysis of variance). No other variable was significantly different from the control group following pretreatment (Table 1). However, arterial pressure and heart rate were significantly reduced and coronary dilator responses to isoprenaline ($0.01\,\mu g\,kg^{-1}$, i.a.) were abolished in Group 4 dogs after administration of propranolol ($P<0.01$, paired t test, data not shown).

Effect of coronary occlusion and reperfusion on baseline variables

Coronary artery occlusion and reperfusion tended to reduce arterial pressure, but this effect was statistically significant only in propranolol-treated dogs at the end of the occlusion period (Table 1, $P<0.05$, one-way analysis of variance). Coronary blood flow in Groups 3 and 4 remained significantly reduced after reperfusion (Table 1, $P<0.05$, one-way analysis of variance). *LV dP/dt* was reduced after coronary

Table 1 Effect of left circumflex coronary artery occlusion and reperfusion on baseline variables

Group	10 min *Pre-occlusion*	60 min *Occlusion*	30 min *Reperfusion*	*End Expt.*
a AP (mmHg)				
1: Untreated	114 ± 5	107 ± 4	104 ± 4	108 ± 4
2: Amlodipine	94 ± 7*	91 ± 8	92 ± 8	87 ± 7
3: Allopurinol	101 ± 6	98 ± 7	106 ± 6	106 ± 6
4: Propranolol	120 ± 3	99 ± 8**	104 ± 6	108 ± 7
b CBF (ml min^{-1})				
1: Untreated	30 ± 4	0**	25 ± 4	27 ± 4
2: Amlodipine	38 ± 7	0**	32 ± 2	36 ± 5
3: Allopurinol	36 ± 7	0**	18 ± 4**	19 ± 4**
4: Propranolol	33 ± 4	0**	20 ± 2**	22 ± 2**
c HR (b min^{-1})				
1: Untreated	134 ± 8	135 ± 8	131 ± 8	131 ± 7
2: Amlodipine	121 ± 11	124 ± 13	127 ± 15	128 ± 16
3: Allopurinol	167 ± 7*	131 ± 7**	121 ± 5**	126 ± 7**
4: Propranolol	121 ± 6	117 ± 5	114 ± 6	111 ± 6
d CVR (mmHg min ml^{-1})				
1: Untreated	4.9 ± 0.8	–	5.9 ± 1.0	5.5 ± 1.0
2: Amlodipine	2.8 ± 0.5	–	2.9 ± 0.3	2.5 ± 0.3
3: Allopurinol	3.4 ± 0.7	–	8.8 ± 3.6	7.6 ± 2.7
4: Propranolol	4.0 ± 0.5	–	5.4 ± 0.6	5.3 ± 0.7

AP: arterial pressure; CBF: coronary blood flow; HR: heart rate; CVR: coronary vascular resistance.
*Significantly different from Group 1; **Significantly different from 10 min pre-occlusion value; $P<0.05$, one-way analysis of variance.

occlusion and reperfusion only in untreated dogs, indicating that both amlodipine and allopurinol prevented the reduction in cardiac contractility caused by ischaemia and reperfusion ($P<0.05$, one-way analysis of variance, data not shown; *LV dP/dt* was not measured in Group 4). No other variable was significantly altered by coronary occlusion and reperfusion. Alterations in the ECG indicative of myocardial ischaemia were observed following coronary artery occlusion in all dogs (data not shown).

Effect of coronary occlusion and reperfusion on coronary dilator responses

Group 1: Untreated dogs Increases in coronary blood flow produced by both acetylcholine and glyceryl trinitrate were significantly reduced after 60 min of coronary occlusion and 30 min of reperfusion (Figure 1; $P<0.05$, two-way analysis of variance). Overall, post-ischaemic increases in coronary blood flow produced by acetylcholine and glyceryl trinitrate were reduced to 39 ± 4% and 42 ± 5% of the pre-ischaemic control response, respectively (Figure 5; both significantly less than control response, $P<0.05$, one-way analysis of variance).

Group 2: Amlodipine-treated dogs In dogs pre-treated with amlodipine, post-ischaemic increases in coronary blood flow produced by glyceryl trinitrate were not significantly different from control responses (Figure 2). Increases in coronary blood flow produced by acetylcholine after ischaemia and reperfusion were significantly reduced in comparison to control (Figure 2; $P<0.05$, two-way analysis of variance). However, post-ischaemic dilator responses to both acetylcholine (58 ± 5% of control) and glyceryl trinitrate (66 ± 6% of control) in amlodipine-treated dogs were significantly greater than those measured in Group 1 dogs (Figure 5; both are $P<0.05$ compared with Group 1, one-way analysis of variance). Thus, amlodipine partially protected against impairment of both endothelium-dependent and endothelium-independent coronary vasodilatation following ischaemia and reperfusion.

Group 3: Allopurinol-treated dogs Allopurinol pretreatment prevented a significant reduction of acetylcholine-induced

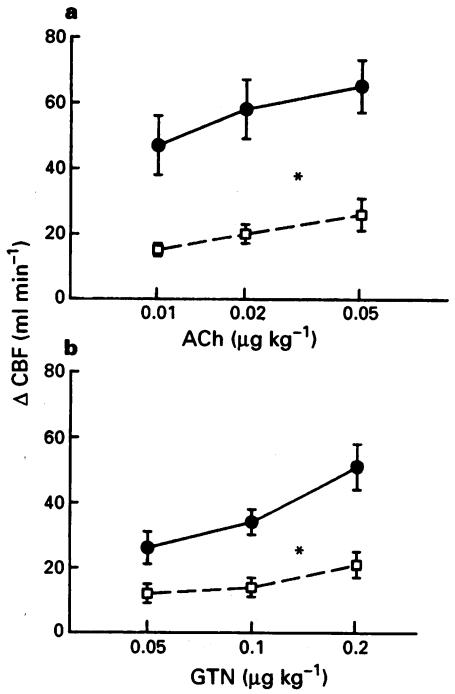

Figure 1 Dose-response effects on coronary blood flow (CBF) produced by intra-coronary injection of acetylcholine (ACh, a) and glyceryl trinitrate (GTN, b) in untreated dogs (Group 1). Control responses for either dilator (●) are significantly greater than those produced after 60 min of coronary artery occlusion and 30 min of reperfusion (□). *$P<0.05$, two-way analysis of variance.

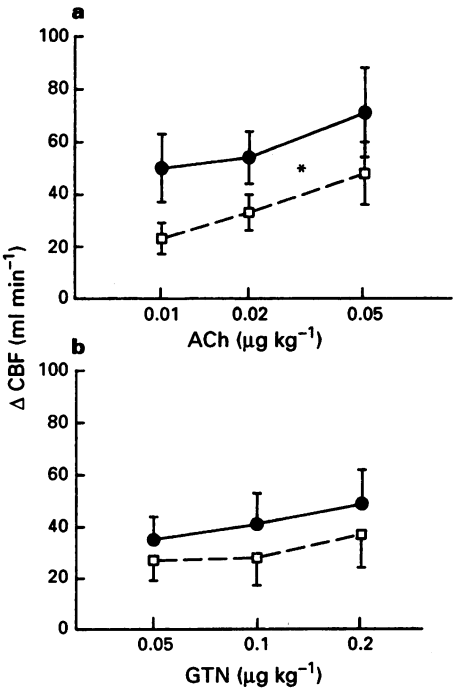

Figure 2 Dose-response effects on coronary blood flow (CBF) produced by intra-coronary injection of acetylcholine (ACh, a) and glyceryl trinitrate (GTN, b) in amlodipine-treated dogs (Group 2). Control responses (●) for ACh are significantly greater than those produced after 60 min of coronary artery occlusion and 30 min of reperfusion (□). *$P<0.05$, two-way analysis of variance; there is no significant difference between control and post-ischaemic responses to GTN.

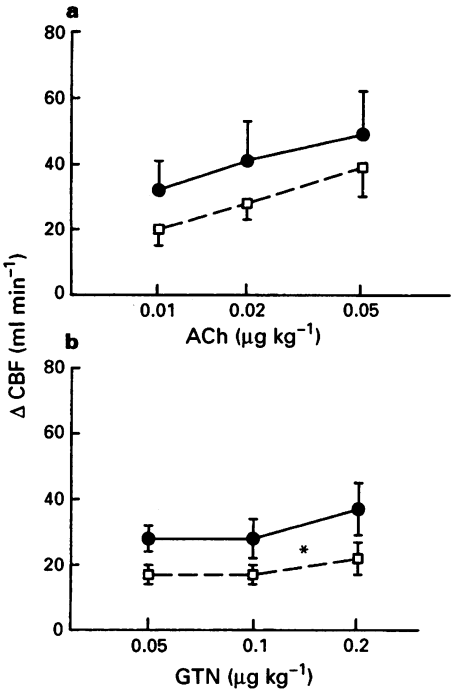

Figure 3 Dose-response effects on coronary blood flow (CBF) produced by intra-coronary injection of acetylcholine (ACh, a) and glyceryl trinitrate (GTN, b) in allopurinol-treated dogs (Group 3). There is no significant difference between control responses (●) to ACh and those obtained after 60 min of coronary artery occlusion and 30 min of reperfusion (□). Control responses to GTN are significantly greater than post-ischaemic responses: $*P<0.05$, two-way analysis of variance.

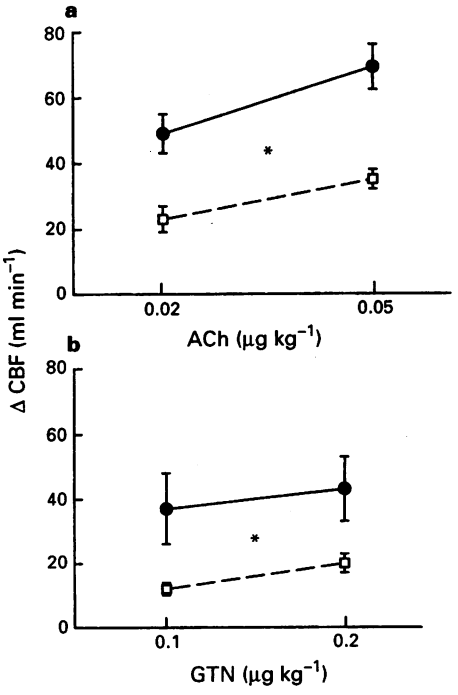

Figure 4 Dose-response effects on coronary blood flow (CBF) produced by intra-coronary injection of acetylcholine (ACh, a) and glyceryl trinitrate (GTN, b) in propranolol-treated dogs (Group 4). Control responses for either dilator (●) are significantly greater than those produced after 60 min of coronary artery occlusion and 30 min of reperfusion (□). $*P<0.05$, two-way analysis of variance.

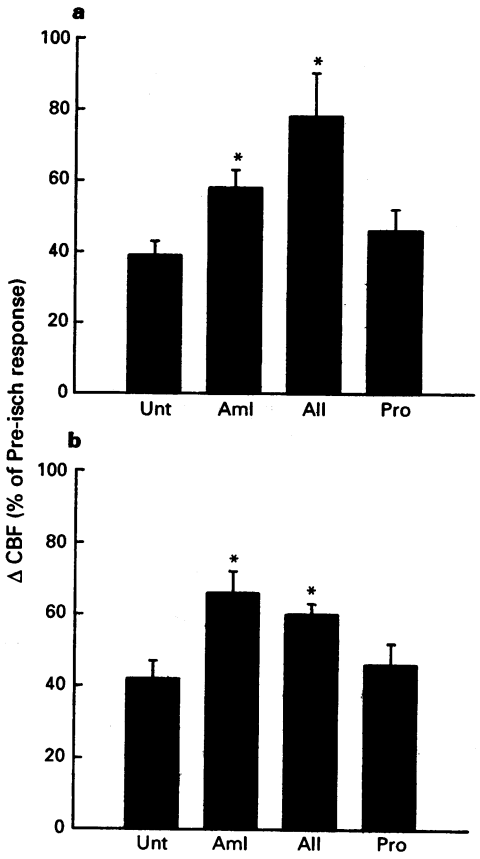

Figure 5 Increases in coronary blood flow (CBF) after 60 min of coronary artery occlusion and 30 min of reperfusion expressed as a percentage of the control response produced before ischaemia (Pre-isch). Post-ischaemic dilator responses to both acetylcholine (a) and glyceryl trinitrate (b) are significantly greater in dogs treated with amlodipine (Aml) and allopurinol (All) than in untreated dogs (Unt) or dogs treated with propranolol (Pro). $*P<0.05$, one-way analysis of variance.

dilator responses after ischaemia and reperfusion (Figure 3). Glyceryl trinitrate-induced responses were significantly reduced in comparison to control (Figure 3; $P<0.05$, two-way analysis of variance), but both acetylcholine- and glyceryl trinitrate-induced responses after ischaemia and reperfusion (78 ± 12% and 60 ± 3%, respectively) were significantly improved by allopurinol treatment (Figure 5; both $P<0.05$ compared with Group 1, one-way analysis of variance).

Group 4: Propranolol-treated dogs Increases in coronary blood flow produced by both acetylcholine and glyceryl trinitrate were significantly reduced after 60 min of coronary occlusion and 30 min of reperfusion (Figure 4; both $P<0.05$). Mean post-ischaemic increases in coronary blood flow produced by acetylcholine and glyceryl trinitrate were both 46 ± 6% of the respective pre-ischaemic control responses (Figure 5; both values not different from Group 1). Thus propranolol did not protect against ischaemia-induced impairment of coronary vasodilatation.

Discussion

This study has confirmed previous reports (Mehta *et al.*, 1989b; Sobey *et al.*, 1990) that increases in coronary blood flow produced by either acetylcholine (an endothelium-dependent dilator) or glyceryl trinitrate (an endothelium-independent dilator) are markedly reduced after an acute period

of ischaemia and reperfusion in anaesthetized dogs. In this model, large coronary artery dilatation by both agents *in vivo* is also impaired after ischaemia and reperfusion (Sobey *et al.*, 1990). However, we (Sobey *et al.*, 1990; 1992) and others (Ku, 1982; Vanbenthuysen *et al.*, 1987; Mehta *et al.*, 1989a; Lefer *et al.*, 1991) have shown that in isolated coronary arteries previously exposed to ischaemia and reperfusion, endothelium-independent relaxation remains intact. Thus, *in vitro* findings do not necessarily reflect the post-ischaemic coronary vasodilator capacity *in vivo*, highlighting the importance of examining the effects of ischaemia on vascular function in the intact circulation. We recently reported that *in vivo* administration of amlodipine (a calcium entry inhibitor), allopurinol (a xanthine oxidase inhibitor) or propranolol (a β-adrenceptor antagonist) significantly protects endothelial function in ischaemic and reperfused large coronary arteries *in vitro* (Sobey *et al.*, 1992). In the present study we have examined whether these pharmacological agents can also prevent the non-specific attenuation by ischaemia and reperfusion of coronary resistance vessel dilatation *in vivo*.

The present findings indicate that amlodipine and allopurinol partially prevent attenuation of both endothelium dependent and endothelium-independent increases in coronary blood flow after ischaemia and reperfusion. Both of these agents also prevented a post-ischaemic impairment in cardiac contractility, consistent with their ability to reduce myocardial infarct size (Werns *et al.*, 1986; Chambers *et al.*, 1987; Hoff *et al.*, 1989). In contrast, protection of endothelial function by propranolol (Sobey *et al.*, 1992) is clearly not sufficient to prevent the attenuation of coronary resistance vessel dilatation *in vivo* after ischaemia and reperfusion. Therefore, in addition to endothelial damage, there must be processes occurring *in vivo* that cause non-specific impairment of post-ischaemic coronary dilator mechanisms.

The protective effects of amlodipine and allopurinol are further evidence that elevated intracellular calcium levels and superoxide anion production, respectively, may be involved in the impairment of vasodilator function after ischaemia and reperfusion. Inhibition of calcium entry by amlodipine during ischaemia and reperfusion may be beneficial in a number of cell types. For example, activation of leukocytes and platelets would be inhibited, and the reduced contractility of cardiac and vascular smooth muscle would limit oxygen demands and hence the hypoxic damage to these cells. As ischaemia and reperfusion does not cause vascular smooth muscle damage in this model (Vanbenthuysen *et al.*, 1987; Mehta *et al.*, 1989a; Sobey *et al.*, 1990; 1992), the protection by allopurinol and amlodipine is probably not mediated by an action on that cell type.

Infiltration of circulating leukocytes into microvessels of the ischaemic myocardium could be an important contributing factor in the reduction of post-ischaemic dilator responses. This event results in the physical obstruction, or 'plugging', of small coronary vessels, and is associated with a gradual reduction of post-ischaemic coronary blood flow known as the 'no-reflow' phenomenon (Kloner *et al.*, 1974; Engler, 1987). A reduced coronary vasodilator reserve then results from the consequent dilatation of non-occluded microvessels to maintain resting coronary blood flow. In addition to these physical effects, the accumulation of activated leukocytes in ischaemic and reperfused coronary arteries (Mullane *et al.*, 1984; Sobey *et al.*, 1990) and myocardium (Romson *et al.*, 1983; Engler *et al.*, 1983; Mullane *et al.*, 1984; Nichols *et al.*, 1988; Sobey *et al.*, 1990; Sheridan *et al.*, 1991; Lefer *et al.*, 1991) is associated with tissue damage that is thought to be mediated by oxygen-derived free radicals. At least part of the protection by amlodipine and allopurinol could therefore involve an inhibitory effect on leukocyte infiltration and/or function. Such an action by amlodipine is possible, since two other dihydropyridine calcium antagonists, nifedipine and nisoldipine, inhibit the generation of several human neutrophil products, including superoxide anion (Jouvin-Marche *et al.*, 1983; Irita *et al.*, 1986). This effect of the calcium antagonists may be independent of their inhibition of calcium influx, for nisoldipine inhibited NADPH oxidase and thus superoxide generation by neutrophils in the absence of extracellular calcium (Irita *et al.*, 1986). Inhibition of leukocyte functions by amlodipine might ultimately produce a number of protective actions resembling those of superoxide dismutase, a well known scavenger of superoxide anions, which inhibits both neutrophil accumulation and the reduction of post-ischaemic coronary vasodilator reserve in ischaemic myocardium of dogs (Mehta *et al.*, 1989c). In combination with the hydrogen peroxide scavenger catalase, superoxide dismutase also improves regional coronary blood flow and decreases microvascular injury after ischaemia and reperfusion in dogs (Przyklenk & Kloner, 1989). Whilst allopurinol inhibits superoxide anion generation by xanthine oxidase, an enzyme localized in the vascular endothelium (Ferrari *et al.*, 1990), it does not inhibit superoxide production, chemotaxis or degranulation by human neutrophils (Jones *et al.*, 1985; Capecchi *et al.*, 1988). Therefore, a direct action by allopurinol on leukocytes in this study is unlikely.

Ischaemia-induced vasoconstriction (Sobey *et al.*, 1990; Chu *et al.*, 1990) could be partly due to a reduction in basal release of the vasodilators nitric oxide and prostacyclin from damaged endothelial cells. However, this process probably did not contribute to the reduction in coronary vasodilatation, since endothelium function *in vitro* (Sobey *et al.*, 1992), but not vasodilatation *in vivo*, was normal in coronary arteries of propranolol-treated dogs. The reason for the sustained reduction in post-ischaemic coronary blood flow in allopurinol- and propranolol-treated dogs only is unclear, but this is unlikely to have influenced the dilator responses to acetylcholine and glyceryl trinitrate because allopurinol, but not propranolol, protected against the impairment of dilatation after ischaemia.

In conclusion, the non-specific attenuation of coronary resistance vessel dilatation that occurs *in vivo* following ischaemia and reperfusion can be inhibited by administration of amlodipine or allopurinol, but not propranolol. These results support the involvement of elevated intracellular calcium levels and superoxide anion generation in the mechanism of post-ischaemic vasodilator dysfunction. Since all three of these agents prevent post-ischaemic endothelial dysfunction (Sobey *et al.*, 1992), the maintenance of vasodilator reserve by amlodipine and allopurinol probably involves additional mechanisms. An inhibitory action of these drugs on leukocyte infiltration and activation is one such possibility that warrants further investigation. Recently reported evidence that nifedipine reduces the incidence of myocardial infarction following coronary bypass surgery (Seitelberger *et al.*, 1991) further emphasises that such protective actions by amlodipine, allopurinol or chemically related pharmacological agents may be of potential clinical benefit.

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Mediators of nonadrenergic, noncholinergic inhibition in the proximal, middle and distal regions of rat colon

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1 The mediators of non-adrenergic non-cholinergic (NANC) relaxation of the longitudinal muscle of rat proximal, middle and distal colon were examined *in vitro*.

2 Electrical transmural stimulation (TMS) of proximal, middle and distal segments of rat colon induced NANC relaxations which were inhibited by tetrodotoxin (1 μM), but not by atropine (1 μM) or guanethidine (4 μM).

3 In the proximal colon, L-nitro-arginine (N^5-nitroamidino-L-2,5-diaminopentanoic acid) inhibited the TMS-induced NANC relaxation and L-arginine (1 mM) reversed this inhibition. Nitric oxide (0.3–10 μM) induced relaxation of the proximal segment.

4 NANC relaxation of the proximal segments was still evident after desensitization to vasoactive intestinal peptide (VIP). A VIP antagonist (VIP 10-28, 10 μM) had no effect on the TMS-induced NANC relaxation, which was also resistant to α-chymotrypsin (2 units ml^{-1}) and a substance P antagonist ([D-Pro^2, D-$Trp^{7,9}$]substance P, 1 μM).

5 In the middle colon, L-nitro-arginine did not inhibit the TMS-induced NANC relaxation in 6 of 9 preparations tested and partially inhibited the relaxation in the other 3 preparations. L-Arginine did not reverse the partial inhibition.

6 Complete desensitization to VIP was not achieved in the middle colon. The VIP antagonist had no effect on the TMS-induced NANC relaxation. After α-chymotrypsin treatment of the segment, desensitization of the segments to substance P, or in the presence of the substance P antagonist, the TMS-induced NANC relaxation was augmented.

7 In the distal colon, L-nitro-arginine did not have any significant effect on the TMS-induced relaxation and nitric oxide did not induce relaxation. The VIP antagonist significantly inhibited TMS-induced NANC relaxation. α-Chymotrypsin-treatment of the distal segments resulted in significant inhibition of NANC relaxation. No desensitization to substance P was achieved. Treatment with the substance P antagonist had no effect.

8 These results suggest that nitric oxide is the mediator of the NANC inhibitory response in the proximal region of rat colon; in the middle colon, substance P acts as an excitatory neurotransmitter, antagonizing the NANC relaxation caused by the mediator of the response, which is still uncertain. Our results also suggest that VIP is the most likely candidate as a NANC transmitter in the distal colon.

Keywords: Nitric oxide; vasoactive intestinal peptide; NANC inhibition; colonic motility; substance P

Introduction

Since Burnstock (1986) first reported a non-adrenergic, non-cholinergic (NANC) inhibitory neural system in the gastrointestinal tract, several possible neurotransmitters have been studied. The distribution and combinations of NANC neurones in the myenteric and submucous plexuses are extremely complex. Investigations on neurotransmission of the enteric inhibitory neurones throughout the gut have indicated that inhibitory neural control varies from one region of the gut to another (Wood, 1987).

Recent reports have indicated a role of nitric oxide in NANC neuromuscular transmission in many tissues other than blood vessels, such as anococcygeus muscle in the rat (Gillespie *et al.*, 1989; Hobbs & Gibson, 1990) and mouse (Gibson *et al.*, 1990), the ileocolonic junction (Bult *et al.*, 1990) and duodenum (Toda *et al.*, 1990) of the dog, the gastric fundus in the rat (Boeckxstaens *et al.*, 1991), the lower oesophageal sphincter in the opossum (Tottrup *et al.*, 1991) and the dog (De Man *et al.*, 1991), and guinea-pig trachea (Tucker *et al.*, 1990; Li *et al.*, 1991). We have also suggested that, in rats, nitric oxide is important in inducing descending relaxation in the circular muscle of the proximal colon (Hata *et al.*, 1990) and relaxation of both the longitudinal and circular muscles of the ileum (Kanada *et al.*, 1992). Furthermore, nitric oxide synthase, an enzyme that produces nitric oxide from L-arginine, has been demonstrated in rats in the brain (Knowles *et al.*, 1990) and nerve fibres in the intestine, retina, adrenal medulla and blood vessels (Bredt *et al.*, 1990). Thus, nitric oxide seems to act as a mediator of the NANC inhibitory response in various tissues.

Another putative neurotransmitter of the enteric nervous system, vasoactive intestinal peptide (VIP), has also been studied extensively. There are numerous reports of VIP-induced relaxation of gastrointestinal muscle (Fahrenkrug *et al.*, 1978; Goyal *et al.*, 1980; Bitar & Makhlouf, 1982; Furness & Costa, 1982; Grider *et al.*, 1985a,b) and inhibition by VIP antiserum of neurally-induced muscle relaxation (Grider & Makhlouf, 1986; Grider & Rivier, 1990). In the present study, we examined the effects of nitric oxide and VIP as possible mediators of the NANC inhibitory responses in longitudinal muscles of the proximal, middle and distal regions of rat colon. We also studied the role of substance P neurones, which have been identified in the gastrointestinal tract of many species of mammals (Pernow, 1951; Pearse & Polak, 1975; Brodin & Nilsson, 1981; Brodin *et al.*, 1983; Bartho & Holzer, 1985), in relation to NANC relaxation in the three regions of rat colon. The results indicated that the mediators of the NANC inhibitory response, and the parti-

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cipation of excitatory and inhibitory peptide neurones, in the motility of the colon differed in the proximal, middle and distal regions of rat colon. A preliminary report of these results has been published (Suthamnatpong *et al.*, 1991).

Methods

Preparations of proximal, middle and distal segments of rat colon

Male Wistar rats (250–380 g) were lightly anaesthetized with ether and then stunned by a blow on the head and bled via the carotid arteries. Proximal, middle and distal segments of the colon were removed and placed in Tyrode solution consisting of (in mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, and glucose 5.6.

The narrow part formed by sphincter in the ascending colon defined the boundary between proximal and middle regions. The portion which is attached by mesentery with the small intestine was defined as the distal region. The contents of the excised segments were gently flushed out with Tyrode solution, and lengths of 2.0–3.0 cm were used for experiments.

Recording of responses of longitudinal muscle to electrical transmural stimulation

Colonic segments were suspended in an organ bath filled with Tyrode solution aerated with 5% CO_2 in O_2 and maintained at 37°C. The oral end of each segment was attached to a transducer and the anal end was mounted on an anodal electrode placed at the bottom of the bath. Responses of the longitudinal muscle to transmural stimulation with trains of 100 pulses of 0.1 ms width at 30 V, and 10 Hz frequency, were recorded isotonically with a 10 min interval between tests. The longitudinal muscle was subjected to a resting load of 1.0 g. The preparations were equilibrated for at least 30 min before experiments. Drugs were added to the organ bath, when responses to TMS became constant, in volumes of less than 1.0% of the bathing solution. These volumes of vehicle of the drugs, redistilled water, did not affect the spontaneous contractile activity or the muscle tone.

Treatment of the colonic segments with α-chymotrypsin

Colonic segments were treated with α-chymotrypsin by adding the agent to the organ bath at a final concentration of 2 units ml^{-1}, which is known to reduce peptide response (De Beurme & Lefebvre, 1987). Also in our preliminary experiments, substance P and VIP did not affect the segments at all after a preincubation of them with this concentration of α-chymotrypsin for 20 min at 37° C.

Drugs

L-Nitro-arginine (N^5-nitroamidino-L-2,5-diaminopentanoic acid), L-arginine, α-chymotrypsin and D-arginine hydrochloride were purchased from Sigma Chemical Co., St. Louis, U.S.A. Atropine sulphate, guanethidine, vasoactive intestinal peptide and tetrodotoxin were from Wako Pure Chemical, Osaka, Japan. Substance P and [D-Pro^2,D-$Trp^{7,9}$]substance P were from the Peptide Institute, Osaka, Japan. Vasoactive intestinal peptide (10–28) was from Peninsula Laboratories, Calif., U.S.A. All other chemicals were of analytical grade. Gaseous nitric oxide was dissolved in Tyrode solution freshly before experiments as described by Gillespie & Sheng (1988). The nitric oxide solution was added to the organ bath in volumes of 0.3–300 μl. These volumes of Tryode solution alone did not affect the spontaneous contractile activity or the muscle tone.

Statistical analysis

Results were analysed statistically by Student's paired *t* test and a *P* value of <0.05 was regarded as significant.

Results

Responses of longitudinal muscle of the proximal, middle and distal colon to electrical transmural stimulation

Longitudinal muscle of the proximal segment of rat colon exhibited spontaneous contractile activity with wider amplitude and higher frequency than muscles of the middle and distal segments. TMS of the proximal segment at 1–50 Hz induced rapid, transient relaxations and then rebound contraction, both in the absence and presence of atropine (1 μM) and guanethidine (4 μM). The amplitude of the relaxation increased with an increase in frequency. In subsequent studies, the segments were stimulated at 10 Hz, because this stimulation gave the most reproducible responses (Figure 1). The frequency of the spontaneous contractile activity of the middle colon was less than that of the proximal and distal segments, and transient rapid relaxation followed by rebound contraction on TMS at 10 Hz was seen only in the presence of atropine and guanethidine (Figure 2). On the other hand, the distal colon exhibited moderate spontaneous contractile activity and very small relaxation on TMS. However, the resting tone of the longitudinal muscle gradually increased during successive trains of TMS at 10 Hz at 10 min intervals. With progresive increase in the tone in this way, the segments began to exhibit clear relaxation followed by a large rebound contraction on TMS over the range of frequencies tested (1–50 Hz), but only in the presence of atropine and guanethidine. In subsequent studies, the segments were stimulated at 10 Hz with good reproducibility of response (Figure 3).

Thus the longitudinal muscles of the proximal, middle and distal colon exhibited different patterns of spontaneous contractile activities, but all three segments exhibited rapid clear transient relaxation on TMS in the presence of atropine and guanethidine. Tetrodotoxin (1 μM) inhibited the TMS-induced responses of the preparations.

Effects of L-nitro-arginine and L-arginine on non-adrenergic, non-cholinergic inhibitory responses of longitudinal muscles obtained from three different regions of the rat colon

Neither L-nitro-arginine (N^5-nitroamidino-L-2,5-diaminopentanoic acid) at concentrations up to 10 μM nor L-arginine at concentrations of up to 1 mM had any significant effect on the spontaneous contractile activity or on the tone of the longitudinal muscle of the rat proximal colon. However, L-nitro-arginine (10 μM) markedly inhibited TMS-induced NANC relaxation observed over the range of frequencies tested (1–50 Hz), causing almost complete inhibition within 20–40 min (Figure 1 and Table 1). Addition of L-arginine (1 mM) to the bathing fluid gradually reversed the effect of L-nitro-arginine, causing complete reversal in 20–30 min (Figure 1 and Table 1). D-Arginine (1 mM) had no effect (data not shown).

In the middle colon, TMS-induced NANC relaxation was partially inhibited by L-nitro-arginine in only 3 of 9 preparations (Figure 2a) and L-arginine did not reverse the effect of L-nitro-arginine. In the other 6 preparations, however, L-nitro-arginine did not have any significant effect (Figure 2b and Table 1).

In contrast to its effect in the proximal colon, L-nitro-arginine did not have any significant effect on TMS-induced NANC relaxation in any of 9 preparations of the distal colon (Figure 3 and Table 1).

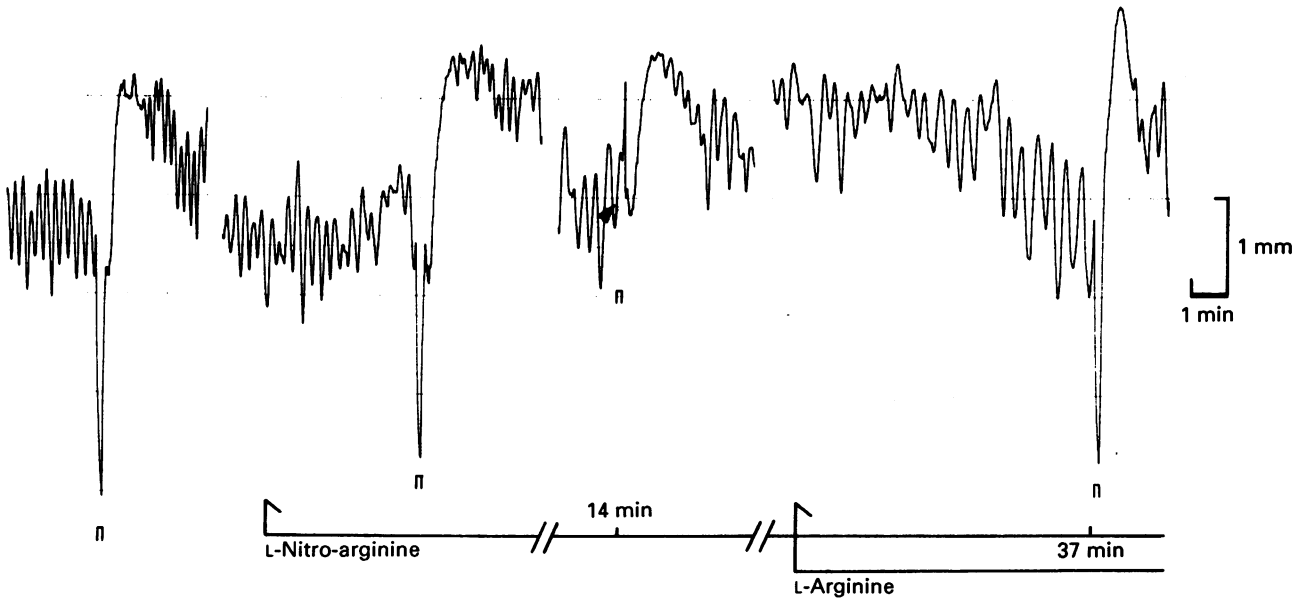

Figure 1 Efects of L-nitro-arginine and L-arginine on transmural stimulation (TMS)-induced relaxation of segments of proximal colon. L-Nitro-arginine (10 μM) and L-arginine (1 mM) were added at the times indicated by the arrows. The continuous lines indicate the presence of L-nitro-arginine and L-arginine in the bathing fluid. Times noted on the lines indicate the time after addition of L-nitro-arginine. Small bars indicate 10 s TMS at 10 Hz. Arrowhead indicates the beginning of the third train of TMS. Atropine (1 μM) and guanethidine (4 μM) were present throughout.

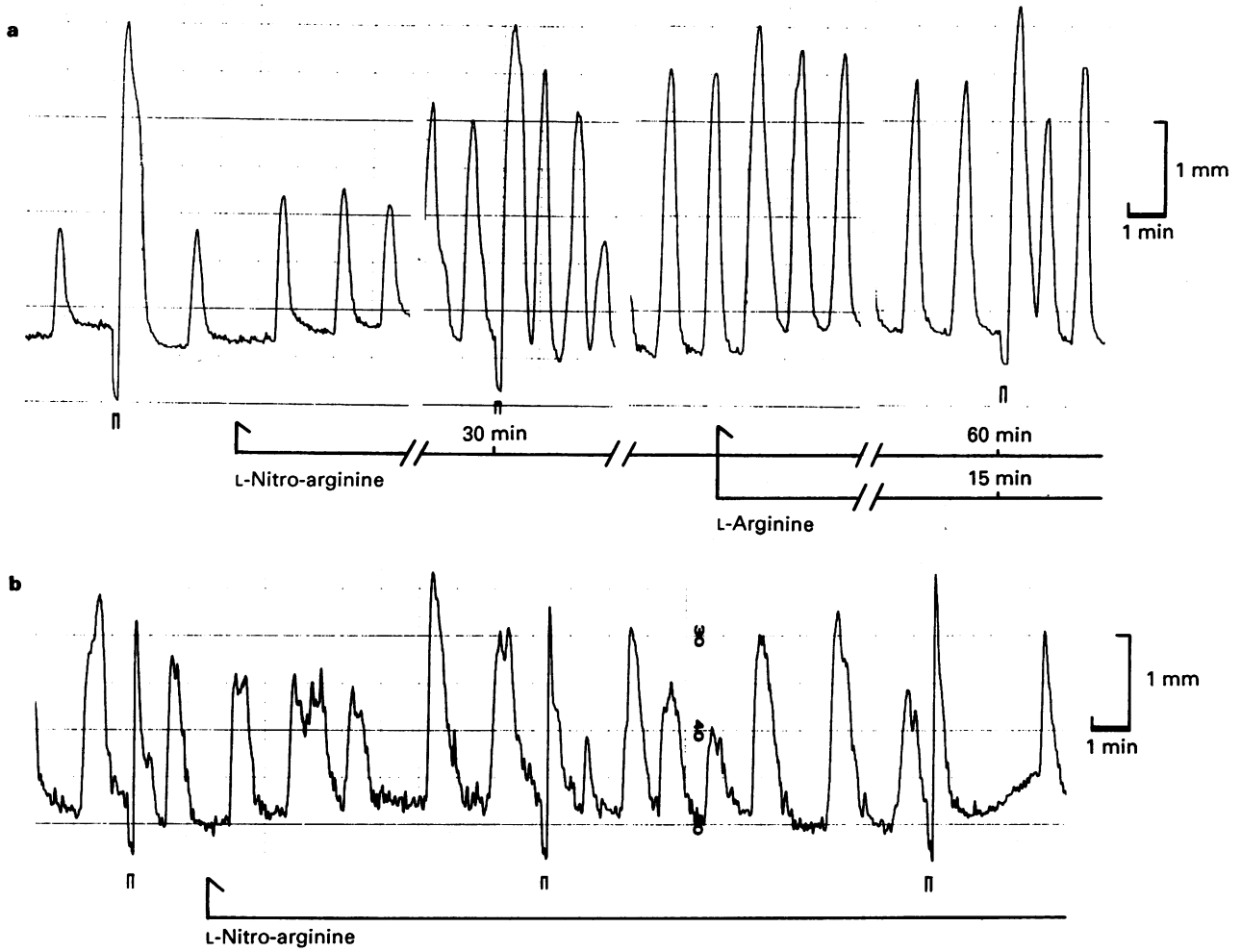

Figure 2 Effects of L-nitro-arginine and L-arginine on transmural stimulation (TMS)-induced relaxation of segments of middle colon. (a) Type A, an example of 3 out of 9 preparations in which responses were reduced by L-nitro-arginine; (b) Type B, an example of 6 out of 9 preparations in which L-nitro-arginine had no effect. The lines indicate the presence of L-nitro-arginine (10 μM) and L-arginine (1 mM). Times noted on the lines indicate time after addition of L-nitro-arginine. Small bars indicate 10 s TMS at 10 Hz.

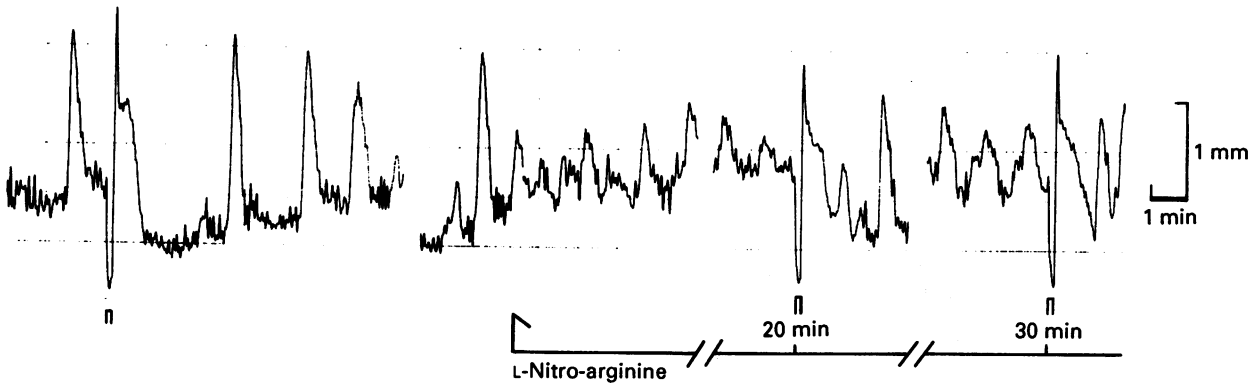

Figure 3 Transmural stimulation (TMS)-induced relaxation of segments of distal colon before and after treatment with L-nitro-arginine. The lines indicate the presence of L-nitro-arginine (10 μM). Times noted on the line indicate time after addition of L-nitro-arginine. Small bars indicate 10 s TMS at 10 Hz.

Effects of nitric oxide on the longitudinal muscles of different regions of rat colon

Nitric oxide (0.3–10 μM) induced relaxation of the proximal colon in a concentration-dependent manner (Figure 4). L-nitro-arginine had no effect on the relaxation induced by nitric oxide (not shown). Nitric oxide slightly inhibited spontaneous contractile activity, but did not induce relaxation of either the middle or distal colon (not shown).

Effects of α-chymotrypsin on TMS-induced NANC relaxation of colonic segments

Next, we examined the effect of α-chymotrypsin treatment to determine the possible contribution of peptidergic neurones to NANC inhibition in the different regions of the colon. Treatment of the proximal colon with α-chymotrypsin (2 units ml^{-1}) for 30 min had no effect on the muscle tone, spontaneous contractile activity, or TMS-induced NANC relaxation (Table 1). In the middle colon, TMS-induced NANC relaxation increased progressively during treatment with α-chymotrypsin for 10–20 min (Table 1) in all 5 preparations tested. In some preparations, however, this treatment moderately inhibited the spontaneous contractile activity and slightly decreased the muscle tone. In the distal colon, treatment with α-chymotrypsin for 20 min resulted in significant decrease of TMS-induced NANC relaxation (Table 1). In some preparations it slightly decreased the muscle tone and inhibited the spontaneous contractile activity.

These data suggested that some excitatory or inhibitory peptide neurones affect NANC inhibition, so we next tested the effects of various peptides.

Effects of substance P and a substance P antagonist on the motility and TMS-induced NANC relaxation of colonic segments

Substance P (1 μM) significantly increased the muscle tone and spontaneous activity of segments of all three regions. Three consecutive applications of substance P to middle colonic segments, without washing, resulted in desensitization to substance P. In contrast, repetitive applications of substance

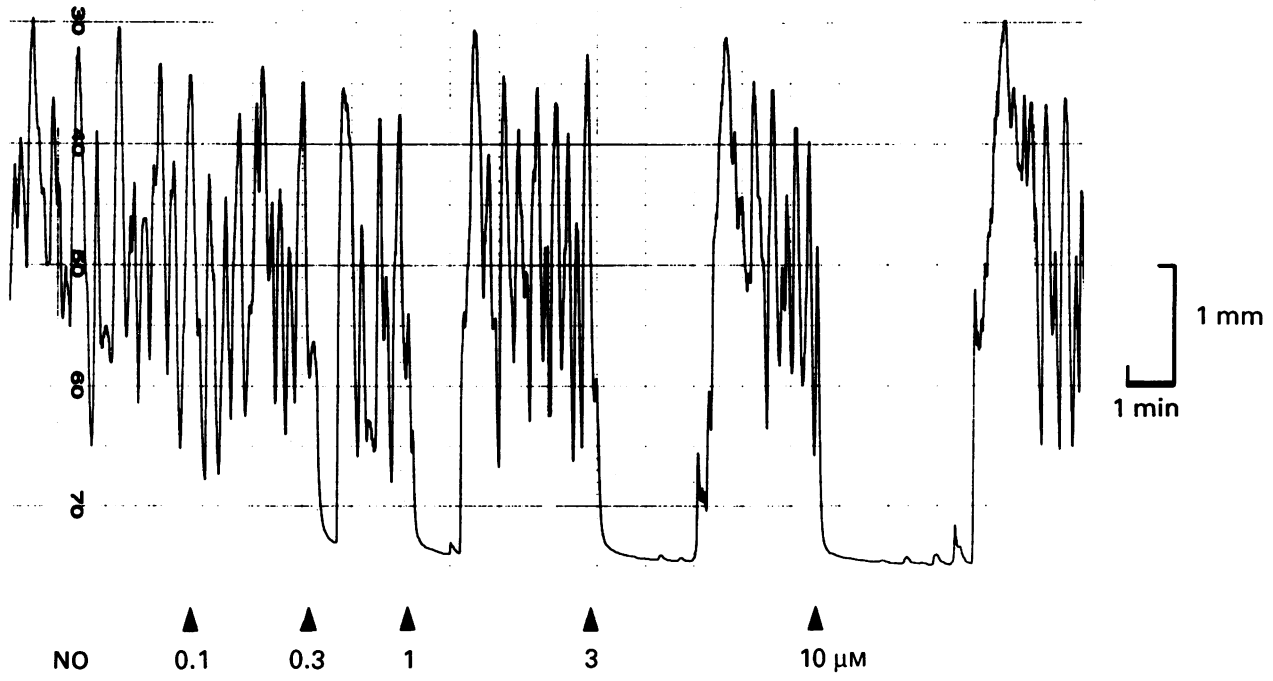

Figure 4 Relaxations of segments of proximal colon to nitric oxide (NO). Various concentrations of nitric oxide were added at the times indicated by the triangles. The bathing fluid was not changed during the time period shown. This record is typical of 10 preparations.

Table 1 Effects of various treatments on electrical transmural stimulation (TMS)-induced relaxation of longitudinal muscle obtained from three regions of the rat colon

Treatment	Relaxation after treatment (% of control) Proximal	Middle	Distal
LNitro-arginine (10 μM)	12.7 ± 6.7 (11)*	Type A 28.3 ± 15.2 (3) Type B 103.0 ± 4.3 (6)	96.7 ± 6.3 (9)
L-Arginine (1 mM) after L-nitro-arginine	92.6 ± 4.3 (8)†	Type A 38.0 ± 7.6 (3)	–
α-Chymotrypsin (2 u ml^{-1})	98.8 ± 2.9 (5)	174.0 ± 26.5 (5)**	69.4 ± 6.4 (9)*
Substance-P desensitization	–	184.0 (167, 200)	–
Substance-P antagonist (1 μM)	100 (93, 106)	200.0 ± 22.0 (7)*	91.3 ± 17.0 (3)
VIP desensitization	98.3 ± 3.8 (3)	–	69.5 ± 11.6 (4)*,***
VIP antagonist (10 μM)	109 (118,100)	97 (100,94)	64.8 ± 7.5 (5)*

Segments were treated with drugs for the durations noted in the text. Responses induced by TMS at 10 Hz after the treatments were expressed as a percentage of those before the treatments (Control). Relaxation in middle colon was partially inhibited (Type A) or not affected (Type B) by L-nitro-arginine. *The preparations were incompletely desensitized to VIP (see text). Values are means ± s.e.mean for the numbers of experiments shown in parentheses. Where only 2 observations were obtained the numbers in parentheses give the individual values.
Significantly different from the value of corresponding control by Student's paired t test: *$P < 0.01$; **$P < 0.02$; ***$P < 0.05$; and from the value with L-nitro-arginine: †$P < 0.01$.

P to proximal and distal colonic segments did not result in desensitization. In two desensitized preparations of the middle colon, TMS-induced relaxation was somewhat augmented (Table 1). This result suggested that substance P counteracts the NANC inhibitory response, so we tested the effect of a substance P antagonist in the following experiment.

A substance P antagonist ([D-Pro^2,D-Trp^{7,9}]substance P, 1 μM) had no effect on the basal tone, spontaneous contractile activity or TMS-induced NANC relaxation of segments of proximal and distal colon, but it gradually increased the muscle tone and spontaneous contractile activity of middle colonic segments and significantly increased TMS-induced relaxation in all 7 preparations tested (Table 1). The antagonist (1 μM) completely inhibited the response induced by exogenously added substance P.

Effects of VIP and a VIP antagonist on motility and TMS-induced NANC inhibition in colonic segments

VIP (0.1 μM) caused transient relaxation of longitudinal muscle of the proximal colon and inhibited the spontaneous contractile activity. However, within 10 min, the spontaneous contractile activity and muscle tone were restored, even in the presence of VIP. Repetitive application of 0.1 μM VIP, every 10 min without washing, markedly decreased the sensitivity of the segments to VIP, and complete desensitization was consistently achieved after 2 or 3 consecutive applications of VIP. TMS-induced NANC relaxation remained unchanged in all 3 desensitized preparations (Figure 5 and Table 1).

In the segments of the middle and distal colon, 0.1 μM VIP inhibited spontaneous contractile activity without having any significant effect on muscle tone. The inhibitory effects of VIP in the middle and distal colon persisted for about 15–20 min and more than 1 h, respectively. Repeated application of the same concentration of VIP after recovery of spontaneous contraction resulted in similar prolonged inhibition, although the duration of inhibition became progressively shorter. Thus, segments of the middle colon were not desensitized to VIP even by 5 to 6 applications of VIP in this way. Moreover, TMS-induced NANC relaxation was not significantly altered after 5–6 applications of VIP in all 5 preparations tested (not shown).

The distal colon was not totally desensitized to VIP even by 3–4 applications of VIP in a period of 2–3 h. However, repeated applications of VIP resulted in partial inhibition of TMS-induced NANC relaxation in all 4 preparations tested (Table 1).

As the middle or distal colon showed no or incomplete desensitization to VIP, respectively, we further examined the

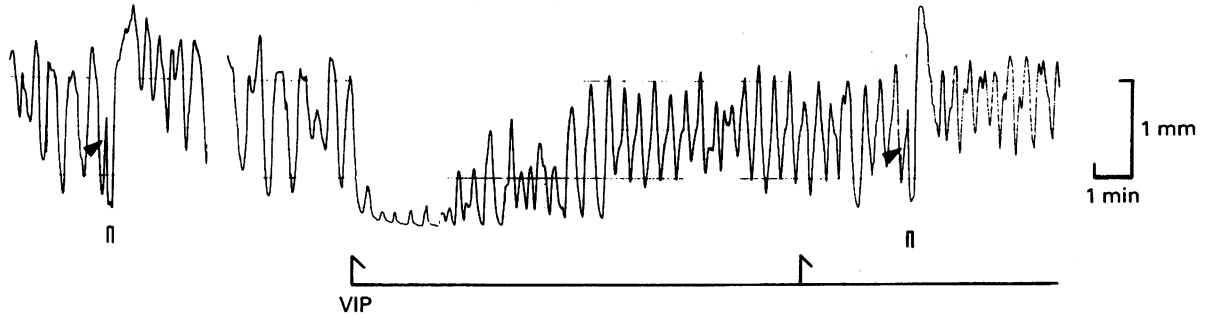

Figure 5 Transmural stimulation (TMS)-induced relaxation of segments of proximal colon before and after desensitisation with vasoactive intestinal peptide (VIP). VIP (100 nM) was added at the times indicated by the arrows. The line indicates the presence of VIP. Small bars indicate 10 s TMS at 10 Hz.

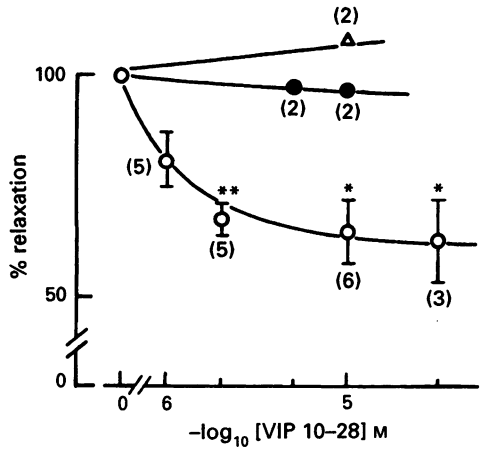

Figure 6 Effect of the vasoactive intestinal peptide (VIP) antagonist, VIP (10–28) on transmural stimulation-induced relaxation of segments of proximal (Δ), middle (●) and distal (○) colon. Relaxations in the presence of the antagonist are expressed as a percentage of those obtained before addition of the antagonist (control). Values are mean ± s.e.mean (vertical bars) of the number of experiments shown in parentheses. $*P < 0.01$; $**P < 0.05$: significantly different from the value of the corresponding control.

effect of a VIP antagonist (VIP 10–28) to study the participation of VIP neurones in these regions. In proximal and middle colonic segments, the relaxation induced by TMS was not altered by this VIP antagonist at concentrations up to 10 μM (Figure 6 and Table 1). The VIP antagonist also had no effect on the basal tone or spontaneous contractile activity. In the distal colon, the VIP antagonist had no effect on the basal tone or spontaneous contractile activity but inhibited the TMS-induced NANC relaxation in a concentration-dependent manner: it inhibited the TMS-induced NANC relaxation slightly at 1 μM, significantly at concentrations of over 2 μM, and by about 40% at 30 μM (Figure 6 and Table 1). The inhibitory effect was pronounced 5–10 min after its application. The antagonist (10 μM) completely inhibited the response induced by exogenously added VIP.

Discussion

The rat colon can be divided into three regions morphologically (Christensen *et al.*, 1984) and functionally (Ferre & Ruckebusch, 1985). In the present study, we found several clear differences in the motilities of these three regions, proximal, middle and distal. First, their frequencies and amplitude of spontanteous contraction were different, being highest in the proximal region, and lowest in the middle region. Second, development of TMS-induced relaxation was different in the distal colon from that in the other two regions: TMS initially induced very little relaxation in distal colon, but on successive TMS at 10 min intervals, relaxation of the distal colon gradually increased and became constant after 4–5 stimulations, whereas the relaxations of the other two regions usually remained constant from the beginning of the experiment. A third difference was the excitatory control by cholinergic neurones: in middle and distal regions, TMS-induced NANC relaxation occurred only in the presence of atropine, whereas, in the proximal colon it occurred in both the presence and absence of atropine. The absence of effect of atropine on NANC inhibition in the proximal colon suggests dominant NANC control or inferior cholinergic control in this region. The inhibitory effect of tetrodotoxin on the TMS-induced relaxation in all three regions of rat colon in the presence of atropine and guanethidine confirmed that the TMS-induced relaxation is mediated by NANC inhibitory neurones.

There were clear differences in NANC inhibitory neurotransmission in the proximal, middle and distal colon. L-Nitro-arginine is a guanidino-substituted arginine derivative that inhibits the synthesis of nitric oxide from L-arginine (Gibson *et al.*, 1990). L-Nitro-arginine inhibited TMS-induced NANC relaxation appreciably in the proximal colon, and this inhibition was reversed by L-arginine. However, L-nitro-arginine inhibited TMS-induced NANC relaxation only slightly in the middle colon and was not inhibitory in the distal colon. The reversibility of the inhibition by L-arginine in the proximal colon suggests that L-nitro-arginine acts competitively, and the absence of effect of the D-isomer indicates that the effect is stereo-specific. In a previous study, we found that nitric oxide mediates relaxation of circular muscle in the proximal colon of rats (Hata *et al.*, 1990). The present results on the effect of nitric oxide on the longitudinal muscle of this region are parallel to this finding.

A tonic inhibitory control mediated by nitric oxide has been suggested in rat anococcygeus muscle (Gillespie *et al.*, 1989) and rat gastric fundus (Grider *et al.*, 1985b): in these tissues, blockade of biosynthesis of nitric oxide raised the basal tension or elicited contraction. However, the inhibitory effect of L-nitro-arginine was selective for TMS-induced NANC relaxation in the proximal colon: this drug had no effect on the spontaneous contractile actvitiy or muscle tone in segments of any of the three regions, in the presence or absence of atropine and guanethidine.

Interestingly, L-nitro-arginine did not inhibit the TMS-induced NANC relaxation in 6 of 9 preparations of middle colon. Furthermore, in the other 3 preparations whose TMS-induced NANC relaxation was partially inhibited by L-nitro-arginine, L-arginine at concentrations of up to 10 mM did not reverse the inhibitory effect of L-nitro-arginine. The reason for this absence of effect of L-arginine is unknown, but the results suggest that nitric oxide has only a minor role, if any, in inducing NANC relaxation in the middle region. The ineffectiveness of nitric oxide in inducing relaxation in this region supports this idea. Similarly, in the distal region, it seems unlikely that nitric oxide mediates NANC relaxation, because L-nitro-arginine did not inhibit NANC relaxation and nitric oxide did not induce relaxation.

It has been suggested that VIP is a neurotransmitter of NANC nerves in the gut (Goyal *et al.*, 1980; Furness & Costa, 1982; Grider & Makhlouf, 1986; Grider & Rivier, 1990). To investigate this possibility, we examined the influence of α-chymotrypsin treatment of the colonic segments, and the effects of a VIP antagonist, and exogenous VIP, on the TMS-induced NANC relaxation of the colonic segments. α-Chymotrypsin is known to cleave peptides at tyrosine, lysine and arginine residues, and these three amino acids are present in the VIP sequence. Therefore, the influence of α-chymotrypsin treatment on VIP-induced or NANC relaxation has been studied in many tissues, such as duodenum (Manzini *et al.*, 1990), ileum (Manzini *et al.*, 1986) and gastric fundus (De Beurme & Lefebvre, 1987) of rats. A VIP antagonist, the C-terminal partial sequence of VIP (VIP 10-28), was reported to be a stronger antagonist than [4-Cl-D-Phe^6, Leu^{17}]VIP or [Ac-Tyr^1, D-Phe^2]GRF1-29 (Grider & Rivier, 1990).

In the proximal colon, TMS-induced NANC relaxation was still observed after desensitization to VIP, and α-chymotrypsin treatment had no effect on TMS-induced NANC relaxation. Thus, VIP is unlikely to be a NANC neurotransmitter in the proximal colon. In the middle colon also, the VIP antagonist and α-chymotrypsin-treatment did not decrease the TMS-induced relaxation. On the other hand, both α-chymotrypsin-treatment and the VIP antagonist inhibited TMS-induced NANC relaxation in the distal colon. Complete desensitization to VIP was not observed, but TMS-induced NANC relaxation was partially inhibited by successive administrations of exogenous VIP. Thus, VIP seems to have a role as a neurotransmitter in TMS-induced NANC

relaxation in the distal colon.

In contrast to the present findings, Grider & Rivier (1990) reported that a VIP antagonist and VIP antiserum abolished descending NANC relaxation in the middle colon of rats. They used segments of 4–6 cm length of the midcolon, while we used segments of 2–3 cm length. Thus, their segments may have included part of the region that we defined as distal colon. Moreover, they induced descending relaxation by mechanical stretch of the circular muscle on the oral side of the segment. Thus, their observed response might have been affected by the response of the distal region.

Interestingly, treatment of only the middle colon with α-chymotrypsin augmented the TMS-induced NANC relaxation and inhibited spontaneous contractile activity. These findings suggest that the activity of some excitatory peptidergic neurones normally masks the NANC inhibitory response to some extent. Substance P is known to cause contraction of smooth muscle of the gut (Pernow, 1983; Bártho & Holzer, 1985). Hou *et al.* (1989) suggested that there are two sites of action of substance P to induce smooth muscle contraction depending on whether the nerves function normally or not. Substance P releases acetylcholine by acting preferentially on cholinergic neurones when the nerves are intact, but acts directly on the smooth muscle when conduction of the nerves is blocked. Therefore, we studied the effect of substance P and a substance P antagonist, [D-Pro^2,-D-Trp^{7,9}]substance P on the NANC inhibitory responses of the colonic segments. Substance P stimulated colonic contractile activity, resulting in prolonged, large-amplitude contraction. As our experiments were carried out in the presence of atropine, the site of action of substance P was its receptor on the smooth muscle. Desensitization of the middle colonic segments to substance P was achieved by successive applications of substance P without washing. In this state the TMS-induced NANC relaxation was significantly augmented. This result is consistent with the finding that the substance P antagonist augmented TMS-induced NANC relaxation of the middle colon. On the other hand, no desensitization to substance P was observed in the proximal or distal colonic segments, and the substance P antagonist had no effect on TMS-induced NANC relaxation in these two regions. Thus, substance P neurones seem to participate mainly in rat middle colon and normally mask the NANC inhibitory response to some extent in this region.

The present results suggest that the mediators of the NANC inhibitory response in longitudinal muscle differ in different regions of rat colon. Our results indicate a role of nitric oxide, but not of VIP, as the mediator in the proximal region, and the participation of VIP neurones but not of nitric oxide in the NANC inhibitory response in the distal region. Our results did not identify the mediator of the response in the middle region, but suggested that activation of substance P neurones had a negative effect on the NANC inhibitory response in this region.

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Lymphocyte β_2-adrenoceptors and adenosine 3′:5′-cyclic monophosphate during and after normal pregnancy

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1 The β_2-sympathomimetics, used to inhibit preterm labour, bind predominantly to β_2-adrenoceptors, activating adenylate cyclase to form adenosine 3′:5′-cyclic monophosphate (cyclic AMP), a messenger substance which inhibits the enzyme cascade triggering smooth muscle contraction. β_2-Adrenoceptor density and cyclic AMP formation can be used as markers of β_2-adrenergic effect.

2 The present study addresses the influence of pregnancy on the β-adrenoceptor system. β_2-Adrenoceptor density and cyclic AMP concentrations (basal and evoked by isoprenaline) in circulating lymphocytes were determined at three points in gestation (16, 29 and 37 weeks) and 9 weeks post partum in 22 normal pregnancies. (−)-[^{125}Iodo]-cyanopindolol was used as the ligand to identify a homogeneous population of β_2-adrenoceptors on lymphocytes. B- and T-cell fractions were estimated from the same samples.

3 β_2-Adrenoceptor density decreased significantly during gestation until week 37 ($P<0.01$), then increased post partum ($P<0.005$). Cyclic AMP concentrations (basal and evoked by isoprenaline) were significantly lower after 16 weeks of gestation than post partum ($P<0.05$).

4 The results, which cannot be explained in terms of a shift in the lymphocyte (B- and T-cell) ratio, indicate that β-adrenoceptor density and function are reduced in normal pregnancy and only return to normal post partum. These findings may be of significance in devising future tocolytic therapy with β_2-adrenoceptor agonists.

Keywords: β_2-Adrenoceptors; cyclic AMP; lymphocytes; normal pregnancy; regulation

Introduction

β_2-Adrenoceptor agonists (β_2-sympathomimetics) relax uterine smooth muscle, which is why they are often used for tocolysis in preterm labour. By binding to myometrial membrane β_2-adrenoceptors, they stimulate adenylate cyclase to form adenosine 3′:5′-cyclic monophosphate (cyclic AMP) from adenosine triphosphate. Cyclic AMP formation, an index of receptor function, inhibits the enzyme cascade resulting in contraction. Animal studies and studies in human subjects, including evidence from clinical experience, have shown a decrease in the tocolytic efficacy of β_2-adrenoceptor agonists during administration (Johansson & Andersson, 1981; Berg *et al.*, 1982; Rydén *et al.*, 1982; Casper & Lye, 1986). However, intermittent as opposed to continuous administration has been shown to prolong the duration of tocolytic effect at least in sheep *in vivo* and in human myometrium *in vitro* (Ke *et al.*, 1984; Casper & Lye, 1986). Berg *et al.* (1982; 1985), and subsequently other investigators (Ekblad *et al.*, 1987; Michel *et al.*, 1989) found a decrease in myometrial β-adrenoceptor density and cyclic AMP formation in women receiving β_2-adrenoceptor agonists. Taken together, these data suggest that β_2-sympathomimetic efficacy is influenced by receptor status and function, and that this in turn is regulated by drug dose and mode of application. However, β_2-adrenoceptor function is additionally influenced by a number of other factors (Motulsky & Insel, 1982; Stiles *et al.*, 1984; Lefkowitz & Caron, 1985), which may influence adrenoceptor function before treatment has even started. The aim of this study was to determine the effect of pregnancy on the β_2-adrenoceptor system, for which the available data are few and partly contradictory.

Twenty-two healthy women with uncomplicated pregnancies were each assessed throughout pregnancy and also post partum. The test material consisted of circulating lymphocytes, which are frequently used to study alterations of β-adrenoceptor density and function in man (Berg *et al.*, 1984; Brodde *et al.*, 1985; 1990; Martinsson *et al.*, 1987; Santala *et al.*, 1990a). As Michel *et al.* (1989) recently showed, there is a positive correlation between β-adrenoceptor density and function in myometrium and in circulating lymphocytes in pregnant women (with or without β_2-adrenoceptor agonist treatment) and we confirmed this correlation in preliminary studies in a group of 16 pregnant women (nontreated $n=13$; fenoterol-treated $n=3$) undergoing caesarean section (ligand: (−)-[^{125}iodo]-pindolol = IPIN; myometrium: K_d 18.8 ± 1.2 pM; B_{max} 9.4 ± 1.1 fmol IPIN mg^{-1} protein); lymphocytes: K_d 42.3 ± 4.5 pM; B_{max} 454 ± 68 IPIN binding sites per cell; correlation between myometrial and lymphocyte B_{max}: $r=0.60$, $n=16$, $P<0.05$. Furthermore, we have taken the precaution of investigating possible changes in lymphocyte B- and T-cell ratio during and after pregnancy, since β-adrenoceptor density is higher on B- than T-cells (Paietta & Schwarzmeier, 1983; Landmann *et al.*, 1984; Griese *et al.*, 1988). The results are discussed in terms of the potential implications for tocolytic therapy using β_2-adrenoceptor agonists.

Methods

Subjects

Twenty-two healthy pregnant women were included in the study. All were of normal weight before the study (mean ± s.d.: 58 ± 7.3 kg; range: 49–79 kg). Weight gain during pregnancy was less than 15 kg. Maternal age at delivery was 30 ± 6 years (mean ± s.d.). Five smoked during pregnancy (>5 cigarettes a day). All were uncomplicated pregnancies ending in fullterm normal deliveries.

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Study design

Subjects were recruited at their first antenatal visit (on average in week 16 of gestation), after providing their informed consent. Whole blood samples were taken concurrently with the blood for routine pregnancy monitoring, so that additional venepuncture was unnecessary. Each subject was sampled three times during pregnancy, in weeks 16 (15.9), 29 (28.6) and 37 (36.4), and once post partum, in week 9 (8.1). This study protocol was approved by the local Ethics Committee.

Analytical procedures

Lymphocyte isolation from venous blood Thirty ml venous blood, collected in 10 ml vacutainer tubes containing 0.34 M EDTA, was mixed with 20 ml phosphate buffered saline (PBS) within 15 min after venepuncture at room temperature. Lymphocytes were separated at room temperature in Ficoll Paque (Pharmacia, Uppsala, Sweden) by the method of Böyum (1969).

β_2-Adrenoceptor density and cyclic AMP (and protein) concentrations were determined in intact lymphocyte suspensions in 20 mM Tris HCl, 105 mM NaCl buffer pH 7.4 containing 5×10^6 cells ml^{-1}.

Determination of β_2-adrenoceptors Determination of β_2-adrenoceptor density in intact lymphocytes was performed by the binding of (−)-[^{125}iodo]-cyanopindolol (ICYP) (Engel *et al.*, 1981), by a method described by Brodde *et al.* (1981) [duplicate estimations in 100 µl lymphocyte suspensions ($= 0.5 \times 10^6$ cells) incubated with 50 µl ICYP at 6 different concentrations between 10 and 250 pM and, for the estimation of nonspecific binding, with 100 µl of the nonselective β-adrenoceptor antagonist (±)-CGP 12177 (Staehelin *et al.*, 1983), 1 µM, in a final volume of 250 µl for 60 min at 37°C].

Cyclic AMP assay Lymphocyte suspension (500 µl = 2.5×10^6 cells) (at least six aliquots) was incubated with 500 µl of 2.2 mM 3-isobutyl-1-methylxanthine (IBMX) solution for 10 min at 37°C. The mixture was then incubated either with 100 µl water (for determination of basal cyclic AMP) or with 100 µl of 0.1 mM isoprenaline (for determination of total cyclic AMP following stimulation) for 5 min in a stirred water bath at 37°C, vortexed and then re-incubated for 5 min. The reaction was stopped by immediate centrifugation at 4°C and 3000 r.p.m. for 5 min. The supernatant was discarded and the residue taken up in 1 ml 0.1 M HCl, sonicated for 5 min and left to stand for 25 min at room temperature. The mixture was then centrifuged at 3000 r.p.m. and room temperature and the residue snap-frozen in 800 µl aliquots in 2 ml Eppendorf tubes. Samples were freeze-dried within a week and stored in sealed containers over periodically renewed silica gel at 4°C for a maximum of 6 months. With this method the samples can be stored without cyclic AMP hydrolysis. Repeated measurements performed within the decribed storage time of samples from the same person under identical conditions did not vary by more than 15%.

Cyclic AMP was determined by a modified version of the method of Tovey *et al.* (1974). The method is basically that of Gilman (1970) which was often used earlier; in contrast to Gilman's method, no prior purification is necessary and the test can be performed with a commercial kit. Freeze-dried samples were redissolved in 20 mM Tris HCl 105 mM NaCl buffer pH 7.4. Buffer volume was adjusted for each sample to give a cyclic AMP concentration on the straight line of the binding curve (4–8 pmol mg^{-1} protein), and the protein-bound [^{3}H]-cyclic AMP was determined in a liquid scintillation counter (Beckmann β-counter LS 1801). Each sample was assayed in duplicate. Measurements of freeze-dried samples from the same person under identical conditions were repeated on a following day.

Protein concentration Protein concentrations were measured in 100 and 200 µl aliquots of the lymphocyte suspension used for the cyclic AMP assay ($= 0.5$ and 1×10^6 cells), by the method of Lowry *et al.* (1951).

B/T-lymphocyte fractions From the blood sampling for lymphocyte isolation, venous blood was collected in heparinized vacutainer tubes to determine the B/T-lymphocyte fractions using double labelled monoclonal antibodies (T-cells: antibody binding to T11 E-rosette receptor associated antigen CD2; B-cells: antibody binding to B1 associated antigen CD20). After antibody labelling, the erythrocytes were lysed and lymphocytes fixed using Q-Prep (Coulter). The results were analysed with a flow cytometer (Coulter). Absolute values in $10^9 l^{-1}$ are calculated from relative values in % and from the absolute number of lymphocytes in the blood sample.

Chemicals

(−)-ICYP was obtained from New England Nuclear (NEX 189, specific activity: 2200 Ci $mmol^{-1}$) (Du Pont de Nemours International S.A., CH-8105 Regensdorf). A kit was used for the [^{3}H]-cyclic AMP protein binding assay (Amersham International plc, cyclic AMP assay kit TRK 432) (H. Rahn & Co, CH-8050 Zurich). Antibodies for B-T-lymphocyte fraction estimation were obtained from Coulter (COULTER CLONE T11 and/or B1) (Instrumentengesellschaft AG, CH-8045 Zurich).

Statistical evaluations

β_2-Adrenoceptor density Given the method used for determining β_2-adrenoceptor density, the parameters of the saturation curve for specific ICYP binding, i.e. the maximum number of binding sites (B_{max}) and the equilibrium dissociation constant (K_d), were determined by simultaneous curve fitting for affinity and nonspecific binding. Nonspecific binding was modelled by linear dependence on the free ligand concentration, and specific binding was calculated by the difference between total and nonspecific binding. The mathematical method used was the same, for example, as that used in the SCTFIT (Feldmann, 1972) and LIGAND programmes (data analysis and curve-fitting for ligand-binding experiments, Munson, P.J., National Institutes of Health, Bethesda, Maryland, U.S.A.) to describe binding to a binding site with nonsubtracted, nonspecific binding. Using the MINSQ PC programme (nonlinear parameter estimation and model development, MicroMath Scientific Software, Salt Lake City, Utah, U.S.A.), a few cases were selected to check that the results matched the SCTFIT programme. The variables of interest, B_{max} and K_d, were obtained from the parameters of the saturation curve. B_{max}, the variable used to describe adrenoceptor density, was expressed as the maximum bond concentration of ICYP in fmol per 10^6 cells and K_d in pM. B_{max} could be multiplied by Avogadro's number ($= 6.023 \times 10^{23}$) and expressed as the maximum number of ICYP binding sites or β_2-adrenoceptors per cell.

Lymphocyte cyclic AMP concentrations Cyclic AMP concentrations were calculated from the protein-bound [^{3}H]-cyclic AMP radioactivity, corrected for lymphocyte protein concentration and buffer volume, by use of an IBM-PC-compatible programme (Dr U. Honegger, Institute of Pharmacology, University, CH-3012 Berne). The programme calculates basal cyclic AMP and total cyclic AMP followed by the stimulation with isoprenaline by averaging values from the same sample in the same assay. The isoprenaline-evoked increase in cyclic AMP was determined by subtracting basal from total cyclic AMP followed by the stimulation with isoprenaline. Means of the concentrations from different assays were used for statistical analysis.

Statistical tests Statistical analysis was performed using the STAT-VIEW II TM programme on a Macintosh. Data were tested in each gestational age group for normality by the Shapiro-Wilk W test. Data for cyclic AMP and for lymphocytes were not normally distributed ($P<0.05$). They represent a log-normally distributed population (Shapiro-Wilk W test: $P>0.05$ after log-transformation). Because of these test results, mean values in the text and table are given both as mean ± standard error and as median (= 50th percentile; in parentheses). The figures show box plots containing the median and the 25th and 75th percentiles and whisker plots (= 10th and 90th percentiles). Where a value was missing (blood sample unavailable for procedural reasons, or deviation between repeated measures exceeding 15%), the number of samples was less than $n = 22$.

Wilcoxon's signed rank test was used to compare data between gestational age groups. The relationship between β_2-adrenoceptor density and the isoprenaline-evoked increase in cyclic AMP was tested by linear regression (given are the equation and the correlation coefficient r) and the Spearman rank correlation (given is the coefficient rs). A P value of <0.05 was used in all tests.

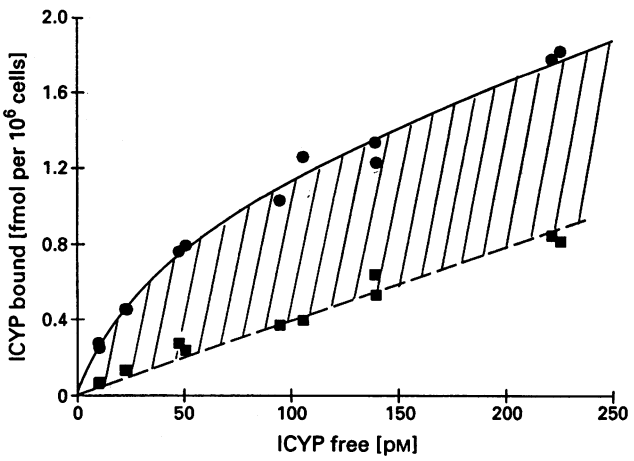

Figure 1 Typical example of simultaneous curve fitting for B_{max} and K_d. Ordinate scale: (−)-[^{125}Iodo]-cyanopindolol (ICYP) bound (fmol per 10^6 cells). Abscissa scale: ICYP free (pM). The continuous curve shows total binding (nonspecific and specific). Nonspecific binding increases linearly with the free ICYP ligand concentration and is denoted by a broken line, while specific binding (the difference between total and nonspecific binding) (hatched area) shows saturation characteristics.

Results

ICYP showed saturable high affinity binding to intact lymphocytes (Figure 1, Table 1).

β_2-Adrenoceptor density (B_{max}) significantly decreased during normal gestation (Table 1 and Figure 2), by 13% between weeks 16 and 29 (NS), by 42% between weeks 16 and 37 ($P<0.01$, $n = 18$) and by 33% between weeks 29 and 37 ($P<0.05$, $n = 19$). There was a mean significant increase post partum of 56% ($P<0.005$ vs week 37, $n = 19$). B_{max} did not differ significantly between early pregnancy (663 ± 83 (696) binding sites per cell) and post partum (605 ± 72 (526) binding sites per cell). K_d showed a slight nonsignificant increase during pregnancy and returned post partum to levels similar to those in early pregnancy (Table 1).

The cyclic AMP increase evoked by isoprenaline (= total concentration following stimulation minus basal concentration) was reduced by 24% from weeks 16 (156 ± 25 pmol mg^{-1} protein) to 29 (119 ± 10 pmol mg^{-1} protein) (NS) and increased significantly post partum ($P<0.05$ vs weeks 29

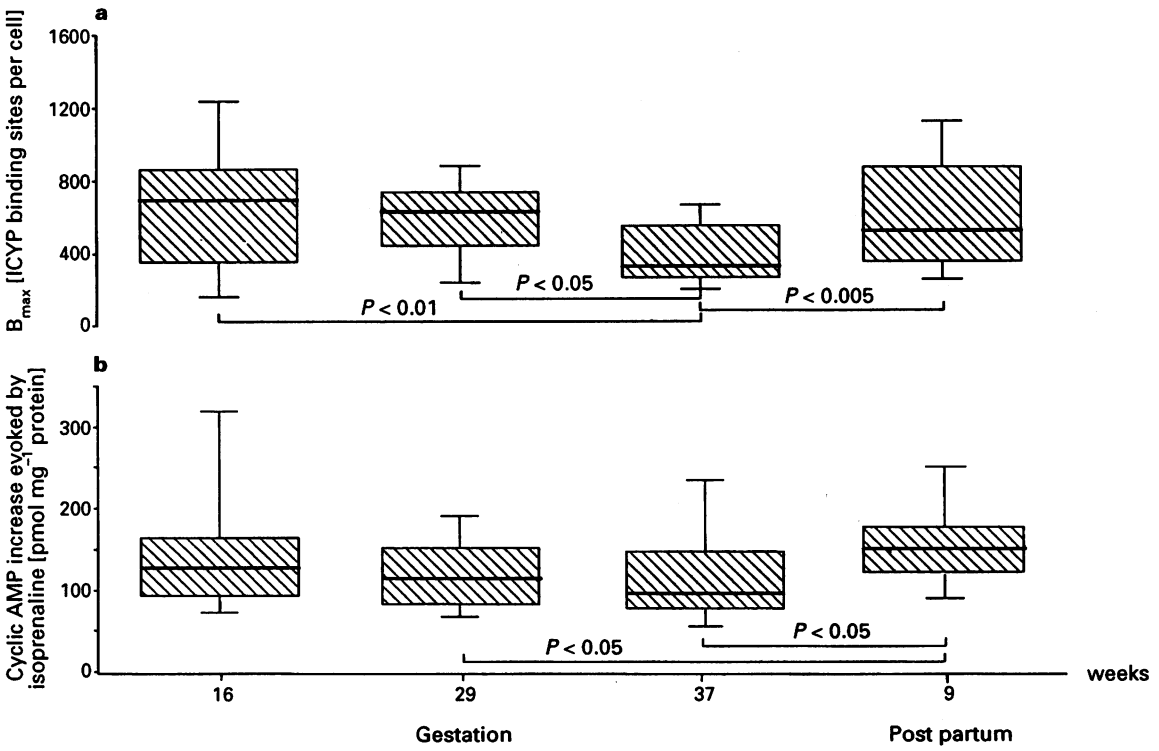

Figure 2 Lymphocyte β_2-adrenoceptor density (a) and lymphocyte cyclic AMP increase evoked by 0.1 mM isoprenaline (b) in 22 healthy women during normal pregnancy and after delivery. Ordinate scale: (a) β_2-adrenoceptor density (B_{max}) in intact lymphocytes in (−)-[^{125}Iodo]-cyanopindolol (ICYP) binding sites per cell; (b) cyclic AMP increase evoked by 0.1 mM isoprenaline in lymphocytes in pmol mg^{-1} protein. Shown: box and whisker plots; medians were compared by Wilcoxon's signed rank test. β_2-Adrenoceptor density and the cyclic AMP increase evoked by isoprenaline are significantly lower at 29 and 37 weeks of gestation than after pregnancy.

Table 1 β_2-Adrenoceptor density (B_{max}), equilibrium dissocation constant (K_d), cyclic AMP basal and cyclic AMP increase (evoked by 0.1 mM isoprenaline) in circulating lymphocytes during and after normal pregnancy

		Weeks of gestation		*Weeks post partum*
	15.1 ± 0.5 (15.9)	28.7 ± 0.4 (28.6)	36.1 ± 0.3 (36.4)	8.3 ± 0.4 (8.1)
Normal pregnancies ($n = 22$)				
B_{max} [ICYP binding sites per cell]	663 ± 83 (696) $n = 21$	579 ± 49 (631) $n = 22$	387 ± 42 (332)* $n = 19$	605 ± 72 (526)† $n = 21$
K_d [pM]	44 ± 7 (42) $n = 20$	57 ± 9 (50) $n = 22$	74 ± 16 (50) $n = 19$	47 ± 8 (35) $n = 19$
Cyclic AMP basal [pmol mg^{-1} protein]	71 ± 12 (55) $n = 14$	48 ± 4 (48) $n = 21$	47 ± 5 (43) $n = 20$	82 ± 11 (59)* $n = 21$
Cyclic AMP increase [pmol mg^{-1} protein]	156 ± 25 (132) $n = 14$	119 ± 10 (115) $n = 21$	127 ± 14 (104) $n = 20$	158 ± 13 (154)* $n = 21$

Wilcoxon's signed rank test for comparison versus preceding value: *$P < 0.05$; †$P < 0.005$.
Values given as mean ± s.e.mean with median in parentheses.

and 37) to values similar to those in early pregnancy (Table 1 and Figure 2). Basal cyclic AMP decreased by 32% from weeks 16 (71 ± 12 pmol mg^{-1} protein) to 29 (48 ± 4 pmol mg^{-1} protein) and increased significantly post partum ($P < 0.05$ vs weeks 29 and 37) (Table 1).

β_2-Adrenoceptor density showed a significant positive correlation with the cyclic AMP increase evoked by isoprenaline at every timepoint studied. At 16 weeks: $y = 0.183\,x + 31.368$, $r = 0.653$, $P < 0.05$; $rs = 0.601$, $P < 0.05$; at 29 weeks: $y = 0.099\,x + 61.314$, $r = 0.509$, $P < 0.05$; $rs = 0.444$, $P < 0.05$.

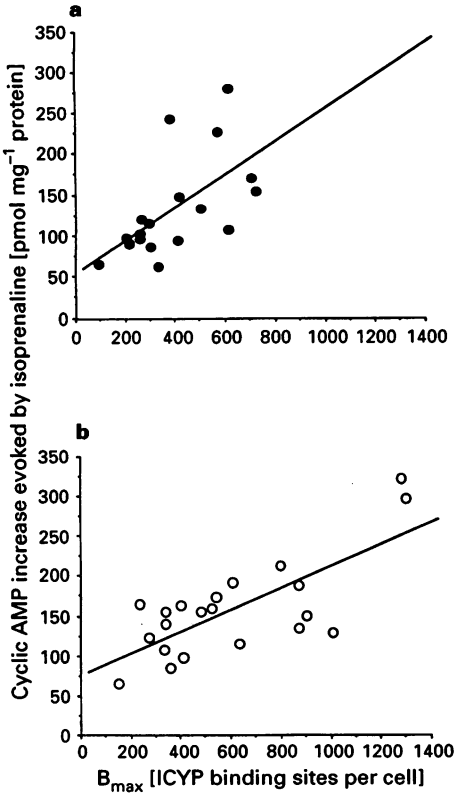

Figure 3 Correlation between lymphocyte β_2-adrenoceptor density and lymphocyte cyclic AMP increase evoked by 0.1 mM isoprenaline in 22 healthy women at 37 weeks of gestation (a, ●) and 9 weeks after delivery (b, ○). Ordinate scales: β_2-adrenoceptor density (B_{max}) in intact lymphocytes in (−)-[^{125}Iodo]-cyanopindolol (ICYP) binding sites per cell. Abscissa scales: cyclic AMP increase evoked by 0.1 mM isoprenaline in lymphocytes in pmol mg^{-1} protein. The β_2-adrenoceptor density correlates significantly with the cyclic AMP increase evoked by isoprenaline (= β_2-adrenoceptor function) at 37 weeks of gestation ($y = 0.205\,x + 50.706$, $r = 0.613$, $P < 0.005$; $rs = 0.689$, $P < 0.005$; $n = 19$) and 9 weeks after delivery ($y = 0.133\,x + 75.08$, $r = 0.737$, $P < 0.0001$; $rs = 0.513$, $P < 0.05$; $n = 21$).

The significant correlations for 37 weeks of gestation and 9 weeks post partum are shown in Figure 3.

The B-lymphocyte fraction increased slightly but not significantly during gestation. Post partum there was a significant reduction versus 37 weeks ($P < 0.005$, $n = 19$, and versus 29 and 16 weeks of gestation: $P < 0.0005$; Figure 4). The T-lymphocyte fraction increased significantly after 29 weeks of gestation and again post partum (29 versus 37 weeks: $P < 0.05$, $n = 20$; 37 weeks versus 9 weeks post partum: $P < 0.05$, $n = 19$; Figure 4). Absolute values of T-lymphocytes increased after 29 weeks of gestation and significantly post partum: 16 weeks: 1.64 ± 0.08 (1.71) × 10^9 l^{-1} versus 37 weeks: 2.03 ± 0.63 (1.97) × 10^9 l^{-1} ($P < 0.05$, $n = 18$); 37 weeks versus 9 weeks post partum (2.31 ± 0.11 (2.24) × 10^9 l^{-1}): $P < 0.005$, $n = 18$. Absolute values of B-lymphocytes showed no significant differences (0.25, 0.23, 0.24 and 0.22 × 10^9 l^{-1} at 16, 29, 37 weeks of gestation and at 9 weeks post partum respectively).

Discussion

Although β_2-adrenoceptor agonists (β_2-sympathomimetics) constitute the conventional tocolytic therapy of preterm labour, the approach is purely symptomatic, hence the outcome is often predetermined by the clinical state of the pregnant women. It also has additional drawbacks, on the one hand because tocolytic effect wanes during administration (desensitization), probably due to a decrease in adenylate cyclase function (Berg *et al.*, 1982; 1985; Rydén *et al.*, 1982; Casper & Lye, 1986), and on the other because the effect may be absent from the outset, despite a promising pretreatment situation.

Different studies have attempted to restore adrenoceptor activity and prevent desensitization. Discontinuation of treatment restores tocolytic efficacy, mirroring a restoration of adrenoceptor density (Brodde *et al.*, 1985; Martinsson *et al.*, 1987). Consonant with this, desensitization is reduced by intermittent versus continuous administration (Ke *et al.*, 1984; Casper & Lye, 1986). These findings indicate that waning efficacy can be avoided by tailoring the dosage schedule to the adrenoceptor status. In this connection the purpose of the present study was to determine whether there is any change in β_2-adrenoceptor density and function during the course of normal pregnancy.

Mean B_{max} values throughout the study period were 563 ± 34 (542) binding sites per cell. In the nonpregnant situation, mean B_{max} is 605 ± 72 (526), and 663 ± 83 (696) binding sites per cell in early gestation. Brodde *et al.* (1985) found similar values in whole lymphocytes from healthy volunteers with a similar cell count (767 ± 66 binding sites per cell, mean ± s.e.mean). Mean basal cyclic AMP throughout the present study was 61 ± 4 (50) and the mean isoprenaline-evoked increase in cyclic AMP was 139 ± 8

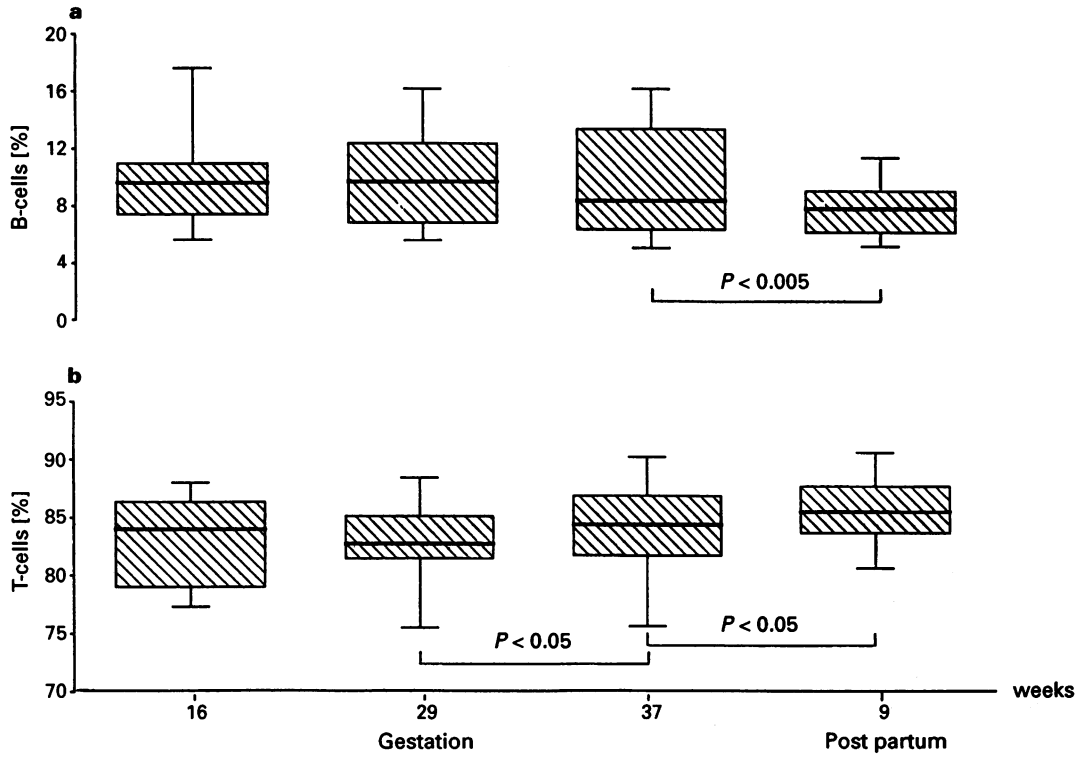

Figure 4 B-cells (a) and T-cells (b) in venous blood samples from healthy women during normal pregnancy and after delivery. Ordinate scales: (a) lymphocyte B-cells (relative) in %; (b) lymphocyte T-cells (relative) in %. Abscissa scales: weeks of gestation and post partum respectively. Shown: box and whisker plots; medians were compared by Wilcoxon's signed rank test. The T-cell fraction was higher at the expense of the B-cell fraction towards the end of pregnancy and post partum than in the first two pregnancy timepoints.

(122) pmol mg^{-1} protein. Using a similar method of measurement and the same Amersham kit, Mäki *et al.* (1988; 1990) found similar values in whole lymphocytes from healthy volunteers: a mean basal cyclic AMP concentration of 50 pmol mg^{-1} protein and an increase evoked by isoprenaline (0.1 mM) of 120 pmol mg^{-1} protein in the β_2-adrenoceptor stimulated group.

Our results show a mean reduction in β_2-adrenoceptor density (B_{max}) by 42% ($P<0.01$) during pregnancy. Reduction was sharpest between weeks 29 and 37, which is precisely when β_2-adrenoceptor tocolytic therapy tends to be performed. Post partum B_{max} rose to a value not differing significantly from that in early pregnancy. Accumulation of cyclic AMP following stimulation by a β-adrenoceptor agonist decreased, although not significantly, in early pregnancy (by 24% between weeks 16 and 29); after that, there was no change until week 37.

However, the correlation between β_2-adrenoceptor density and the isoprenaline-evoked increase in cyclic AMP was significantly positive at every timepoint studied, strongly implying that adrenoceptor function depends on density of available receptors (Figure 3). Basal cyclic AMP showed a similar pattern of change obtained with isoprenaline-evoked cyclic AMP, again implying diminished β-adrenoceptor function after 16 weeks of gestation.

That the changes in β_2-adrenoceptor density are the result of uneven β_2-adrenoceptor distribution on B- and T-lymphocytes can be excluded. Although our findings show a decrease in the B- and T-cell ratio during pregnancy, further significant decrease occurs post partum (Figure 4). Moreover, no significant differences were found in the absolute values of B-lymphocytes. T-lymphocytes increased after weeks 16 (absolute values) and 29 (relative values, Figure 4) of gestation and post partum. Hence, during the period investigated, changes in the B- and T-cell populations are unlikely to account for the observed changes in β_2-adrenoceptor density and function.

Although there is a substantial body of data on the regulation of adenylate cyclase, studies on pregnancy-related changes are comparatively few. Padbury *et al.* (1981) using sheep placenta, and Legrand *et al.* (1987) using rat myometrium, showed a fall in β-adrenoceptor density with increasing gestational age, as did Breuiller *et al.* (1987) using human placenta. In these early studies, receptor density changes could not be attributed exclusively to β_2-adrenoceptors, since nonspecific ligands measuring the total β-adrenoceptor population were used. Nevertheless, in support of a general decrease in β-adrenoceptor function, Cemerikic *et al.* (1985) found less cyclic AMP being formed in placentas of high rather than low gestational age following *in vitro* addition of ritrodrine. Nisell *et al.* (1988) found lymphocyte β-adrenoceptor densities in pregnant women of similar gestational age (weeks of gestation not specified) to be 40% lower than in non-pregnant women of matching age. The recently published study by Santala *et al.* (1990b), the sole study to date performed with women tested throughout pregnancy, showed a slight but non-significant fall in lymphocyte β_2-adrenoceptor density during gestation ((−)-ICYP ligand also used; cyclic AMP not studied). In contrast to these findings, which seem unanimous in indicating a fall in β- or β_2-adrenoceptor density, Dattel *et al.* (1986) found no difference in myometrial β-adrenoceptor density in women of differing gestational age. However, these latter results call for careful critical appraisal, since comparisons were made between patient populations with widely differing features (patients with and without β_2-adrenoceptor treatment not investigated separately).

In summary, our results support the contention that β_2-adrenoceptor density and function decrease during pregnancy, although the pregnancy-specific influences or mechanisms responsible for these changes remain unknown. Assuming that lymphocyte β_2-adrenoceptor density and function correlate with effects in uterine smooth muscle, the data imply that the ability of uterine smooth muscle to react to

β_2-adrenoceptor stimuli, and thus to relax, decreases with increasing gestational age. Assuming additionally that β_2-adrenoceptor desensitization occurs rapidly with increasing doses of β_2-adrenoceptor agonists, our results also indicate that the conventional dosage schedule for tocolysis should be revised. This may consist of doses as low as possible to suit the available status of β_2-adrenoceptors and to prevent rapid desensitization. Fewer side-effects from β_1-adrenoceptor stimulation would also be expected from a lower dose regime. Controlled clinical studies are now needed to test this hypothesis.

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Potentiation of aggregation and inhibition of adenylate cyclase in human platelets by prostaglandin E analogues

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1 The 16-phenoxy prostaglandin E analogue sulprostone consistently potentiates primary aggregation waves induced by adenosine 5'-diphosphate (ADP), PAF and 11,9-epoxymethano PGH_2 (U-46619) in platelet-rich plasma from human donors. The effect is not blocked by the TP-receptor antagonists, EP 092 and GR 32191. The high potency of sulprostone (threshold concentration = 4–10 nM) and the weak block of sulprostone potentiation by the EP_1-receptor antagonist, AH 6809 ($pA_2 = 4.3$) suggest the involvement of EP_3-receptors as opposed to EP_1- or EP_2-subtypes.

2 Eight prostaglandin E (PGE) analogues were compared against sulprostone for their effects on PAF-induced aggregation in human platelet-rich plasma (PRP) in the presence of GR 32191 and the DP-receptor antagonist, BW A868C. PGE_2 and 11-deoxy PGE_2-1-alcohol showed evidence of both potentiating and inhibitory actions and butaprost showed only inhibitory activity at high concentrations. The remaining analogues always elicited potentiation, with the following potency ranking: sulprostone = 16,16-dimethyl PGE_2 > MB 28767 > misoprostol > GR 63779X = 17-phenyl-ω-trinor PGE_2. The results again indicate that EP_3- rather than EP_1- or EP_2-receptors are involved. However, relative potentiating potency could be affected by differences in plasma protein binding and the very high sensitivity of the human platelet to prostacyclin (IP)-receptor-mediated inhibition (IC_{50} for the specific IP-receptor agonist cicaprost = 0.8 nM).

3 On human washed platelet suspensions the PGE analogues, with the exception of butaprost, inhibited the rise in adenosine 3':5'-cyclic monophosphate (cyclic AMP) induced by cicaprost (8 nM). PGE_2 produced a monophasic inhibition curve (IC_{50} = 5.4 nM, 92% inhibition at 600 nM). The potency ranking was 16,16-dimethyl PGE_2 > sulprostone > MB 28767 = PGE_2 > misoprostol > GR 63778X > 17-phenyl-ω-trinor PGE_2 > 11-deoxy PGE_2-1-alcohol. AH 6809 inhibited the effect of sulprostone and 17-phenyl-ω-trinor PGE_2 with pA_2 values of 5.75 and 5.32 respectively; these values are at least one log unit lower than those found for EP_1-receptor block in smooth muscle.

4 There is a statistically significant correlation between IC_{50} values for the PGE analogues on the human platelet cyclic AMP assay and the guinea-pig vas deferens (standard EP_3 preparation): slope = 1.00, $r = 0.80$, $P < 0.05$. However the correlation is far from ideal and GR 63779X in particular has a lower potency in the cyclic AMP assay. At this time we suggest that it is prudent to describe the human platelet receptor as 'EP_3-like'.

5 We believe that our results provide further evidence for linking PGE-induced potentiation of aggregation to inhibition of adenylate cyclase. Sulprostone is a suitable agonist for further study of this system and in particular the nature of the G-protein linkage(s) involved. In addition the necessity to consider potentiation of platelet aggregation in relation to the clinical use of PGE analogues in man is emphasised.

Keywords: Prostaglandin E_2; prostacyclin; sulprostone; cicaprost; potentiation of platelet aggregation; inhibition of adenylate cyclase; EP-receptors

Introduction

In the early phase of prostanoid research, prostaglandin E_1 (PGE_1) was shown to inhibit platelet aggregation in platelet-rich plasma (PRP) from man, pig and rat (Kloeze, 1967). This effect was associated with a rise in the adenosine 3':5'-cyclic monophosphate (cyclic AMP) level in the platelet (Vigdahl *et al.*, 1969; Robison *et al.*, 1969). Following the discovery of prostacyclin (PGI_2) (Moncada *et al.*, 1976), it became clear that PGE_1 probably behaves as an agonist at prostacyclin (IP-) receptors on platelets (Whittle *et al.*, 1978). This view was strengthened by the demonstration that PGE_1 competes with either [^{3}H]-PGI_2 (Siegl *et al.*, 1979; Eggerman *et al.*, 1986) or [^{3}H]-iloprost (Schillinger & Losert, 1980) for a saturable binding site on the human platelet. PGE_1 is about an order of magnitude less potent than PGI_2 in both functional and binding assays.

The effect of PGE_2 on platelet function is more variable and more difficult to analyse. In PRP from pig and rat, PGE_2 potentiated adenosine diphosphate (ADP)-induced aggregation with threshold activity present at 100 and 10 nM respectively (Kloeze, 1967). In the same studies PGE_2 at 5–10 μM inhibited aggregation in human PRP and Robison and co-workers (1969) showed that cyclic AMP levels were raised at these high concentrations. Inhibition of primary aggregation and enhancement of secondary aggregation to ADP in human PRP have been reported for PGE_2 (Shio & Ramwell, 1972; McDonald & Stewart, 1974), while enhancement of primary phase aggregation has also been observed (Andersen *et al.*, 1980). More recently Gresele and co-workers (1988) showed that PGE_2 (0.5–5 μM) potentiated arachidonate-induced aggregation in human PRP from all 'non-responders to thromboxane synthetase inhibitors', whereas aggregation was inhibited in 80% of the 'responder' group.

It has been suggested that PGE_2 interacts with a specific receptor on the platelet surface to enhance aggregation and its variable effects are due to an opposing (albeit weak) anti-aggregatory action operating through the IP-receptor (Anderson *et al.*, 1980; Tynan *et al.*, 1984). The same

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research group (Eggerman *et al.*, 1986) went on to provide evidence of a specific binding site for [^{3}H]-PGE_2 on human platelets; the radioligand was displaced equally well by PGE_1 and PGE_2 (IC_{50}~10 nM) whereas PGI_2 was relatively ineffective (IC_{50}~3 μM). Gray & Heptinstall (1985) suggested that activation of this receptor leads to inhibition of adenylate cyclase. The studies of Ashby (1988) support this view and point to the regulatory G-protein G_i as the link between receptor and cyclase molecules.

It has been known for some time that certain PGE analogues (e.g. 16-*p*-fluorophenoxy-ω-tetranor PGE_2) can induce irreversible aggregation of human platelets (Jones *et al.*, 1979; MacIntyre *et al.*, 1978) and that this can be attributed (at least in part) to their agonist action at thromboxane (TP-) receptors (Jones *et al.*, 1982). During testing of prostanoids for potential TP agonist activity in human PRP, we observed that another 16-phenoxy PGE analogue, sulprostone, occasionally produced a slow aggregation wave which was not preceded by a shape change signal; the response was not typical of a TP-receptor agonist (e.g. 11,9-epoxymethano PGH_2 (U-46619)), but was similar to that produced by adrenaline in some PRP samples (see Hourani & Cusack, 1991). On further investigation we found that sulprostone at low concentrations potentiated primary aggregation responses to ADP, PAF and U-46619 in PRP from all donors examined. This led us to suggest that sulprostone might be a specific agonist for the 'pro-aggregation receptor' negatively linked to adenylate cyclase. This paper describes experiments designed to test this hypothesis and to characterize the receptor involved.

Methods

Platelet aggregation

The preparation of PRP and washed platelet suspensions (using PGI_2) from human blood and the measurement of shape change and aggregation responses were carried out as recently described by us (Tymkewycz *et al.*, 1991). GR 32191 (500 nM) and BW A868C (200 nM) were added to the bulk of the PRP at the start of the aggregation measurements. For the study of potentiation, the PGE analogue was added to an aliquot of PRP, 2 min before a fixed dose of the aggregating agent and aggregation was measured for a further 1–2 min. The concentrations of ADP (500–800 nM) and PAF (15–50 nM) were chosen to give an aggregation response between 15–20% of the maximum aggregation response for each agent (obtained with 10 and 1 μM respectively). Sulprostone was used as the standard potentiating agonist and a concentration-response curve involving duplicate measurements was obtained for each PGE analogue over a time period when a submaximal potentiation response to sulprostone remained constant. Responses were calculated as a percentage of the control response and an EC_{150} value, representing a 50% increase in aggregation response over control was calculated. Measurements were completed within 2 h of aspiration of the PRP. For the study of inhibition of aggregation in PRP by cicaprost, PGE_1 and PGE_2, ADP at a concentration of 2 μM (in a few instances 4 μM) was used to produce a 75% maximal aggregation response.

Cyclic AMP measurements

Fresh human PRP or time-expired (5 days at room temperature) human platelet concentrates were treated with 10 μM indomethacin and then centrifuged at 450 *g* for 20 min. The pellet was suspended in sufficient 0.05 M Tris buffer pH 7.4 containing 4 mM EDTA at 37°C to give an absorbance at 600 nm of about 1.4 (10 mm pathlength). In 10 experiments using 180 ml of fresh blood, the protein content of the suspension (45 ml), as measured by the method of Bradford (1976), was found to be 1.5 ± 0.1 mg ml^{-1} (mean ± s.e.mean). GR 32191 (0.5 μM) was added to the platelet suspension. Duplicate 0.5 ml aliquots of the suspension were incubated with the varying concentrations of PGE analogue for 2 min, followed by 8 nM cicaprost for 1 min. In each experiment a single concentration of sulprostone (6.45 nM) was included to ensure that the cyclase inhibitory effect was functioning. After quenching with 1 ml ethanol, the precipitated material was sedimented by centrifugation at about 1000 *g* for 30 min. The supernatant was removed, evaporated to dryness and the residue dissolved in 0.25 ml assay buffer. Insoluble material was sedimented by centrifugation at 2300 *g* for 30 min at 4°C. The cyclic AMP content of duplicate 50 μl aliquots of supernatant was assayed by competition with [8-^{3}H]-cyclic AMP ammonium salt (21 Ci mmol^{-1}, Amersham) for binding to cyclic AMP-dependent protein kinase from bovine adrenal cortex (BDH). Recovery of cyclic AMP (60 pmol ml^{-1}) added to the washed platelet suspension, extracted and assayed as above was 85 ± 5% (n = 5). Also recovery of radioactivity following addition of labelled cyclic AMP to the washed platelet suspension, extraction and dissolution in buffer was 94 ± 3% (n = 5). Log concentration-inhibition curves were plotted and IC_{50} values calculated; for fresh platelets these corresponded to 55% of control cyclic AMP (taking maximum inhibition as 10% of control) and for stored platelets to 60% of control (maximum = 20%).

Compounds

11-Deoxy PGE_2-1-alcohol (starting material *nat* PGA_2) and EP 092 (*rac* 9α,11α-ethano-ω-heptanor-13-methyl-13-phenylthiocarbamoylhydrazino-prosta-5Z-enoic acid) were prepared in our laboratory. The following compounds were gifts: sulprostone, PGI_2 sodium salt and cicaprost from Prof. H. Vorbruggen, Schering AG, Berlin, Germany; MB 28767 (*rac* 15S- hydroxy-9-oxo-16-phenoxy-ω -tetranor-prost- 13E- enoic acid) from Dr M. Caton, Rhone-Poulenc, U.K.; misoprostol from Dr P. Collins, G.D. Searle, U.S.A.; butaprost from Dr P. Gardiner, Bayer, U.K.; AH 6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid), GR 32191 (9α-(biphenylyl)methoxy-11β- hydroxy-12β -(N-piperidinyl)-ω -octanor- prost-4Z -enoic acid) and GR 63799X (13-oxa-13,14-dihydro-16-phenoxy-ω-tetranor PGE_2-4-(benzoylamino)phenyl ester) from Dr R.A. Coleman, Glaxo, U.K. PGE_2, 16,16-dimethyl PGE_2, 17-phenyl-ω-trinor PGE_2, 11-deoxy PGE_1 and U-46619 (11,9-epoxymethano PGH_2) were purchased from Cayman Chemicals, U.S.A. Ethanolic stock solutions of the prostanoids (10–30 mM) were stored at −20°C and diluted with 0.9% NaCl solution (saline) for use. Due to its low water solubility, GR 63779X was stored in ethanol at a concentration of 0.4 mM. Saline dilutions of 20 μM and less were prepared and used for one day only. An aliquot of a 2 mg ml^{-1} chloroform solution of PAF (L-isomer, Sigma) was evaporated to dryness with a nitrogen jet and the residue dissolved in saline to give a 10^{-4} M stock solution. A 10^{-3} M ADP stock solution was prepared by dissolving ADP (Grade III, Sigma) in saline and adjusting the pH to 6.8 by addition of solid $NaHCO_3$.

Results

Potentiation of aggregation in human PRP

Initial observations on sulprostone and PGE_2 Shape change and aggregation in citrated human PRP were recorded by the conventional light scattering method. In about half of the 31 donors examined, sulprostone between 4 and 400 nM had no effect on the oscillatory signal associated with stirring a suspension of non-activated (discoid) platelets. In the remaining donors, 4–50 nM sulprostone also had no effect, but at 100–400 nM a slow increase in light transmission with some decrease in oscillation was seen (see Figure 1a, sulprostone 100 nM). This type of response was quite different from the rapid shape change (decrease in light transmission with loss

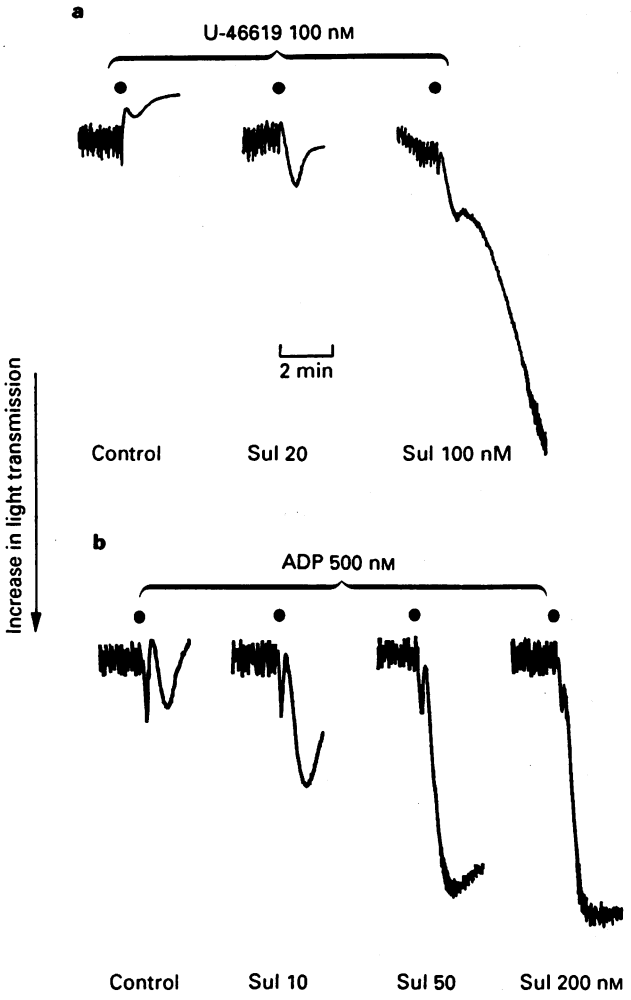

Figure 1 Light transmission records showing the potentiating effect of sulprostone (Sul) on platelet aggregation in human PRP induced by (a) U-46619 (no antagonists present) and (b) ADP in the presence of 1 μM EP 092. U-46619/ADP were added at (●) and sulprostone was added 2 min before each dose of aggregating agent.

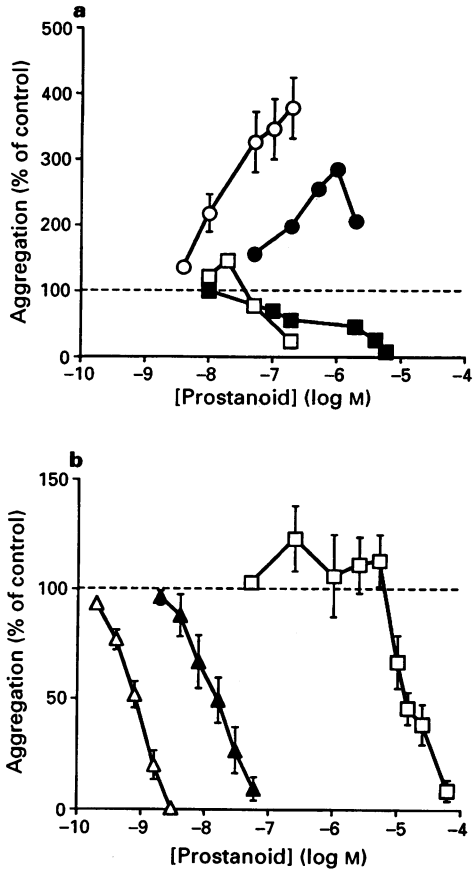

Figure 2 Log concentration-response curves for platelet aggregation in human platelet-rich plasma (PRP) in the presence of GR 32191 (0.5 μM). Control response = 100%. Vertical bars indicate s.e.mean. (a) Effects of sulprostone (○) (mean of 8 donors) and prostaglandin E_2 (PGE_2, ●, □, ■) (three individual donors) on small primary waves induced by ADP. (b) Effects of cicaprost (△) ($n = 8$), PGE_1 (▲) ($n = 4$) and PGE_2 (□) ($n = 4$) on 75% maximal aggregation responses induced by ADP.

of oscillation) and reversible aggregation induced by low concentrations of ADP (500 nM), PAF (20 nM) or U-46619 (100 nM) (Figure 1). 'Slow waves' to sulprostone were unaffected by the TP-receptor antagonists EP 092 (1 μM) and GR 32919 (0.5 μM); both antagonists blocked shape change/reversible aggregation responses to U-46619, but not those to ADP and PAF.

Irrespective of whether slow waves were seen, sulprostone (4–400 nM) consistently potentiated aggregation responses to ADP, PAF and U-46619. At the lower sulprostone concentrations the small reversible aggregation wave to a fixed concentration of each aggregating agent was increased in size. At the higher concentrations the potentiation was great enough to elicit secondary aggregation, particulary with U-46619 (Figure 1a). Although EP 092 (1 μM) and GR 32191 (0.5 μM) abolished secondary aggregation, they did not inhibit sulprostone potentiation of primary aggregation waves to ADP (Figure 1b) and PAF. The log concentration-response curve (mean values using PRPs from 8 donors) for sulprostone potentiation of ADP-induced aggregation in the presence of 0.5 μM GR 32191 is shown in Figure 2a.

Unlike sulprostone, PGE_2 showed variable effects on small primary waves induced by ADP. In PRPs from 2 of the 8 donors investigated PGE_2 (10–5000 nM) only inhibited aggregation, the log concentration-response curves being rather shallow (Figure 2a, filled squares). In the remaining 6 PRP samples, potentiation of aggregation was seen at the lower concentrations of PGE_2 tested (10–50 nM). As the concentration of PGE_2 was increased, either a bell-shaped potentiation curve was obtained (Figure 2a, filled circles) or a switch to inhibition of aggregation was observed (Figure 2a, unfilled squares).

For comparison, the effects of cicaprost (a stable prostacyclin analogue), PGE_1 and PGE_2 on 75% maximal aggregation responses to ADP in the presence of GR 32191 (0.5 μM) are shown in Figure 2b. Cicaprost was a highly potent inhibitor of aggregation ($IC_{50} \sim 0.8$ nM). PGE_1 also inhibited aggregation, being 15–20 times less potent than cicaprost and having a slightly shallower log concentration-response curve. PGE_2 (50–5000 nM) potentiated aggregation such that maximum aggregation was often produced; between 10 and 62.5 μM PGE_2 always inhibited aggregation.

Effect of AH 6809 In three experiments, the EP_1-receptor antagonist, AH 6809, at 10 μM had no effect on sulprostone potentiation of ADP-induced aggregation. With 100 μM AH 6809, there was a modest parallel shift to the right of the sulprostone log concentration-response curve, giving dose-ratios of 2.0, 3.2 and 4.4.

Activities of other PGE analogues Preliminary experiments appeared to show that the potentiating effect of PGE_2 was favoured over its inhibitory effect when PAF was used as the aggregating agent in place of ADP. The effects of nine PGE

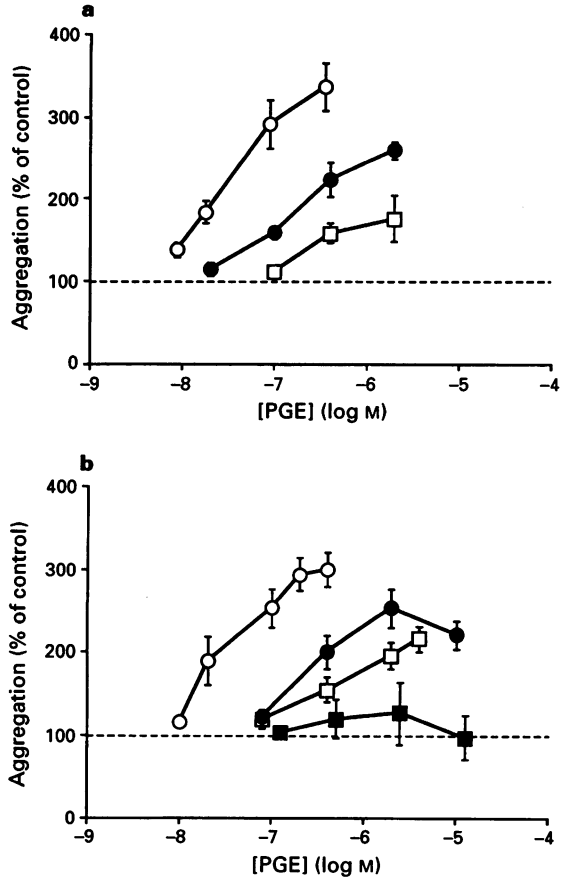

Figure 3 Effects of prostaglandin E (PGE) analogues on PAF-induced aggregation in human platelet-rich plasma (PRP) in the presence of GR 32191 (0.5 μM) and BW A868C (0.2 μM). Control response = 100%. Vertical bars indicate s.e.mean. (a) Sulprostone (○) ($n = 6$); MB 28767 (●) ($n = 4$); GR 63779X (□), ($n = 4$). (b) 16,16-dimethyl PGE_2 (○); misoprostol (●); 17-phenyl-ω-trinor PGE_2 (□); 11-deoxy PGE_2-1-alcohol (■) (all $n = 4$).

analogues were therefore tested for their ability to potentiate PAF-induced aggregation in PRP. In addition to GR 32191 (0.5 μM), BW A868C (0.2 μM) was added to prevent any inhibition of aggregation through activation of DP-receptors. GR 32191 was preferred to EP 092 because the block of TP-receptors produced by the former is more difficult to surmount (see Tymkewycz *et al.*, 1990). Six of the analogues, sulprostone, 16,16-dimethyl PGE_2, MB 28767, misoprostol, GR 63779X and 17-phenyl-ω-trinor PGE_2 showed consistent potentiating activity (Figure 3), although the log concentration-response curves for misoprostol, MB 28767 and in particular GR 63779X and 17-phenyl-ω-trinor PGE_2 were shallower than those of sulprostone and 16,16-dimethyl PGE_2. Equi-effective molar ratios (EMR) calculated at the 150% control level are given in Table 1. The testing of GR 63779X was affected by the high proportion of ethanol required to solubilize this prostanoid. Thus 20 mM ethanol (corresponding to 0.4 μM GR 63779X) did not affect PAF aggregation; 100 mM ethanol (2 μM GR 63779X) reduced the PAF response by 11 ± 3% ($n = 4$) and 200 mM ethanol by 51 ± 6%. At the highest concentrations of misoprostol and 11-deoxy PGE_2-1-alcohol used, the ethanol concentration in the PRP was 20 mM.

PGE_2 (20–1000 nM) and 11-deoxy PGE_2-1-alcohol (100–10,000 nM) still showed variable actions. In some donors, potentiation with a shallow log concentration-response curve was seen and in others there was evidence of inhibition of the PAF aggregation. In the final analysis it appears that the use of PAF may not have a significant advantage over ADP. Butaprost (10–3000 nM) inhibited only PAF aggregation ($IC_{50} = 0.95$, 1.3, 1.5 μM, 3 donors).

Inhibition of cyclic AMP production

Due to difficulties in obtaining large quantities of fresh human blood, some experiments were performed on time-expired platelet concentrates supplied by the local blood transfusion service. Cyclic AMP levels in suspensions of the washed platelets were measured with a radiolabelled cyclic AMP/protein binding assay. Elevations of cyclic AMP induced by cicaprost in the presence of 0.5 μM GR 32191 are shown in Figure 4a; Fresh and stored platelets gave similar log concentration-response curves; EC_{50} values were about 10 and 8.5 nM respectively. For all subsequent inhibition experiments, a cicaprost concentration of 8 nM was chosen to induce a submaximal (control) rise in cyclic AMP.

The effects of pre-incubation of the PGE analogues on the cicaprost-induced rise in cyclic AMP in the presence of 0.5 μM GR 32191 are shown in Figure 4. All of the PGE analogues inhibited cyclic AMP accumulation with the exception of butaprost; IC_{50} values and EMR are given in Table 1. The slopes of the inhibition curves for the stored platelets were somewhat shallower than those of the fresh platelets, although sensitivities to sulprostone were similar ($IC_{50} = 2.2$ and 2.0 nM respectively).

AH 6809 at a concentration of 10 μM shifted the log concentration-response curves to sulprostone and 17-phenyl-ω-trinor PGE_2 to the right (dose-ratio = 7.2 and 3.9 respectively) (Figure 4b). pA_2 values calculated using the Schild equation are 5.75 and 5.32 respectively.

Table 1 Agonist potencies of prostaglandin E (PGE) analogues relative to sulprostone

	Equi-effective molar ratio			
PGE analogue	*Potentiation of PAF aggregation in human PRP*	*Inhibition of cyclic AMP in human washed platelets*	*Contraction of guinea-pig trachea*	*Inhibition of guinea-pig vas deferens*
Sulprostone	1.0 (EC_{150} = 10 nM)	1.0 (IC_{50} = 2.2/2.0 nM*)	1.0 (EC_{50} = 7 nM)	1.0 (IC_{50} = 0.2 nM)
16,16-Dimethyl PGE_2	1.3	0.34*	0.022	0.93
MB28767	6.8	2.1*	>150	5.0
PGE_2	↓	2.5	↓	7.1
Misoprostol	13	10.3*	↓	3.6
GR 63779X	26	20	14	1.7
17-Phenyl-ω-trinor PGE_2	29	37	0.32	45
11-Deoxy PGE_2-1-alcohol	↓	180	↓	79
Butaprost	↓	>350	↓	>7000

Human platelet data were obtained in the present study. Guinea-pig trachea and vas deferens values are from Lawrence *et al.*, 1992, except those for GR 63779X which are our unpublished values, in agreement with Bunce *et al.*, 1990. *Values for stored platelets. ↓ Inhibitory effect observed – assessment of stimulant potency not possible.

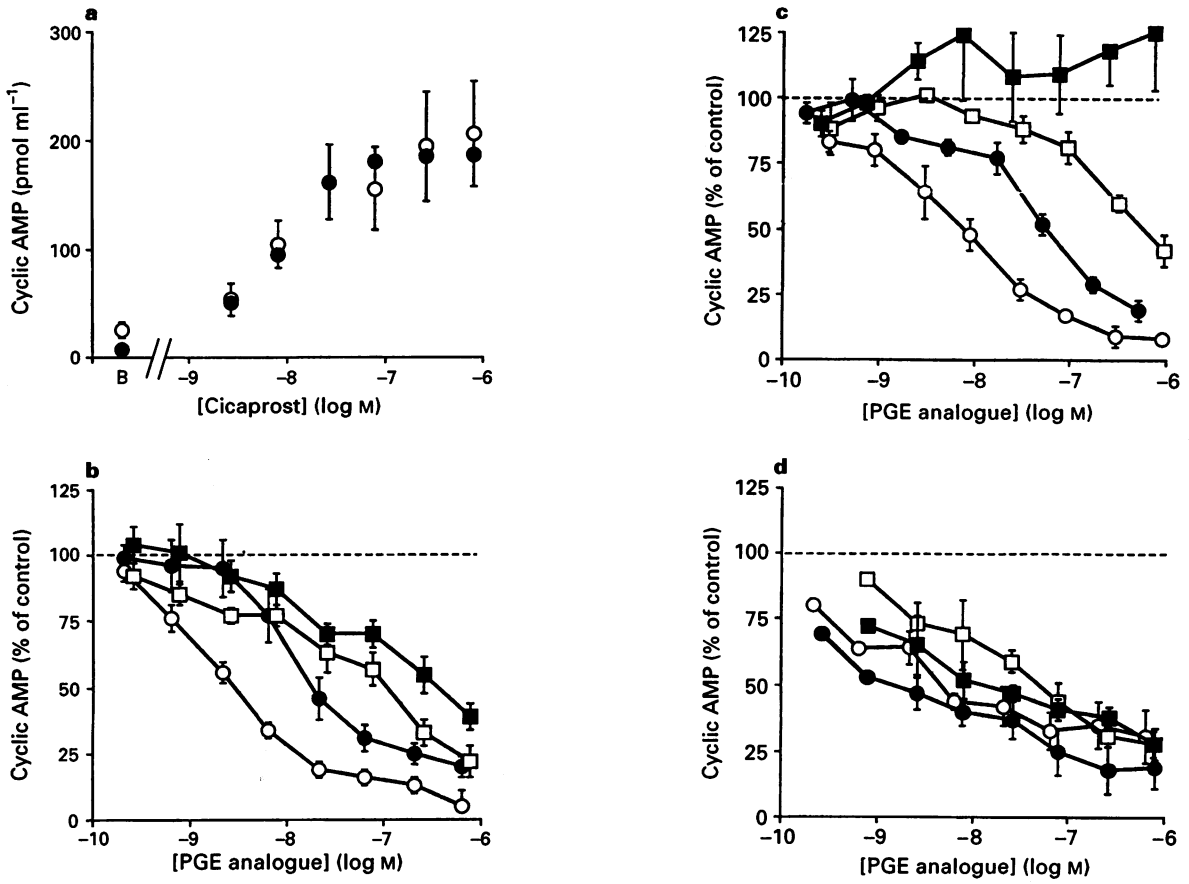

Figure 4 Effects of prostaglandin E (PGE) analogues on cicaprost-induced elevation of cyclic AMP in human washed platelets. GR 32191 (0.5 μM) was present in all experiments. Vertical bars indicate s.e.mean; $n = 4$ in (b) (c) and (d). (a) Elevation of cyclic AMP by cicaprost in fresh (○) ($n = 6$) and stored (●) ($n = 3$, no error bars) platelets. B = basal condition; 8 nM cicaprost was used in (b) (c) and (d); cyclic AMP generated = 100%. (b) Fresh platelets: inhibition curves for sulprostone (○); sulprostone in the presence of 10 μM AH 6809 (●); 17-phenyl-ω-trinor PGE_2 (□) and 17-phenyl-ω-trinor PGE_2 in the presence of 10 μM AH 6809 (■). (c) Fresh platelets: inhibition curves for PGE_2 (○); GR 63779X (●); 11-deoxy PGE_2-1-alcohol (□) and butaprost (■). (d) Stored platelets: inhibition curves for sulprostone (○); 16,16-dimethyl PGE_2 (●); misoprostol (□) and MB 28767 (■).

Discussion

We have shown that several analogues of PGE_2 consistently potentiate ADP- or PAF-induced aggregation in human PRP in the presence of a specific TP-receptor antagonist such as EP 092 (Armstrong *et al.*, 1985) or GR 32191 (Lumley *et al.*, 1989). Sulprostone and 16,16-dimethyl PGE_2 were the most active agents, with threshold responses seen at about 2 nM in some PRPs. However sulprostone was chosen as the standard agonist since it is a weaker TP-receptor agonist than 16,16-dimethyl PGE_2. For example, 16,16-dimethyl PGE_2 can elicit irreversible aggregation in human PRP and is a medium potency full agonist on the rat aorta, rabbit aorta and dog saphenous vein (see Tymkewycz *et al.*, 1991), whereas sulprostone does not induce typical thromboxane-like aggregation and is a very weak agonist on the rat aorta (Coleman *et al.*, 1988) and rabbit aorta (R.L. Jones, unpublished observations).

Two factors may affect estimations of the relative potentiating potencies of the PGE analogues under investigation. First, there may be differences in the amounts of the PGE analogues bound to plasma protein in the PRP. Secondly, and probably of greater importance, the human platelet contains a highly sensitive IP-receptor-adenylate cyclase system which when activated inhibits aggregation. This is illustrated in our experiments by the high potency of cicaprost (IC_{50} against ADP-induced aggregation = 0.8 nM). Thus the mixed effects of PGE_2 on PAF aggregation in the presence of the potent and specific DP-receptor antagonist BW A868C (Giles *et al.*, 1989) are most easily explained by a concurrent activation of EP- and IP-receptors over the 50 nM to 5 μM concentration-range. Previous studies with the weak DP-receptor antagonist, N-0164, have produced conflicting results for PGE_2. In the earlier study, N-0164 suppressed the platelet inhibitory action of PGD_2 and PGE_2, but did not affect the inhibitory actions of PGE_1 (MacIntyre & Gordon, 1977), whereas in a later study the inhibitory actions of PGI_2, PGE_1 and PGE_2 were unaffected (Tynan *et al.*, 1984). The shallower log concentration-potentiation curves for MB 28767, misoprostol, 17-phenyl-ω-trinor PGE_2 and 11-deoxy PGE_2 1-alcohol could also reflect IP-receptor activation by these analogues at concentrations of 1 μM and above.

Taking the EC_{150} as a measure of potentiating potency, then the ranking is as follows: sulprostone = 16,16-dimethyl PGE_2 > MB 28767 ≥ misoprostol > GR 63779X = 17-phenyl-ω-trinor PGE_2. The high potency of sulprostone as a potentiator implies that an EP_2-receptor is unlikely to be involved. EP_2-receptors are associated with the relaxant actions of PGE_2 on vascular and respiratory smooth muscle and sulprostone is virtually inactive in these systems (Coleman *et al.*, 1987a,b; Lawrence & Jones, 1992). Furthermore it is likely that the EP_3 rather than the EP_1 subtype mediates the potentiating effect, since sulprostone and MB 28767 are more potent than 17-phenyl-ω-trinor PGE_2 on the guinea-pig vas deferens (EP_3 preparation) and *vice versa* for the guinea-pig trachea (EP_1 preparation) (Lawrence *et al.*, 1992) (see Table 1). It should be noted that we are using the guinea-pig vas deferens as the standard EP_3 preparation, since there is

now evidence (Lawrence & Jones, 1992) that the chick ileum, the original EP_3 preparation (Coleman *et al.*, 1987a,b), contains more than one EP-receptor.

PGE_1 is a highly potent EP_3 agonist, being slightly more potent than PGE_2 and about 5 times less active than sulprostone (see Coleman *et al.*, 1990). But for its prostacyclin-like activity we would expect it to potentiate aggregation at concentrations of 30 nM and above. The slightly shallower inhibition curve for PGE_1, also observed previously by Andersen and colleagues (1980), may reflect the existence of an opposing potentiation.

Preliminary experiments on human washed platelets showed that sulprostone produced marked potentiation of aggregation, but mixed effects were still seen with PGE_2 and 11-deoxy PGE_2-1-alcohol. Because of this and the greater difficulties of working with washed platelets, we felt that it was hardly worthwhile attempting to obtain potentiating potencies on washed platelets. Instead we concentrated our efforts on developing an assay based on the putative second messenger mechanism, namely inhibition of adenylate cyclase in a low protein system. Our aim was to accentuate the EP agonist action of a PGE analogue at the expense of its IP agonist action. Cicaprost was chosen as the agent to raise the cyclic AMP level since it is a stable and specific IP-receptor agonist. In particular it lacks the potent EP_1-receptor agonist activity shown by other carbacyclins, such as iloprost and 6a-carba PGI_1 (Dong *et al.*, 1986; Lawrence *et al.*, 1992). Furthermore it has less EP_3-agonist activity than iloprost and carbacyclin on the guinea-pig vas deferens (Lawrence *et al.*, 1992). Ashby (1992) has suggested from analysis of the time course of cyclic AMP accumulation in intact human platelets that both iloprost and cicaprost activate an inhibitory receptor to reduce cyclic AMP accumulation. However the initial rise in cyclic AMP is only rapidly suppressed at high concentrations (3 μM) of each prostacyclin analogue and it is easily possible that the slow fade of the cyclic AMP level seen with lower concentrations (30 nM) is due to a mechanism other than activation of an inhibitory receptor. In our experiments we deliberately used a low concentration of cicaprost (8 nM, $\sim EC_{50}$, Figure 4a), which is unlikely to activate the EP-receptor. In addition submaximal stimulation of adenylate cyclase should allow the greatest opportunity for PGE-induced inhibition of the enzyme complex. The success of our strategy can be judged by the high sensitivities and monophasic inhibition curves obtained for both sulprostone and PGE_2 (IC_{50} = 2.2 and 5.4 nM respectively).

Reconsidering the nature of the EP-receptor involved, the low potency of 17-phenyl-ω-trinor PGE_2 relative to sulprostone and MB 28767 again indicates that the EP_1-receptor subtype is unlikely to be involved. Furthermore AH 6809 shows a much weaker blocking effect (pA_2 = 5.3/5.76) than would be expected for EP_1-receptor antagonism (pA_2 on guinea-pig trachea = 7.35, 17-phenyl-ω-trinor PGE_2 as agonist, Lawrence *et al.*, 1992). The even lower pA_2 value ($\sim$4.3) obtained for block of sulprostone potentation by AH 6809 in the aggregation assay is probably due to plasma protein binding of the antagonist. Coleman and colleagues (1985) have shown that about 98% of AH 6809 is bound to 4% defatted bovine serum albumen. In our PRP system, 90–95% binding of AH 6809 to the 2% total plasma protein present would account for the difference between the pA_2 values. AH 6809 is not a particularly specific EP_1 antagonist and will also block DP- and TP-receptors on human washed platelets at a concentration of 10 μM (pA_2 = 6.3 and 5.9, calculated from data in Keery & Lumley, 1988).

Finally, how good is the correlation between the potencies of the PGE analogues for inhibition of adenylate cyclase in human platelets and for inhibition of the twitch response of the guinea-pig vas deferens, the standard EP_3 preparation (Table 1)? Using log EMRs, the least squares regression line has a slope of 1.00 and a correlation coefficient of 0.80 ($P < 0.05$). However, this is not a particularly convincing correlation. The greatest discrepancy is the higher potency of GR 63779X on the vas deferens compared to the adenylate cyclase system (and also the aggregation assay). GR 63779X has an agonist specificity similar to sulprostone ($EP_3 > EP_1 >> EP_2$) (Bunce *et al.*, 1990) and although there are clearly difficulties in its use owing to its low water solubility, we feel that our potency estimations are valid. Consequently we consider it prudent to err on the side of caution and pending further studies to refer to the EP-receptor in the human platelet as 'EP_3-like'.

It seems reasonable to propose from our experiments and the studies of Ashby (1988) that activation of EP_3-like receptors on the platelet membrane inhibits adenylate cyclase through the intermediary of G_i, and this leads to potentiation of aggregation. Other human platelet receptors known to operate in a similar manner are the ADP receptor (a type of P_2-purinoceptor) and the α_2-adrenoceptor (see Hourani & Cusack, 1991, for a review of this area). In an elegant study Brass and colleagues (1988) have investigated the nature of the G-proteins mediating pertussis toxin-sensitive phospholipase C activation (G_p) and adenylate cyclase inhibition (G_i) in human platelets. Thrombin stimulated PI hydrolysis and inhibited cyclic AMP accumulation and this was associated with >90% ADP-ribosylation of the extracted G-protein. In contrast, adrenaline only inhibited cyclic AMP accumulation in concert with 50% ADP-ribosylation; U-46619 neither inhibited cyclic AMP nor induced pertussis toxin-sensitive PI hydrolysis. It appears that the G-proteins regulating both PI hydrolysis and inhibition of cyclic AMP formation in this system are very similar in nature and the possibility that they are the same chemical species has been raised. Clearly it would be of interest to investigate by similar methods the nature of the G-protein(s) interacting with the EP_3-like receptor of the human platelet; from our studies sulprostone would be the most useful agonist for this purpose.

In conclusion, our results are relevant to the therapeutic use of PGE analogues in man. TP-agonist activity in a PGE analogue is an obvious risk factor. However, the possibility of a PGE analogue potentiating platelet aggregation via EP_3-like receptors should also be taken into consideration.

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Central α_2-autoreceptors: agonist dissociation constants and recovery after irreversible inactivation

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1 Rats received an intraperitoneal injection of 1.6 mg kg^{-1} N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) to achieve an irreversible inactivation of presynaptic release-modulating α_2-autoreceptors. Cerebral cortex slices were prepared at different times after the injection (24, 48, 96, 192, 336, 774 h), incubated with [^{3}H]-noradrenaline ([^{3}H]-NA), superfused and stimulated electrically with 4 pulses at 100 Hz (= autoinhibition-free condition). Overflow of radioactivity was used to measure release. Furchgott analysis was used to estimate agonist dissociation constants (K_A) and pool size of resynthesized receptors (q).

2 The K_A values of the three α_2-autoreceptor agonists, bromoxidine (UK-14304), clonidine and noradrenaline (NA) were 187 nM, 72 nM, and 1202 nM, respectively.

3 The release-inhibiting effects of the agonists returned considerably faster than the receptor pool. The calculated half-lives for the recovery of the maximal release-inhibiting effects of bromoxidine, clonidine and NA were 30.7, 63.6 and 20.8 h, respectively, whereas the half-life for the recovery of the receptor pool was 445 h.

4 The data indicate a large receptor reserve at presynaptic α_2-autoreceptors for the agonists used and validate the use of EEDQ as a tool for the determination of agonist dissociation constants.

Keywords: Presynaptic α_2-adrenoceptors; receptor reserve; agonist affinities; noradrenaline release; rat brain cortex; N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)

Introduction

Superfusion and electrical stimulation of brain slices prelabelled with [^{3}H]-noradrenaline ([^{3}H]-NA) are widely used experimental procedures to study prejunctional inhibitory α_2-autoreceptors *in vitro* (for review see Starke *et al.*, 1989). The determination of agonist affinities at these receptors, however, is complicated by the release of endogenous noradrenaline (NA) causing autoinhibition in addition to the release-modulating effect of the drug under investigation. Thus, potency and affinity of an agonist will be underestimated unless care is taken to avoid interference caused by transmitter in the biophase of the receptor. In a previous publication (Agneter *et al.*, 1991) a method was presented allowing the determination of agonist dissociation constants (K_A) and relative efficacies at presynaptic α_2-autoreceptors in the absence of ongoing autoinhibition. The brain slices were stimulated with a short burst of 4 pulses delivered at 100 Hz (pseudo-one-pulse, POP-stimulation) resulting in an easily measurable overflow of radioactivity in the virtual absence of autoinhibition (Singer, 1988; Zier *et al.*, 1988). The equilibrium dissociation constants of the three α_2-autoreceptor agonists bromoxidine (UK-14304), clonidine and NA were determined according to the method of Furchgott (1966) after pretreatment of the rats with a low dose of the irreversible α-adrenoceptor antagonist EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline). The under-estimation of the K_A values of the agonists as a consequence of ongoing autoinhibition during electrical stimulation was also demonstrated in those experiments.

In the present investigation the dissociation constants (K_A) of the same three agonists were determined at newly synthesized α_2-autoreceptors after irreversible inactivation following a maximally effective dose of EEDQ (Adler *et al.*, 1985). At the same time, information on the velocity of α_2-autoreceptor recovery and the magnitude of the receptor reserve at the α_2-autoreceptors was obtained.

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Methods

Animals and pretreatment

Male Sprague-Dalwey rats (180–220 g; Forschungsinstitut für Versuchstierzucht, Himberg, Austria) were used. In order to inactivate α_2-autoreceptors the animals were treated with the irreversible α-adrenoceptor antagonist, EEDQ (Belleau *et al.*, 1968). The animals received 1.6 mg kg^{-1} EEDQ i.p. and were killed at various times after the injection (24, 48, 96, 192, 336, 744 h). All control rats received an i.p. injection of saline (1 mg kg^{-1}).

Preparation of tissue, incubation and superfusion

The brains were removed rapidly after decapitation of the rats. A longitudinal strip of parieto-occipital cortex (about 6 × 3 mm) was prepared and cut into 0.4 mm thick slices with a McIlwain tissue chopper. The slices were then incubated for 30 min at 37°C in 2 ml of medium containing 0.125 μM (−)-[^{3}H]-NA (specific activity 40 Ci mmol^{-1}), washed twice and transferred to superfusion chambers which were equipped with platinum wire electrodes 5 mm apart (Valenta *et al.*, 1988). The slices were placed on a polypropylene mesh between the electrodes and superfused at 37°C and a flow rate of 0.7 ml min^{-1} (one slice per chamber, 12 chambers in parallel; Ismatec IPS-16 peristaltic pump, Zürich, Switzerland). The tissues were superfused for 40 min to establish a stable basal efflux of radioactivity, after which superfusion samples were collected at 4-min intervals. The superfusion medium contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, D-glucose 11, ascorbic acid 0.57 and disodium EDTA 0.03; aerated with 95% O_2/5% CO_2 at 37°C, final pH 7.4. The superfusion, but not the incubation medium used for labelling with [^{3}H]-NA, also contained 1 μM desipramine in order to inhibit neuronal reuptake of NA (in experiments in which NA was used as agonist, the medium contained 3 μM desipramine). At the end of each experiment the slices were sonicated in 1.2 ml 2% (v/v) perchloric acid (Branson sonifier B 15; Branson Sonic

Power, Danbury, CT). Radioactivity was determined in slices and superfusate fractions by liquid scintillation counting (Packard Tri-Carb 460 CD) with a counting efficiency of 24.3% (estimated using internal standard).

Stimulation and drug administration

The slices were stimulated electrically with monophasic rectangular pulses of 2 ms duration and a voltage drop between the electrodes of 12 V cm^{-1} yielding a current strength of 18 mA (Stimulator T, Hugo Sachs Elektronik, Hugstetten, Germany). An initial period of electrical stimulation, not used for determination of tritium overflow, was applied during the 40 min washout period (18 pulses at 0.3 Hz after about 20 min of superfusion). Electrical stimulation was applied for four test periods (S1–S4) at 8, 36, 64 and 92 min, respectively, after commencement of the collection of 4 min fractions. The test periods consisted of 4 pulses at 100 Hz, which is also denoted 'POP' (pseudo-one-pulse; Singer, 1988).

The procedure for the determination of agonist affinities was as follows: Six slices prepared from the brain of an EEDQ-treated animal and six slices from a control animal were used in a single experiment. Four slices from each group were used for the generation of cumulative concentration-response curves for α_2-autoreceptor agonists (one agonist per experiment). The drugs were added at increasing concentrations 20 min before S2, S3 and S4, respectively (thus, a six-point concentration-response curve was generated with two slices). Two slices of each group served as control, i.e. they did not receive the agonist. Although this procedure was followed in most experiments, some superfusions were performed with control tissue from EEDQ-treated rats only. It had been established in a separate set of experiments in which the drugs were added at different time points before stimulation that the 20-min period allowed the drugs to reach equilibrium concentrations in the tissue. Furthermore, there appeared to be no receptor desensitisation during exposure to the agonists (see also Limberger *et al.*, 1989).

Final concentration-response curves for each agonist, one under control conditions and one after EEDQ-pretreatment, were generated from pooled data of at least three (up to five) separate superfusion experiments and were used for the calculations described below (K_A, q).

Calculation of release

The outflow of tritrium per fraction was expressed as percentage of the radioactivity in the slice at the start of the respective 4-min collection period. The stimulation-evoked overflow was calculated as the difference between the total overflow during and after stimulation and the estimated basal outflow which was assumed to decline linearly; the difference was expressed as a percentage of the tritium content of the tissue at the time of stimulation (%S1, %S2, %S3, %S4). Drug effects on evoked tritium overflow were evaluated by calculation of the ratio between the overflow evoked by Sx (S2, S3, S4) and the overflow evoked by S1 (Sx/S1). The Sx/S1 ratios were expressed as a percentage of the corresponding mean Sx/S1 control ratios (no agonist). Effects of drugs on the basal outflow of tritium were calculated from the ratio between fractional outflow during the 4-min collection period preceding S2, S3, S4 and the 4-min collection period preceding S1 (L2/L1, L3/L1, L4/L1).

Receptor affinity and receptor reserve analysis

Concentration-response curves for the agonists were generated at various times after the pretreatment of the rats with saline (controls) or EEDQ (irreversible receptor inactivation). The determination of the agonist affinity constants (K_A) at steady-state conditions and the fraction of receptors resynthesized (q) after the inactivation was performed according to the method of Furchgott (1966). The following equation was used:

$$\frac{1}{[A]} = \frac{1}{q} \times \frac{1}{[A']} + \frac{1\text{-}q}{q \times K_A}$$

where A is the concentration of agonist needed to give a specific percentage of the maximal response, and A′ the concentration of agonist needed to give the same percentage of the control maximal response after receptor inactivation.

Equieffective concentrations A and A′ of the agonist were obtained by generating the best fit concentration-response curves by use of the ALLFIT computer programme of De Lean *et al.* (1978). A′ values were taken from the concentration-response curve after EEDQ pretreatment, between 40–90% of its maximum (see Thorn, 1970; Kenakin, 1987, page 171). The values were calculated at 1% increments using the fitted curve parameters, and corresponding A values were obtained from the control curve. The reciprocals of these values, 1/A and 1/A′, were plotted and analysed by least-squares linear regression yielding a slope of 1/q and a y-intercept of (1-q)/(q × K_A) (Furchgott, 1966). These values served for the calculation of the fraction of resynthesized receptors, q = 1/slope, and the affinity constant, K_A = (slope-1)/y-intercept.

Statistics

All data are given as means ± s.e.mean; n = number of observations; one observation = one slice. Student's unpaired t test was used for determination of statistical significance.

Drugs

N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was purchased from Research Biochemicals (ANAWA Trading, Wangen, Switzerland); (−)-noradrenaline from Sigma (Munich, Germany); (−)-ring-2,5,6-[^{3}H]-noradrenaline (specific activity 40 Ci mmol^{-1}) from NEN (Dreieich, Germany). Gifts: 5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline (bromoxidine, UK-14304) from Pfizer (New York, NY, U.S.A.); clonidine HCl from Bender (Vienna, Austria); desipramine HCl from Ciba-Geigy (Basel, Switzerland).

EEDQ was dissolved in absolute ethanol and this solution was sequentially diluted with propylene glycol and distilled water (0.2:0.25:0.55; v/v/v) immediately before injection.

Results

Basal outflow and stimulation-evoked overflow of radioactivity

The spontaneous efflux of radioactivity from control tissues (saline pretreatment) during the 4 min period immediately before the first stimulation was 1.42 ± 0.02% (n = 157) corresponding to 2.63 ± 0.05 nCi. The corresponding values in the treated animals were in the range of 1.32%–1.51%.

The stimulation-evoked overflow of tritium (%S1; 4 pulses/100 Hz) from control tissues was 1.21 ± 0.03% (n = 157) corresponding to 2.36 ± 0.07 nCi, and the range of values in EEDQ-treated tissues was 1.05%–1.65%. Thus, there was no influence of EEDQ pretreatment on spontaneous outflow or stimulation-evoked overflow of radioactivity.

The L4/L1 ratios calculated from superfusion experiments performed at various times after EEDQ pretreatment and without agonist addition did not show significant differences when compared to the L4/L1 ratios obtained in control experiments. The control L4/L1 ratio (saline pretreatment, all pretreatment times pooled) was 0.82 ± 0.01 (n = 39), and the

range of values after EEDQ pretreatment was 0.82–0.86. The α_2-adrenoceptor agonists bromoxidine and clonidine slightly and at some time points significantly decreased spontaneous efflux in the highest concentrations tested (L4/L1 values 0.71–0.77), whereas NA had no significant influence (L4/L1 values 0.81–0.92).

The S2/S1, S3/S1, and S4/S1 ratios in agonist-free control experiments in tissue from saline-treated rats were 1.00 ± 0.02, 0.98 ± 0.02, and 0.92 ± 0.05 ($n = 39$), respectively. The S2/S1, S3/S1, and S4/S1 ratios in agonist-free control experiments in tissue from EEDQ-treated rats were in the range of 0.96–1.02, 0.92–0.99, and 0.92–0.99, respectively. Thus, there was no significant change in the electrically evoked overflow of tritium over time (see also Limberger *et al.*, 1989).

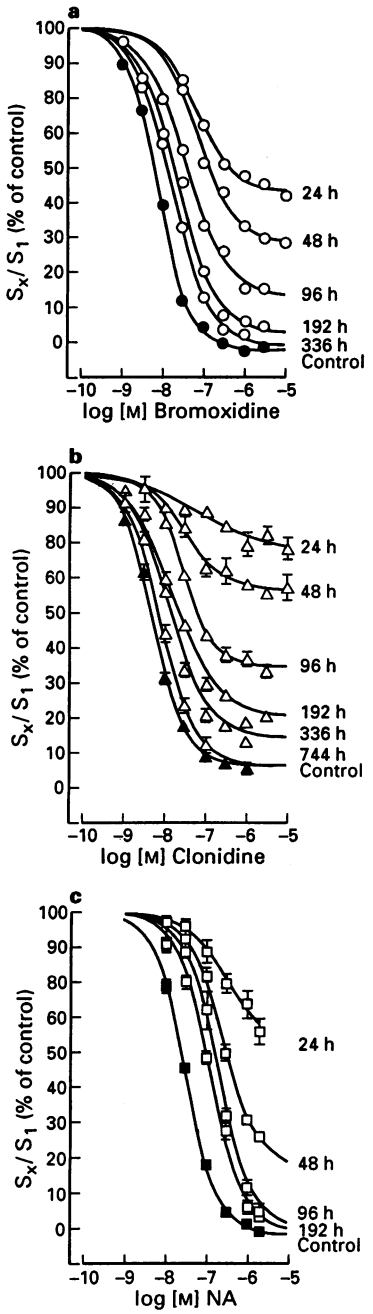

Figure 1 Concentration-response curves to (a) bromoxidine, (b) clonidine and (c) noradrenaline (NA) on electrically evoked overflow of [^{3}H]-NA from cortex slices of rats. The animals were killed 24, 48, 96, 192, 336 or 744 h after an injection of saline (1 mg kg^{-1} i.p., controls) or N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, 1.6 mg kg^{-1}). Slices of parieto-occipital cortex were incubated with [^{3}H]-NA and then superfused in the presence of 1 μM desipramine (3 μM in NA experiments). Four periods of electrical stimulation (S1-S4) were applied at 8, 36, 64 and 92 min after the start of sample collection using 4 pulses/100 Hz. Cumulative concentration-response curves were generated by adding increasing concentrations of the drugs 20 min before S2, S3 and S4. The ratios Sx/S1 (S2/S1, S3/S1, S4/S1) obtained in the presence of agonist were expressed as percentage of the mean ratios of the respective drug-free Sx/S1 control value. Symbols represent means ± s.e.mean (vertical bars, if greater than symbols) of 6 to 12 determinations from three to five separate experiments; curves were fitted using the ALLFIT computer programme (see Methods). Closed symbols, controls (all saline pretreatment times pooled); open symbols, EEDQ pretreatment.

Determination of q and K_A

The concentration-response curves for the three α-adrenoceptor agonists in tissue from saline-pretreated rats and from EEDQ-pretreated rats obtained at different times after pretreatment are shown in Figure 1. Maximal effects in control tissue, as derived from sigmoidal curve fitting with the ALLFIT computer programme, and expressed as Sx/S1 ratios (Sx/S1max), were 0.075, 0.159 and 0.080 for bromoxidine, clonidine, and NA, respectively. Pretreatment with EEDQ caused a pronounced rightward shift of the concentration-response curves and a decrease in the maximal effects of all three agonists. For instance, 96 h after EEDQ the IC_{50} values for bromoxidine, clonidine, and NA were shifted to the right by factors of 4.5, 5.3, and 5.4, respectively (controls = 8, 5, and 31 nM), and the respective computer-calculated Sx/S1max ratios amounted to 0.22, 0.44 and 0.07.

Equieffective concentrations of agonists were calculated (see Methods) and used to construct double reciprocal plots by the method of Furchgott (1966). The resulting estimates for K_A and q are summarized in Table 1.

Equilibrium dissociation constants and q values were calculated only from those curves in which the maximal effect of the agonist after EEDQ pretreatment was clearly separated from its maximal effect in the control situation (Kenakin, 1987; pp. 171 and 177). The lowest K_A values were found for clonidine, whereas those for bromoxidine and NA were higher by factors of about 3 and 17, respectively. For a given agonist, there was reasonable agreement between the K_A values obtained at the different time points, and, for a given time point, there was good agreement of the q values obtained with different agonists. The mean K_A values were 187, 72, and 1202 for bromoxidine, clonidine and NA, respectively.

Table 1 Agonist dissociation constants of bromoxidine, clonidine and noradrenaline (NA) determined at presynaptic α_2-autoreceptors of the rat brain cortex at various times after irreversible receptor inactivation

Time after EEDQ	*Bromoxidine* K_A (nM)	q	*Clonidine* K_A (nM)	q	*NA* K_A (nM)	q
24 h	128	0.059	84	0.022	i.d.	
48 h	199	0.073	47	0.067	1202	0.094
96 h	139	0.195	70	0.125	ND	
192 h	282	0.323	81	0.266	ND	
336 h	ND		77	0.375	No Exp.	
744 h	No Exp.		ND		No Exp.	

Agonist dissociation constants (K_A) and fractions of active receptors after EEDQ pretreatment (q) were derived from the concentration-response curves shown in Figure 1. Equieffective concentrations were calculated from each pair of curves (controls vs. EEDQ; Figure 1) and K_A and q were estimated using the double reciprocal plot (1/A and 1/A′) according to Furchgott (1966); i.d., insufficient data for calculation of maximum effect (results not included in Figures 2 and 3); ND, not determined due to limitations of Furchgott's method (see text); No Exp., no experiment performed, because agonist reached 100% of control effect at earlier time point.

Receptor reserve and recovery

In Figure 2 the maximal inhibition obtained with each of the three agonists at each point of time after EEDQ pretreatment

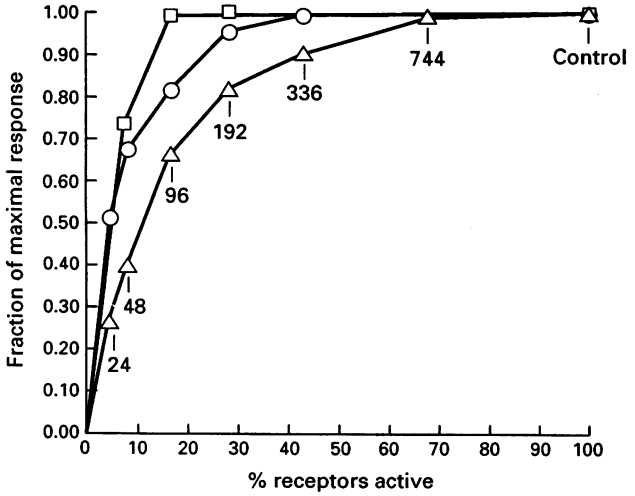

Figure 2 Inhibition of evoked overflow as function of resynthesized α_2-adrenoceptor pool (experiments of Figure 1). Ordinate scale: calculated maximum inhibition of each agonist expressed as a fraction of its control maximum inhibition. Abscissa scale: percentage of active receptors calculated from means of q-values in Table 1; those q-values which could not be estimated due to the limitations of Furchgott's method (see text and ND in Table 1) were calculated from the regression line in Figure 3 (◆). Numbers next to symbols refer to the time points after EEDQ-pretreatment (h). Control = maximum inhibition in saline-pretreated animals; (○) bromoxidine; (△) clonidine; (□) noradrenaline.

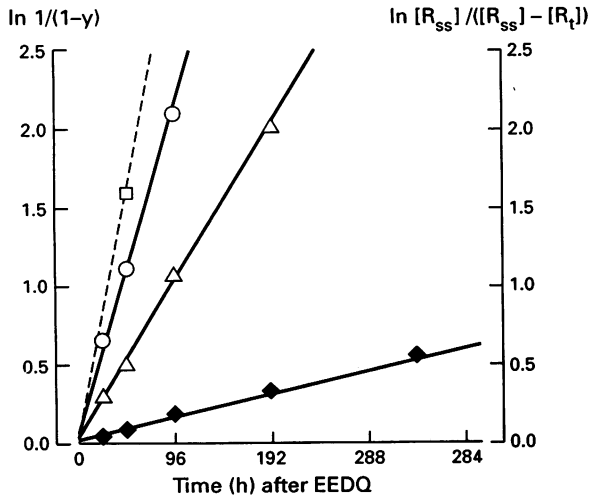

Figure 3 Time course of the reappearance of α_2-adrenoceptors and maximal agonist effects after inactivation with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). Semilogarithmic plot of fractions of active receptors (◆) and maximal effects of agonists (open symbols). Right-hand ordinate scale: ln ($[R_{ss}]/(1-[R_t])$); $[R_{ss}] = 1$ = total pool of active receptors; $[R_t]$ = fraction of active receptor pool at time t after EEDQ injection (= mean of q-values in Table 1). Left-hand ordinate scale: ln (1/(1-y)); 1 = is defined as the maximal agonist-induced decrease of stimulation-evoked overflow, and y is the fraction of this response obtained at the different time points after EEDQ-injection. Abscissa scale: time (h) after EEDQ injection. Lines were calculated by linear regression analysis; since there was only one experimental point for noradrenaline (NA), no analysis was performed and a dashed line was drawn through this point and the origin. (○) Bromoxidine; (△) clonidine; (□) NA.

is expressed as fraction of the respective maximal inhibition in saline-treated animals and plotted against the estimated fraction of active receptors. The analysis yielded rectangular hyperbolae for all three agonists indicating receptor reserve (Ruffolo, 1982). For instance, 90% of the agonist-mediated inhibition of ^{3}H-overflow occurred at fractions of active receptors of approximately 12%, 25%, and 45% for NA, bromoxidine, and clonidine, respectively.

A kinetic analysis of receptor reappearance as well as reappearance of maximal agonist effects is given in Figure 3. A plot of $\ln[R_{ss}]/([R_{ss}] - [R_t])$ vs time with $[R_{ss}]$ = receptors at steady state and $[R_t]$ = receptors at time t after EEDQ injection yielded a straight regression line with a correlation coefficient of $r = 0.988$ ($n = 5$; ◆). This result indicates that receptor repopulation occurred in a monoexponential fashion (Mauger *et al.*, 1982). A simliar result was obtained when this type of plot was performed with the maximal agonist effects; plotting ln(1/(1-y)) vs time with 1 = maximal agonist-induced inhibition under control conditions (= saline pretreatment) and y = fraction of this response at different times after EEDQ injection also yielded a set of straight regression lines (open symbols). The correlation coefficients were 0.982 ($n = 4$) and 0.954 ($n = 5$) for bromoxidine and clonidine, respectively. (Regression analysis of NA data was not possible, since there was only one experimental point). The calculated half-lives ($t_{1/2}$) for the recovery of maximal agonist effects were 20.8 h, 30.7 h and 63.6 h for NA, bromoxidine and clonidine, respectively. The $t_{1/2}$ for the recovery of the active receptor pool was considerably longer and amounted to 445 h.

Discussion and conclusions

The present study extends a previous investigation on the determination of agonist affinities at presynaptic α_2-autoreceptors under autoinhibition-free conditions (Agneter *et al.*, 1991). Whereas in the previous study a partial receptor inactivation was achieved with a low dose of EEDQ (0.8 mg kg^{-1}, i.p.), and all analyses were performed at a single time point (18 h) after pretreatment, a maximally effective dose of this agent was used in the present experiments and the analyses were performed at several time points after pretreatment. The dose of 1.6 mg kg^{-1} EEDQ can be considered maximally effective for α-adrenoceptor inactivation, since Adler *et al.* (1985) found 80%–85% loss of α_2-adrenoceptors 6 h after this dose using [^{3}H]-idazoxan binding and Pilc *et al.* (1992) found a somewhat lower inactivation (70%) 48 h after doses of 5 and 10 mg kg^{-1} EEDQ using [^{3}H]-clonidine binding. In binding assays, however, pre- and postsynaptic α_2-adrenoceptors are labelled and the measured overall loss of receptors after EEDQ treatment will not correctly reflect the loss of α_2-adrenoceptors if the irreversible ligand shows preference for the pre- or postsynaptic binding site. In fact, the percentage of receptors remaining active 48 h after EEDQ (Table 1) was 6–9% in the present experiments, which is less than could be expected on the basis of the published binding data (Adler *et al.*, 1985; Pilc *et al.*, 1992). Extrapolation to time zero after EEDQ from the regression analysis in Figure 3 yields a value of 2.7% active receptors indicating an almost complete inactivation of the α_2-adrenoceptor pool. Thus, the q-values determined according to the method of Furchgott represent largely the fractions of newly synthesized receptors and the K_A values determined for the three α_2-adrenoceptor agonists bromoxidine, clonidine and NA represent the dissociation constants at these receptors. However, a small influence of α_2-adrenoceptors not inactivated by EEDQ cannot be excluded in the present analyses.

Another issue that may be brought up in context with the K_A estimates are the the theoretical considerations published by Leff *et al.* (1990; see also Black & Leff, 1983). Operational models of pharmacological agonism involving the isomerization of the ternary complex mechanism predict that K_A

values tend to be underestimated when determined according to the method of Furchgott. This possibility cannot be excluded in the present experiments. Some evidence against it lies in the fact that the K_A estimates did not become smaller with decreasing receptor number (i.e. with a shorter time interval after EEDQ treatment), as would be predicted by the model (Black & Leff, 1983; Leff *et al.*, 1990).

There was reasonable agreement of the K_A values in the present study and those of the previous study with partial receptor inactivation. The respective values for bromoxidine, clonidine and NA were 136, 50, and 625 (previous study) vs. 187 (range 128–282), 71 (range 47–84), and 1202 (present study). The apparently divergent values for NA may be explained on the basis of method-inherent difficulties: reliable results are obtained with the method of Furchgott only when the maximal effect of the agonist after receptor inactivation is clearly separated from its effect in the control situation (Kenakin, 1987; pp. 171 and 177). For NA, this was the case at a single time point only after EEDQ administration, and hence, a considerable variability may apply for the determined K_A value. However, the value is identical with the one published by Fuder *et al.* (1986) for presynaptic α_2-adrenoceptors of sympathetic nerves in rat heart. The fact that the K_A values estimated from partial receptor inactivation and from near-complete inactivation after receptor resynthesis are the same, would suggest that either method is valid to determine the K_A for agonists at α_2-adrenoceptors (at least when EEDQ is used as receptor-inactivating agent). Adler *et al.* (1987) used the same approach of irreversible receptor inactivation with a maximally effective dose of EEDQ to determine the K_A for bromoxidine at newly synthesized α_2-adrenoceptors in rat cerebral cortex. The value is in the range of 2400–3600 nM and is therefore considerably higher than the value estimated in the present experiments. Probable reasons for this difference may be the presence of autoinhibition and the use of potassium as depolarizing agent in the experiments of Adler *et al.* (1987). It is well known that α_2-adrenoceptor agonist display a lower potency under such experimental conditions. For instance, the IC_{50} of bromoxidine in decreasing [^{3}H]-NA overflow from cortex slices of the rat in the study of Adler *et al.* (1987) was about 3 times higher than its IC_{50} in the present study.

When the q values (percentages of resynthesized receptors) were plotted against the maximal inhibition of ^{3}H-overflow at the same time points (Figure 2), it was found that receptor response returns faster than the estimated fraction of receptors. The analysis of repopulation kinetics (Figure 3) yielded a $t_{1/2}$ of 18.5 days for the resynthesis of α_2-adrenoceptors, whereas the $t_{1/2}$ for the recovery of full agonist effects were considerably shorter by factors of about 14, 7 and 21 for bromoxidine, clonidine, and NA, respectively. Adler *et al.* (1987) arrived at an estimate of 10 days for the $t_{1/2}$ of receptor repopulation and of 2.4 days for recurrence of the overflow-decreasing effects of bromoxidine (factor of about 4). The longer $t_{1/2}$ for resynthesis and shorter $t_{1/2}$ for recovery of functional response in our experiments may be a consequence of differences in methodology. The estimation of q according to the method of Furchgott and, hence, the estimation of receptor recovery is extremely sensitive against small changes in maximal effects. The method of stimulation with 4 pulses at 100 Hz, which produces a large overflow of tritium but avoids autoinhibition, results in steep concentration-response curves for the agonists with good resolution of maximal effects. This allows the detection of rather small differences in agonist effects which are observed at the later time points after receptor inactivation.

McKernan & Campbell (1982) reported a $t_{1/2}$ for α_2-adrenoceptor repopulation of 14 h as measured by [^{3}H]-rauwolscine and [^{3}H]-clonidine binding (receptor inactivation by phenoxybenzamine), and Adler *et al.* (1985) reported a $t_{1/2}$ of 4.1 days as measured by [^{3}H]-idazoxan binding (receptor inactivation by EEDQ). Both studies indicate a much faster resynthesis of α_2-adrenoceptors than the present study. It must be kept in mind, however, that [^{3}H]-α_2-adrenoceptor ligands incubated with membrane preparations also bind to postsynaptic receptors, and the observed rates may therefore not reflect the resynthesis rate of presynaptic α_2-adrenoceptors.

Taken together, the results of the present study corroborate and extend the results of our previous investigation on determination of agonist dissociation constants at presynaptic α_2-adrenoceptors. The fact that the receptor pool recovers considerably slower after extensive irreversible inactivation than the functional response to bromoxidine, clonidine or NA suggests a large receptor reserve for these receptors.

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The action of SDZ 205,557 at 5-hydroxytryptamine (5-HT_3 and 5-HT_4) receptors

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1 The interaction of the novel antagonist, SDZ 205,557 (2-methoxy-4-amino-5-chloro benzoic acid 2-(diethylamino) ethyl ester), at 5-HT_3 and 5-HT_4 receptors has been assessed *in vitro* and *in vivo*.

2 In guinea-pig hippocampus and in the presence of 0.4 μM 5-carboxamidotryptamine, 5-HT_4-mediated stimulation of adenylyl cyclase was competitively antagonized by SDZ 205,557, with a pA_2 value of 7.5, and a Schild slope of 0.81. In rat carbachol-contracted oesophagus, 5-HT_4-receptor mediated relaxations were surmountably antagonized by SDZ 205,557 with a similar pA_2 value (7.3). This value was agonist-independent with the exception of (**R**)-zacopride, against which a significantly lower value (6.4) was observed.

3 In functional studies of 5-HT_3 receptors, SDZ 205,557 exhibited an affinity of 6.2 in guinea-pig ileum compared with 6.9 at binding sites labelled by [^{3}H]-quipazine in NG108-15 cells. In the anaesthetized, vagotomized micropig, SDZ 205,557 produced only a transient blockade of 5-HT_4-mediated tachycardia. This contrasted with tropisetron, which was active for over 60 min after administration. The half-lives for the inhibitory responses of SDZ 205,557 and tropisetron were 23 and 116 min, respectively.

4 In conclusion, SDZ 205,557 has similar affinity for 5-HT_3 and 5-HT_4 receptors. The apparent selectivity observed in guinea-pig is due to the atypical nature of the 5-HT_3 receptor in this species. The short duration of action of this novel antagonist may complicate its use *in vivo*. SDZ 205,557 should, therefore, be used with appropriate caution in studies defining the 5-HT_4 receptor.

Keywords: SDZ 205,557; 5-HT_3 receptors; 5-HT_4 receptors

Introduction

The 5-hydroxytryptamine (5-HT) receptor family may be classified into 5-HT_1, 5-HT_2, 5-HT_3 (Bradley *et al.*, 1986) and 5-HT_4 subgroups (Bockaert *et al.*, 1992). The 5-HT_4 receptor (Dumuis *et al.*, 1988) is stimulated by indoles (such as 5-HT, 5-methoxytryptamine, 5-carboxamidotryptamine), substituted benzamides (such as renzapride, zacopride, cisapride) and azabicycloalkyl benzimidazolones (such as BIMU-1, BIMU-8), but is insensitive to antagonists of 5-HT_1, 5-HT_2, and 5-HT_3 receptors, such as methysergide, ketanserin and ondansetron, respectively (see Craig & Clarke, 1990; Bockaert *et al.*, 1992 for reviews). The 5-HT_4 receptor is competitively antagonized by tropisetron (ICS 205,930; $-\log K_B = 6.5$) and DAU 6285 ($-\log K_B = 6.8$; Dumuis *et al.*, 1992). Receptors exhibiting this pharmacological profile have been demonstrated in embryonic mouse colliculi neuronal cultures (Dumuis *et al.*, 1992), guinea-pig ileum (Craig & Clarke, 1990; Eglen *et al.*, 1990) and colon (Elswood *et al.*, 1991), rat oesophagus (Baxter *et al.*, 1991; Waikar *et al.*, 1992), porcine myocardium (Kaumann, 1990), human myocardium (Kaumann *et al.*, 1990) and amphibian adrenal gland (Idres *et al.*, 1991). More precise definition of 5-HT_4 receptors is, however, hampered by the lack of selective, competitive antagonists (see Turconi *et al.*, 1991; Bockaert *et al.*, 1992 for reviews).

Buchheit *et al.* (1991) reported that SDZ 205,557 (2-methoxy-4-amino-5-chloro benzoic acid 2-(diethylamino) ethyl ester) selectively antagonized 5-HT_4 receptors in guinea-pig isolated ileum with an affinity ($-\log K_B$) of 7.5. This affinity was greater than that at 5-HT_3 receptors in this tissue ($-\log K_B = 6.0$). SDZ 205,557 lacked affinity at 5-HT_1 or 5-HT_2 receptors and was, therefore, suggested as a useful ligand to define the 5-HT_4 receptor (Buchheit *et al.*, 1992). Pharmacological evidence suggests that 5-HT_3 receptors are a family of species variants. The guinea-pig 5-HT_3 receptor, notably, exhibits lower affinities for ligands than either rat or mouse 5-HT_3 receptors (see Kilpatrick & Tyers, 1992, for review). This may lead to an overestimation of the selectivity of antagonists, including SDZ 205,557, in favour of the 5-HT_4 receptor (Bockaert *et al.*, 1992). In the present study, SDZ 205,557 has been further evaluated by use of *in vitro* and *in vivo* responses in various species, on preparations sensitive to 5-HT_4 stimulation. This receptor, in contrast to the 5-HT_3 receptor, appears to exhibit affinities that are not species-dependent (Bockaert *et al.*, 1992).

A preliminary account of these studies has been previously published (Eglen *et al.*, 1992).

Methods

Guinea-pig hippocampal adenylyl cyclase

Male guinea-pigs (Hartley, 250–500 g) were killed by CO_2 asphyxiation. The hippocampi were then rapidly dissected, and homogenized (20 strokes) in a glass homogenizer in 9 ml of ice-cold buffer, of the following composition (mM): sucrose 300, Tris-HCl 20, EGTA 1, Na_2EDTA 2.5 and dithiothreitol 1 (pH 7.4, 23°C). This homogenate was then diluted, 1:8 (v/v) with buffer and centrifuged (10 min, 39,000 *g*, 4°C). The pellet was resuspended in 9 ml of buffer and the membrane suspension used directly in the adenylyl cyclase assay. Antagonists were added 30 min prior to addition of agonist. Measurements of adenylyl cyclase activity were performed, three times, according to the method of Alvarez & Daniels (1990) with each sample run in triplicate. The final composition of the incubation medium was (mM): [α^{32}P]-adenosine 5'-triphosphate ([α^{32}P]-ATP, 0.25 μCi) 0.5, $MgSO_4$ 5, Tris-HCl 44 (pH 7.4), 1-methyl-3-isobutylxanthine 1.0, sucrose 50, EDTA 1.0, EGTA 0.2, dithiothreitol 0.2, adenosine 3':5'-cyclic monophosphate (cyclic AMP) 2, guanosine 5'-triphosphate (GTP) 0.1, phosphoenol pyruvate 20 and pyruvate

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kinase 6 units ml^{-1}. The reaction was initiated by the sequential addition of the membrane suspension (40 µl) to the incubation medium in a total reaction volume of 200 µl. Incubations were performed for 30 min at 37°C and terminated by the addition of 20 µl of a stock solution of [^{3}H]-cyclic AMP (0.005 µCi) in 2.2 N HCl. Labelled cyclic AMP was added to estimate and correct for recovery of the nucleotide following column chromatography. The tube contents were heated at 95°C for 4 min and then cooled in an ice-water bath. Neutral alumina (1.3 g) was dispensed into disposable polypropylene columns with a Uniflow adjustable powder measure. The columns were placed on a plexiglas rack designed to hold the columns and to fit over a box of 100 scintillation vials. An aliquot (200 µl) of the solution contained in each tube was pipetted onto the columns and allowed to flow into the dry alumina. Cyclic AMP was then eluted with 4 ml of 0.1 M ammonium acetate, pH 7. The effluent (3.2 ml) was collected into scintillation vials, mixed with 15 ml scintillation fluid and counted for ^{3}H and ^{32}P in a liquid scintillation spectrometer.

Rat isolated oesophageal muscularis mucosae

The method used was that previously described by Baxter *et al.* (1991). Male rats (Sprague-Dawley, 200–250 g) from Charles River were killed by CO_2 asphyxiation, the thoracic oesophagus removed and placed in Tyrode solution (composition, mM: NaCl 139.0, KCl 2.7, $MgCl_2 \cdot 6H_2O$ 1.1, NaH_2PO_4 0.4, glucose 5.6, $NaHCO_3$ 11.8 and $CaCl_2 \cdot 6H_2O$ 1.8). The outer proprial muscle coat was cut longitudinally and gently peeled away, leaving the inner muscularis mucosae. Silk threads were then tied through the lumen on both ends of the tissues, which were then mounted vertically in 10 ml tissue baths containing Tyrode solution with 1 µM methysergide, 30 µM cocaine and 30 µM corticosterone. The baths were maintained at 37°C and constantly aerated with a mixture of 95% O_2 and 5% CO_2. An initial resting tension of 1 g was applied to the tissues, then adjusted to 0.5 g tension at 15 min intervals thereafter. After 1 h of equilibration, 100 µM pargyline was added to the baths for 30 min, followed by a 15 min washout. At this point, the tissues were exposed to 50 mM KCl for 5 min, washed four times, followed by an additional 30 min equilibration period with a 15 min wash cycle.

Carbachol (3 µM) was added to the baths to contract the preparations. Once a stable contraction had been established (usually 30 min) the agonist was then added cumulatively to the bath to induce relaxation. After establishing the control concentration-response curve for the agonist, the preparations were then washed. SDZ 205,557 (1–10 µM) was applied to the baths and allowed to equilibrate with the tissues for 60 min before the second agonist concentration-response curve. Since all 5-HT$_4$ agonists at high concentrations have the propensity to antagonize muscarinic receptors (Baxter *et al.*, 1991), a final concentration-response curve was constructed in the presence of both 10 µM 5-methoxytryptamine and SDZ 205,557 after a further 60 min. This procedure established the agonist concentration-range attributable to 5-HT$_4$ agonism alone as distinct from additional relaxation due to muscarinic receptor antagonism.

Agonist potencies were determined by nonlinear iterative curve fitting procedures (Michelson *et al.*, 1992), using the relationship described by Parker & Waud (1971). Apparent antagonist affinities ($-\log K_B$) were estimated by the relationship $-\log K_B = -\log$ [Antagonist]/(concentration ratio − 1) (Furchgott, 1972). Concentration ratios were measured at the agonist concentration which elicited 30% of the maximal relaxation, since under some conditions the effects observed at higher concentrations may have reflected muscarinic receptor antagonism. Against 5-HT itself, the apparent affinity was determined by the method of Arunlakshana & Schild (1959), wherein three concentrations of SDZ 205,557 were used, and the slope of the Schild plot determined by regression analysis.

Competition radioligand binding studies

Membranes were prepared from NG108-15 cells cultured under conditions described previously (Sharif *et al.*, 1991). In competition binding studies, 5-HT$_3$ receptors in NG108-15 cell membranes were labelled with 0.5 nM [^{3}H]-quipazine and non-specific binding was defined by 1 µM (S)-zacopride. The membrane homogenates were incubated in 25 mM Tris-Krebs (pH 7.4 at 25°C) with radioligand and varying concentrations of SDZ 205,557, or other standard 5-HT$_3$ receptor antagonists, in a final assay volume of 0.5 ml. The incubations were carried out for 45 min at 25°C and were terminated by rapid vacuum filtration over Whatman GF/B filters using a Brandell 48 cell harvester. This was immediately followed by 8 s of washing with ice-cold 0.1 M NaCl. The filters were pretreated with 0.3% polyethyleneimine in order to reduce filter binding of the radioligand and the radioactivity retained on the filters was determined by liquid scintillation spectrometry. All competition data were analyzed by iterative curve fitting procedure as described previously (Michel & Whiting, 1984). The apparent dissociation constant (K_i) of competing ligands was calculated from IC_{50} values by the Cheng-Prusoff equation (Cheng & Prusoff, 1973).

Anaesthetized micropig studies

The method used was modified from that described by Villalon *et al.* (1990). Yucatan micropigs (male and female; 14.9–20.8 kg, Charles River Laboratories Inc., Wilmington, MA, U.S.A.) were pretreated with ketamine HCl (approx. 30 mg kg^{-1}, i.m.), anaesthetized with pentobarbitone sodium (20 mg kg^{-1}) via a marginal ear vein, intubated, and mechanically ventilated with room air by an animal respirator (Harvard, Model 613). A femoral artery was cannulated for the measurement of arterial blood pressure via a Gould/Statham pressure transducer (P23ID). Dual cannulae were inserted into the ipsilateral femoral vein, one cannula for continuous infusion of supplemental anaesthetic (pentobarbitone sodium 8–15 mg kg^{-1} h^{-1}) and the second cannula for compound administration. A limblead II ECG was monitored via subcutaneously placed electrodes and heart rate (HR) was determined by a cardiotachometer triggered by the aortic pressure signal form. Following a midline cervical incision, the vagus nerves were bilaterally transected. Blood gas parameters were periodically monitored via a blood gas analyzer (Corning, Model 168) and blood gas values were stabilized within a normal (pH 7.2; P_{CO_2}, 33 mmHg; P_{O_2}, 96 mmHg) physiological range by adjustments of ventilatory rate, tidal volume, and positive end-expiratory pressure prior to continuing an experiment.

5-HT, tropisetron and SDZ 205,557 were administered in base equivalent doses. 5-HT, dissolved in 0.154 M NaCl, was administered at bolus i.v. doses of 1, 3, 10, 30 and 100 µg kg^{-1} (0.05 ml kg^{-1} doses) in each animal and a dose-response curve constructed. An ED_{50} dose for 5-HT was determined visually from each dose-response curve. SDZ 205,557 and tropisetron were dissolved in a 3:7 propylene glycol:normal saline (v/v) mixture. Animals were assigned randomly to 3 treatment groups: vehicle (3:7 propylene glycol:normal saline), SDZ 205,557 (6 mg kg^{-1}, i.v.), or tropisetron (5 mg kg^{-1}, i.v.). Doses for SDZ 205,557 and tropisetron were determined in preliminary dose-finding experiments (data not shown). These doses represented those that maximally antagonized 5-HT-induced tachycardia and/or were the maximum feasible dose based on compound supply and micropig weight. The ED_{50} dose of 5-HT (determined from a previous experiment) was administered 3 times at 10–15 min intervals to determine a control response (mean of 3 responses). Following administration of vehicle, SDZ 205,557, or tropisetron in a volume of 0.1 ml kg^{-1}, the ED_{50}

dose of 5-HT was administered in a volume of 0.03 ml kg^{-1} at 3, 10, 20, 30, 45, 60, 75, 90, 105, and 120 min thereafter.

Statistical analysis

Unless stated otherwise, all values are mean ± s.e.mean. Statistically significant differences between values estimated from *in vitro* studies were determined by Students *t* test, with $P<0.05$ being considered significant. The data from *in vivo* studies were analysed as follows: for the SDZ 205,557 duration of action study, the SDZ 205,557 treatment group was compared to its vehicle group with respect to the changes from baseline or the ranked changes from baseline. These ranks were used whenever the data exhibited different variances from group to group, using Bartlett's test for heteroscedasticity. A repeated measures two-way analysis of variance (ANOVA) including effects for treatment, time, and treatment by time interaction was used. One-way ANOVAs were performed at each time point to determine statistical significance for individual differences between treatment groups and the data were expressed as % inhibition of control tachycardia response. Pairwise contrasts were adjusted for multiple comparisons by Dunn's procedure. The critical value for each pairwise comparison was adjusted by a Bonferroni correction, if no overall treatment or treatment-by-time effect was noted. Analysis of tachycardic dose-response curves for 5-HT, renzapride, and (**R,S**)-zacopride, as well as determinations of rates of return of responses to baseline in the SDZ 205,557 duration of action study, were conducted by nonlinear iterative curve fitting procedures (Michelson *et al.*, 1992) with statistically significant differences assessed from a *t* test.

Materials

NG108-15 cells were obtained from the Institute of Cancer and Developmental Biology, Syntex Research, Palo Alto, CA, U.S.A. SC-53116 was donated by Searle, Skokie, Ill., U.S.A. BIMU-1 (endo-N-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazol-1-carboxamide), BIMU-8 (endo-N-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-isopropyl-2-oxo-1H-benzimidazol-1-carboxamide HCl), renzapride, MDL 72222 (1αH, 3α, 5αH-tropan-3yl-3,5-dichlorobenzoate), SDZ 205,557, ondansetron, tropisetron and zacopride (**R + S**; **R,S**) were synthesized in the Institute of Organic Chemistry, Syntex Research, Palo Alto, CA, U.S.A. 5-Hydroxytryptamine, 2-methyl-5-HT and phenylbiguanide were obtained from Research Biochemicals Incorporated, Natick, MA, U.S.A. [^{3}H]-quipazine (specific activity 55 Ci $mmol^{-1}$) was purchased from DuPont NEN (Boston, MA, U.S.A.). [2,8-^{3}H]-adenosine 3′:5′-cyclic monophosphate (30–50 Ci $mmol^{-1}$), [α^{32}P]-adenosine 5′-triphosphate (10–50 Ci $mmol^{-1}$) and Aquasol were purchased from New England Nuclear Corp. (NEN). Neutral alumina (ICN, Alumina N, activity grade 1) was purchased from ICN Biomedicals, Eschwege, Germany. All remaining compounds were obtained from Sigma Chemical Co, St. Louis, Mo, U.S.A.

Results

Guinea-pig hippocampal adenylyl cyclase

Adenylyl cyclase activity (basal activity = 1.8 nmol 30 min^{-1} mg^{-1} protein) in membranes isolated from the guinea-pig hippocampus was stimulated by 5-HT (~2 fold) with a $-\log EC_{50}$ value of 6.4 (Figure 1). The sigmoidal concentration-response curve to 5-HT appeared to be monophasic. By contrast, 5-carboxamidotryptamine (5-CT) produced a biphasic response curve. Low concentrations (0.4 μM) of 5-CT were sufficient to stimulate maximally the initial phase (Figure 1). Previous studies have demonstrated

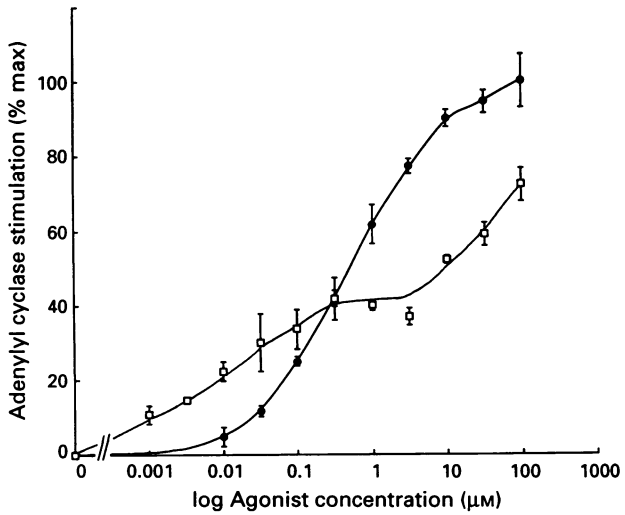

Figure 1 Effect of 5-hydroxytryptamine (●) and 5-carboxamidotryptamine (□) on guinea-pig hippocampal adenylyl cyclase. Values are mean ± s.e.mean (vertical bars), with the experiment repeated three times and each sample analysed in triplicate.

that the increase in adenylyl cyclase activity elicited by 5-HT in the presence of 0.4 μM 5-CT is due to the stimulation of 5-HT_4 receptors (Shenker *et al.*, 1987). In the absence of 5-CT, maximally effective concentrations (100 μM) of SDZ 205,557 and spiperone reduced the stimulation in response to 10 μM 5-HT by 44% and 64%, respectively. Stimulation of adenylyl cyclase by 5-HT was abolished when the membrane preparation was incubated in the presence of both antagonists (Figure 2).

In the presence of 5-CT, responses to 5-HT were antagonized, in a concentration-dependent manner, by both tropisetron and SDZ 205,557, with $-\log IC_{50}$ values of 6.4 and 5.1 (mean, s.e.mean < 10%), respectively. In contrast, responses to 5-HT were unaffected by propranolol (0.1 mM), ketanserin (1 μM) or methysergide (1 μM; data not shown). The apparent affinity of SDZ 205,557 at the 5-HT_4 receptor

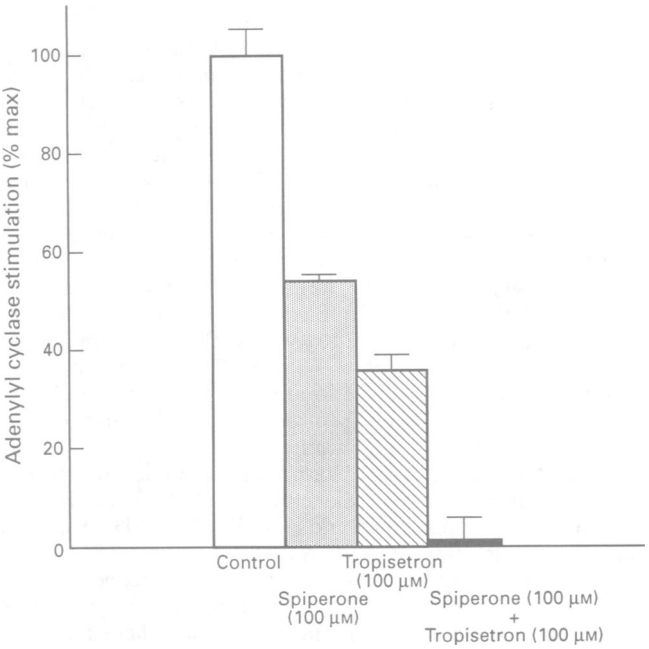

Figure 2 Effect of spiperone, tropisetron (100 μM) and in combination on the stimulation of hippocampal adenylyl cyclase by 5-hydroxytryptamine. Values are mean ± s.e.mean (vertical bars), with the experiment repeated three times and each sample analysed in triplicate.

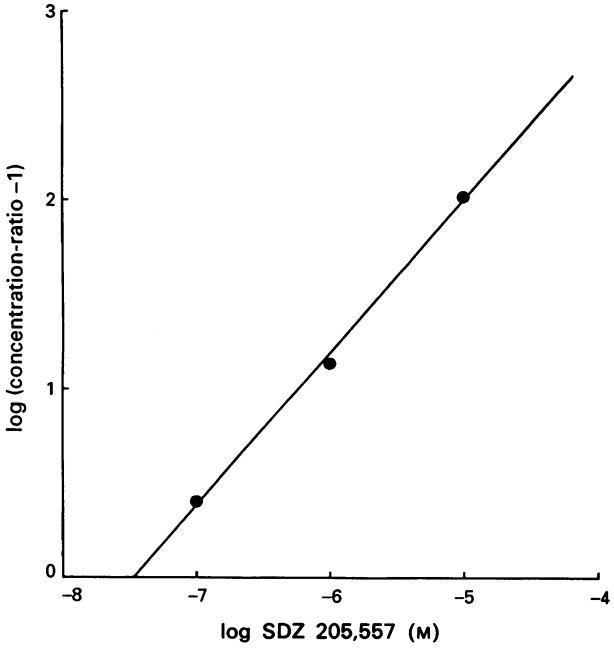

Figure 3 Schild analysis of antagonism by SDZ 205,557 of 5-hydroxytryptamine-stimulated adenylyl cyclase in guinea-pig hippocampus. Values are means of three experiments, each sample analysed in triplicate.

mediating the stimulation of adenylyl cyclase was derived by Schild regression analysis. The pA_2 value was 7.5 with a Schild slope of 0.81 (Figure 3).

Rat oesophageal muscularis mucosae

In preparations pre-contracted with 10 μM carbachol, all agonists induced a concentration-dependent relaxation. The potencies and intrinsic activities to agonists studied are shown in Table 1. The rank order was 5-HT = 5-MeOT > BIMU-1 ≥ SC-53116 ≥ BIMU-8 > (S)-zacopride > (R)-zacopride. SDZ 205,557 (1 nM–10 μM) lacked intrinsic activity at the oesophageal 5-HT$_4$ receptor in that no relaxations were seen. In the presence of SDZ 205,557, concentration-response curves to all agonists were shifted to the right in a competitive fashion. Schild regression analysis performed with SDZ 205,557 (1–10 μM) and using 5-HT as the agonist, resulted in a Schild slope not significantly different from unity (1.24, 95% confidence limits 0.98–1.50) (Figure 4). When the unity constraint was imposed, a pA_2 value of 7.3 (95% confidence limits: 7.2–7.4) was determined. The apparent affinities ($-\log K_B$) obtained with a single concentration of SDZ 205,557 against the other agonists were similar to this value (Table 1). The exception to this was the affinity

Table 1 Potencies ($-\log EC_{50}$) and intrinsic activities (α) of compounds and affinity estimates for SDZ 205,557 ($-\log K_B$) at 5-HT$_4$ receptors mediating oesophageal relaxation

Compound	$-\log EC_{50}$	α	$-\log K_B$
5-HT	8.3 ± 0.03	1.0	7.4 ± 0.1
5-Methoxytryptamine	8.2 ± 0.03	1.1	6.9 ± 0.2
BIMU-1	7.9 ± 0.1	0.7	7.3 ± 0.2
SC-53116	7.7 ± 0.1	0.6	7.1 ± 0.3
BIMU-8	7.6 ± 0.2	0.9	7.1 ± 0.2
(S)-zacopride	6.7 ± 0.1	0.9	6.8 ± 0.3
(R)-zacopride	6.2 ± 0.2	0.9	6.4 ± 0.5*

Values are mean ± s.e.mean, $n = 4–8$ animals. SDZ 205,557 was employed at a concentration of 10 μM.
*Significantly different ($P < 0.05$) from $-\log K_B$ value estimated using 5-HT.

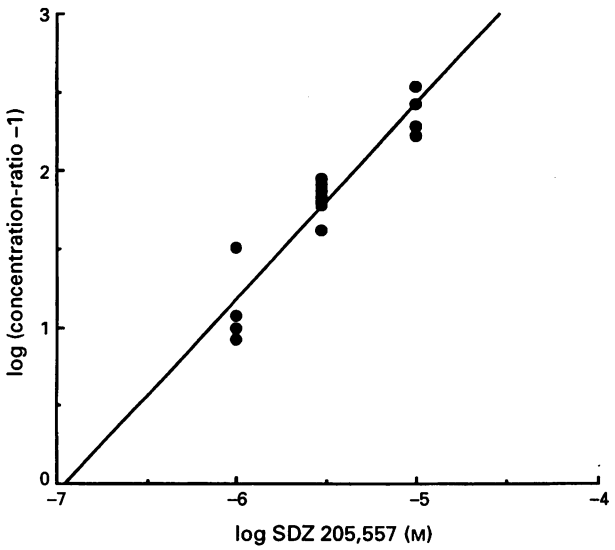

Figure 4 Schild analysis of antagonism by SDZ 205,557 of 5-hydroxytryptamine-mediated relaxation in rat oesophagus. Values are individual concentration-ratios.

when (R)-zacopride was used as the agonist. In this case the $-\log K_B$ value was significantly lower.

Tropisetron (3 μM) antagonized the relaxations induced by 5-HT with a concentration-ratio of 35 (95% confidence limits, 27–45) and an apparent affinity ($-\log K_B$) of 7.0 ± 0.04. SDZ 205,557 (3 μM) antagonized responses to 5-HT with a concentration-ratio of 98 (41–237). In the presence of both SDZ 205,557 (3 μM) and tropisetron (3 μM), responses to 5-HT were shifted to the right with a combined concentration-ratio of 153 (99.8–234; mean, 95% confidence limits). This was not significantly different from that predicted by the combination of two competitive antagonists interacting at the same site (i.e. 133 fold).

Interactions at 5-HT$_3$ receptors

Experiments in guinea-pig isolated ileum were conducted in the presence of methysergide (1 μM) and 5-methoxytryptamine (10 μM) to desensitize selectively 5-HT$_1$, 5-HT$_2$ and 5-HT$_4$ receptors. At ileal 5-HT$_3$ receptors SDZ 205,557 (10 μM) antagonized responses to 5-HT, with an apparent affinity ($-\log K_B$) of 6.2 ± 0.08. Affinities for other 5-HT$_3$ antagonists reported by Eglen *et al.* (1990), under similar conditions are included for comparison (Table 2). The rank order of antagonist affinity at ileal 5-HT$_3$ receptors was therefore: (S)-zacopride > tropisetron > ondansetron > SDZ 205,557. In binding studies, at 5-HT$_3$ receptors identified by [^{3}H]-quipazine in NG108-15 cell membranes (Table 3), SDZ

Table 2 Apparent affinity in competition radioligand binding and functional studies of compounds for 5-HT$_3$ receptors

	NG108-15 cells		*Guinea-pig ileum*[a]
	$-\log pK_i$	Hill coefficient	$-\log K_B$
(S)-zacopride	9.5 ± 0.1	1.1 ± 0.2	8.1
Ondansetron	8.4 ± 0.1	1.0 ± 0.1	6.9
Tropisetron	8.3 ± 0.4	0.9 ± 0.2	7.6
MDL-72222	7.6 ± 0.1	1.1 ± 0.1	ND
SDZ 205,557	6.9 ± 0.2	1.3 ± 0.1	6.2[b]
Metoclopramide	6.7 ± 0.1	1.0 ± 0.1	ND

Binding values in NG108-15 cells are mean ± s.e.mean, $n = 3–4$ separate determinations; functional values in guinea-pig ileum are mean ± s.e.mean, $n = 6$ animals.
[a]Eglen *et al.* (1990).
[b]Unsurmountable.
ND = not determined.

Table 3 Potencies and maximum increase in heart rate to intravenous administration of 5-HT_4 agonists in the anaesthetized, bilaterally-vagotomized Yucatan micropig

Compound	*ED_{50}* ($\mu g\ kg^{-1}$)	*Maximum tachycardic increase* (beats min^{-1})
5-HT	4.2 (3.3–5.3)	95 (90–100)
Renzapride	20[a] (17–25)	76[a] (70–81)
(R,S)-zacopride	28[a] (20–41)	100 (98–110)

Values are mean, with 95% confidence limits in parentheses, $n = 6$ animals.
[a]Significantly different ($P < 0.05$) compared to 5-HT.

205,557 displacement isotherms yielded an apparent affinity ($-\log K_i$) of 6.9 ± 0.2. The Hill coefficient (1.3 ± 0.1) was not significantly different from unity, suggesting an interaction of SDZ 205,557 at a homogeneous population of sites. The rank order of apparent affinities was (S)-zacopride > ondansetron > tropisetron > MDL 72222 > SDZ 205,557 > metoclopramide. The values estimated in binding sites were greater then those estimated in functional studies (Table 2), in agreement with previous reports by Butler *et al.* (1990).

Tachycardia in anaesthetized pig

5-HT, renzapride and (R,S)-zacopride elicited a dose-dependent tachycardia in the anaesthetized pig with 5-HT being significantly more potent than either benzamide (Figure 5). Preliminary studies showed that consecutive, reproducible dose-response curves to 5-HT in the pig can be constructed at intervals of 90–120 min. Heart rate responses returned to baseline within 10–15 min after each dose of 5-HT, with responses treated cumulatively after successive doses of the benzamides. 5-HT and (R,S)-zacopride acted as full agonists

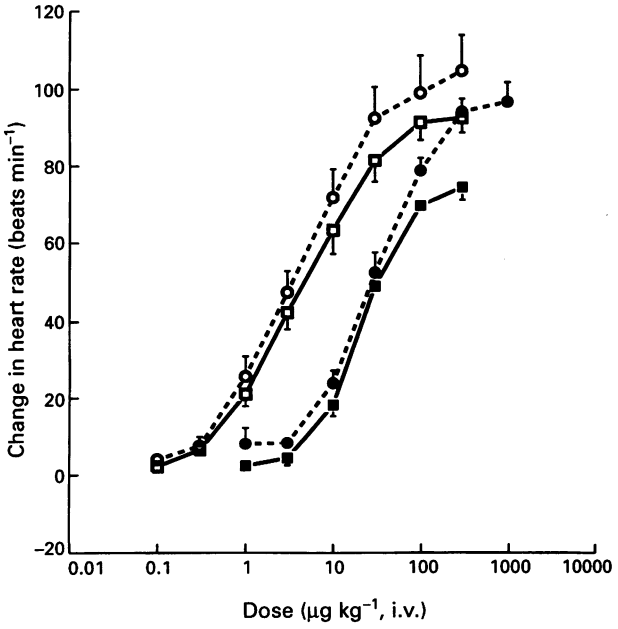

Figure 5 Dose-response curves for 5-hydroxytryptamine (5-HT, ○ and □), renzapride (■) and (R,S)-zacopride (●) in the anaesthetized, vagotomized micropig. Two curves for 5-HT are shown, conducted in separate series of experiments. The curve to renzapride was established in animals following construction of the 5-HT curve shown with (○), whereas the curve to (R,S)-zacopride was established following construction of the 5-HT curve shown with (□). Values are mean ± s.e.mean (vertical bars), $n = 6$ animals.

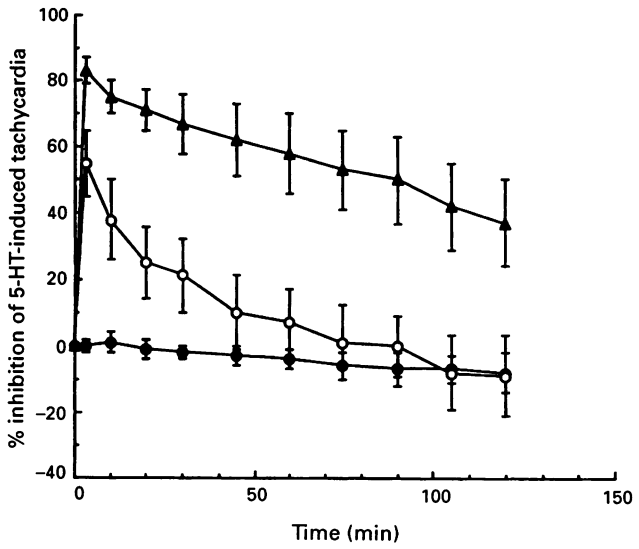

Figure 6 Time-course of the inhibition of tachycardic responses to 5-hydroxytryptamine (5-HT) by vehicle (●), tropisetron (▲) or SDZ 205,557 (○) in the anaesthetized, vagotomized micropig. Values are mean ± s.e.mean (vertical bars), $n = 6$.

zacopride acted as full agonists and renzapride as a partial agonist (Table 3). The responses to all these agonists were antagonized by tropisetron (data not shown). To assess the inhibitory activity of tropisetron and SDZ 205,557, single doses of the antagonists were administered against the ED_{50} dose for 5-HT (3–10 $\mu g\ kg^{-1}$, i.v.), previously obtained by individual titration. The variances of the control and treatment groups did not differ sufficiently to utilize ranked changes from baseline; therefore a parametric repeated measures ANOVA was used to analyze this data. In these overall ANOVAs, the effects of treatment, time and treatment × time interaction were all highly significant ($P < 0.0001$). Subsequent pairwise contrasts (one-way ANOVAs) showed that SDZ 205,557, administered at 6.0 mg kg^{-1}, i.v., significantly ($P < 0.05$) inhibited the tachycardic response to 5-HT for only 3 min following administration whereas no significant effect was seen for the remainder of the experiment. In contrast, tropisetron (5 mg kg^{-1}, i.v.) administered against similar doses of 5-HT, significantly ($P < 0.05$) inhibited responses for 120 min following administration (Figure 6). The half-lives for the inhibitory responses were 23 (17–35) and 116 (85–175) min for SDZ 205,557 and tropisetron, respectively (values are mean with 95% confidence intervals).

Discussion

Receptors can be defined on the basis of selective antagonism. Tropisetron and DAU 6285 are mixed 5-HT_3 and 5-HT_4 receptor antagonists (Dumuis *et al.*, 1992). SDZ 205,557 may be useful in 5-HT_4 characterization since it exhibits a higher affinity at guinea-pig ileal 5-HT_4 receptors (7.5) relative to 5-HT_3 receptors (6.2; Buchheit *et al.*, 1991; 1992).

In guinea-pig hippocampal membranes, two 5-HT receptors are positively coupled to adenylyl cyclase (Shenkar *et al.*, 1987). At low 5-HT concentrations (1 nM–1 μM), stimulation is mediated by an, as yet undefined 5-$HT_{1\text{-like}}$ receptor, whereas higher 5-HT concentrations interact with a 5-HT_4 receptor (Dumuis *et al.*, 1988). The 5-HT_1 receptor component is selectively blocked by spiperone and can be maximally stimulated by 5-CT at concentrations that fail to stimulate the 5-HT_4 receptor (Shenker *et al.*, 1987; this study). The 5-HT_4 receptor component is selectively blocked

by tropisetron but not by spiperone (Dumuis *et al.*, 1988; this study). Selective inhibition by SDZ 205,557 of the 5-HT$_4$ but not the 5-HT$_1$ component was observed in the present study. At the former, SDZ 205,557 acted in a competitive surmountable fashion, as judged by the Schild slope. The pA$_2$ value was similar to the value reported at 5-HT$_4$ receptors mediating contraction of guinea-pig ileum (Buchheit *et al.*, 1992).

SDZ 205,557 in guinea-pig hippocampus and rat oesophagus lacked intrinsic activity and Buchheit *et al.* (1992) reported similar findings in guinea-pig ileum. In rat isolated oesophageal muscularis mucosae, relaxant responses to 5-HT$_4$ agonists were surmountably antagonized by SDZ 205,557 and this was also observed in the hippocampus. In this respect, these findings differ from those reported by Buchheit *et al.* (1992) in guinea-pig ileum, in which unsurmountable antagonism was evident. The reason for these differences in the kinetics of antagonism by SDZ 205,557 between 5-HT$_4$ receptors in ileum and oesophagus is unclear. The $-\log K_B$ values for SDZ 205,557 in oesophagus were, in general, also agonist-independent suggesting an interaction by these compounds at a common site (see below). Similar observations have been made with tropisetron (Baxter *et al.*, 1991) or DAU 6285 (Waikar *et al.*, 1992) in this tissue. In guinea-pig ileum (Buchheit *et al.*, 1992), lower pA$_2$ values were observed for SDZ 205,557 against (**R,S**)-zacopride (6.8) and metoclopramide (5.4), in comparison to those against 5-HT (7.4). The reason for the agonist-independence in rat oesophagus (this study) but not in guinea-pig ileum (Buchheit *et al.*, 1992) is unclear, but the unsurmountable nature of the antagonism in the latter makes direct comparison difficult. SC-53116 is a substituted pyrrolizidine reported to be a potent and selective 5-HT$_4$ agonist in the rat oesophagus (Flynn *et al.*, 1992). The antagonism by SDZ 205,557 of responses to SC-53116 on the rat oesophageal muscularis mucosae (this study) is in agreement with this suggestion, since it was antagonized with a similar affinity to that observed against 5-HT.

The lower $-\log K_B$ value against (**R**)-zacopride observed in the present study is difficult to explain although Buchheit *et al.* (1992) reported a lower affinity for SDZ 205,557 in guinea-pig ileum when (**R,S**)-zacopride was used as the agonist. Combination concentration-ratio experiments (Paton & Rang, 1965) however, showed that SDZ 205,557 and tropisetron interacted at the same oesophageal site (this study) since the observed combined concentration-ratio was not significantly different from the predicted combined ratio.

The affinity of SDZ 205,557 for 5-HT$_3$ receptors in NG108-15, cells estimated in binding studies, was similar to the affinity obtained at 5-HT$_4$ receptors in guinea-pig ileum (Buchheit *et al.*, 1991; 1992), hippocampal adenylyl cyclase or rat oesophagus (this study; Tables 1 and 3). In contrast, the affinity at the 5-HT$_3$ receptor in guinea-pig ileum was lower (Buchheit *et al.*, 1992; this study), a feature shared by other antagonists at this site (Table 2). Indeed, using the novel 5-HT$_3$ receptor ligand [^{3}H]-RS-42358-197 to label the 5-HT$_3$ site in guinea-pig ileum, SDZ 205,557 gave a pK_i of 7.3 and Hill coefficient of 1.04 (Wong & Stefanich, unpublished data). These disparities suggest that the guinea-pig possesses a species variant of the 5-HT$_3$ receptor (see Kilpatrick & Tyers, 1992, for review; Wong *et al.*, 1992). Apart from the guinea-pig, therefore, SDZ 205,557 had equal affinity at 5-HT$_3$ and 5-HT$_4$ receptors. When using SDZ 205,557, appropriate care should therefore be taken to exclude 5-HT$_3$ function in bioassays in which both 5-HT$_3$ and 5-HT$_4$ receptors are involved.

In the anaesthetized, vagotomized micropig, 5-HT elicited a tachycardic response in the presence of 5-HT$_1$, 5-HT$_2$, 5-HT$_3$, M$_2$-muscarinic and β-adrenoceptor blockade. These responses were antagonized by high doses of tropisetron and mimicked by (**R,S**)-zacopride and renzapride. Similar observations have been reported by Villalon *et al.* (1990) in the Yorkshire pig and concur with biochemical data showing that porcine myocardial 5-HT$_4$ receptors stimulate adenylyl cyclase (Kaumann, 1990). The tachycardia may be due to subsequent activation of a kinase (Kaumann *et al.*, 1991) and closure of potassium channels (Bockaert *et al.*, 1992). 5-HT-induced tachycardia in the micropig, thus, provides a suitable *in vivo* assay to study 5-HT$_4$ compounds. The administration of SDZ 205,557 failed to antagonize, except briefly (3 min) after injection, the tachycardic responses to 5-HT. The doses of 5-HT were submaximal and adequately blocked by tropisetron. The short-lived antagonism by SDZ 205,557 *in vivo* contrasts with the sustained antagonism seen *in vitro* (at least 60 min; see Methods). The compound, at least at the dose tested, appears to be subject to rapid metabolism, probably due to hydrolysis at the ester moiety in the SDZ 205,557 molecule. It should be noted, however, that such rapid degradation may not be so marked at higher doses and additional experiments are required to study this.

In conclusion, SDZ 205,557 acted as a surmountable 5-HT$_4$ receptor antagonist in guinea-pig hippocampus and rat oesophagus, although it had no selectivity between mouse neuroblastoma 5-HT$_3$ and 5-HT$_4$ receptors. In guinea-pig, a 5-HT$_4$/5-HT$_3$ selectivity was evident due to the atypical nature of the guinea-pig 5-HT$_3$ receptor. The short duration of action *in vivo* together with this limited selectivity, suggests that caution should be exercised in its use to define the 5-HT$_4$ receptor.

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NK_2 receptors mediate plasma extravasation in guinea-pig lower airways

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1 Neurokinin (NK) receptor-mediated extravasation has been examined in guinea-pig airways by use of a recently described marker for microvascular protein leakage, ^{125}I-labelled human fibrinogen.

2 Neurokinin A (NKA) caused a dose-dependent increase in plasma [^{125}I]-fibrinogen extravasation in trachea, main bronchi, secondary bronchi and intraparenchymal airways. In contrast, the NK_2 selective agonist [β-Ala^8]NKA(4–10) only caused extravasation in the secondary and intraparenchymal airways.

3 The NK_2 selective antagonist, SR 48968, caused a dose-dependent inhibition of NKA and [β-Ala^8]NKA(4–10)-induced extravasation of fibrinogen in guinea-pig secondary bronchi and intraparenchymal airways. SR 48968 was without effect on the NKA-induced extravasation in trachea and main bronchi.

4 NKA- or [β-Ala^8]NKA(4–10)-induced plasma extravasation was not modified by pretreatment with histamine H_1- or H_2-receptor antagonists.

5 It is concluded that NK_2 receptors mediate plasma [^{125}I]-fibrinogen extravasation in guinea-pig secondary bronchi and intraparenchymal airways. This effect is direct and does not depend upon histamine released from mast cells.

Keywords: NK_2 receptor; [β-Ala^8]NKA(4–10); SR 48968; plasma extravasation; ^{125}I-labelled human fibrinogen; guinea-pig airways

Introduction

Neurokinin A (NKA) is a decapeptide belonging to a family of peptides named tachykinins, which are characterized by a common C-terminal amino acid sequence. It is now generally accepted that the diverse actions of the tachykinins (substance P, NKA, neurokinin B) are mediated by at least three types of receptors currently termed NK_1, NK_2 and NK_3 (Helke *et al.*, 1990; Nakanishi, 1991; Guard & Watson, 1991). It has been proposed recently (Maggi *et al.*, 1990; Patacchini *et al.*, 1991) that subtypes of the NK_2 receptor may also exist. Each NK receptor binds several naturally occurring tachykinins though their affinities for each receptor can differ appreciably. Thus, among the endogenous mammalian tachykinins, substance P has a preferential affinity for NK_1 receptors, NKA for NK_2 receptors and neurokinin B for NK_3 receptors (Watson, 1984; Inversen *et al.*, 1988; 1990; Quirion & Dam, 1988). Notwithstanding, each tachykinin exhibits appreciable activity at the other neurokinin receptors. This is exemplified by NKA which has 25% of the affinity of substance P on the dog carotid artery, a preparation that contains only NK_1 receptors (Regoli *et al.*, 1988). Modifications of specific amino acids on the tachykinin peptide chains have resulted in the development of selective agonists {[Sar^9,Met(O$_2$)^{11}]-SP at NK_1; [β-Ala^8]NKA(4–10) at NK_2; [MePhe^7] NKB at NK_3} for neurokinin receptors as demonstrated *in vitro* on peripheral tissues (Regoli *et al.*, 1988). The recent discovery of a potent, selective non-peptide antagonist for NK_2 receptors (SR 48968; Advenier *et al.*, 1992; Emonds-Alt *et al.*, 1992) has allowed more definitive evaluation of the physiological role(s) of NKA and its analogues.

In the respiratory system, tachykinins exhibit a variety of effects on airway function that include bronchoconstriction, plasma extravasation, mucus secretion and vasodilatation (Barnes *et al.*, 1990). Increased microvascular permeability with accompanying plasma exudation is the root cause of tissue oedema observed in asthma (Chung *et al.*, 1990). In guinea-pig upper airways (trachea and main bronchi) it has been suggested that neurogenic plasma extravasation is mediated via NK_1 receptors (Abelli *et al.*, 1991; Lei *et al.*, 1992). In contrast, however, bronchoconstriction in guinea-pig and human airways is mediated principally by NK_2 receptors (Emonds-Alt *et al.*, 1992; Ellis & Undem, 1992).

In the present study, we have used the NK_2 selective agonist [β-Ala^8]NKA(4–10) and antagonist SR 48968 (Advenier *et al.*, 1992) to determine whether NK_2 receptors mediate microvascular leakage in guinea-pig airways. Using a recently described marker of plasma protein extravasation, ^{125}I-labelled human fibrinogen (Pedersen *et al.*, 1991), we have been able to show quite clearly that NK_2 receptors are capable of promoting microvascular leakage but only in guinea-pig lower airways (secondary bronchi and intraparenchymal airways).

Methods

Plasma protein extravasation

Male guinea-pigs weighing 350–400 g were anaesthetized with ketamine hydrochloride (25 mg kg^{-1}, i.m.) and xylazine (5 mg kg^{-1}, i.m.). The right jugular vein was cannulated for the intravenous administration of drugs. The trachea was intubated to permit mechanical ventilation of the animal (tidal volume 8 ml kg^{-1}; rate 55 strokes min^{-1}) with room air at 22°C and 42% humidity. The animals were injected with succinylcholine chloride (5 mg kg^{-1}, i.p.) and equilibrated for 10 min before the intravenous injection of [^{125}I]-fibrinogen (50 μCi kg^{-1}). Either saline or the neurokinin agonist (NKA or [β-Ala^8]NKA(4–10)) were given 5 min after the administration of [^{125}I]-fibrinogen. In the experiments in which the receptor antagonists were used (SR 48968, mepyramine,

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cimetidine) they were injected intravenously 1 min after the administration of [^{125}I]-fibrinogen i.e. 4 min before challenge with either saline or NK agonist.

Twenty seconds before the aminal was killed, heparin (1,400 units kg^{-1}) was injected intravenously to prevent non-specific [^{125}I]-fibrin formation. In order to determine a time course for NK-induced extravasation, the guinea-pigs were killed at different time points (5,15 or 30 min) after injection of the agonist. The 15 min time point was chosen in order to establish a dose-response relationship for inhibition of NK-induced leakage by SR 48968. A blood sample was taken by cardiac puncture for determination of the amount of radioactivity in the plasma (c.p.m. μl^{-1} plasma). The heart and lung were rapidly removed and a small incision made at the right ventricle for the insertion of a blunt tip feeding needle. The lung was perfused through the pulmonary artery via the feeding needle with 50 ml of gravity-fed saline (0.9%). Leaking of saline from the ventricle was prevented by clamping the opening with a haemostat. After the perfusion, the heart was removed and the airways were then cleaned and dissected out into four regions: (a) trachea; (b) main bronchi, defined as the region between the first branch off the trachea and the second branch; (c) secondary bronchi, defined as the region between the main bronchi and the airways imbed in the lung parenchyma; (d) intraparenchymal airways, defined as the airways imbed in the parenchyma. Parenchymal tissues, which increase background value, were removed by gently scrubbing the lung lobes with a pair of tweezers. This procedure keeps the whole length of intraparenchymal airways intact. The tissues were blotted, weighed and the radioactivity counted in a gamma counter (Beckman). Microvascular leakage is expressed as μl plasma 100 mg^{-1} of tissue.

Drugs

^{125}I-labelled human fibrinogen (1 μCi μg^{-1} protein: McMaster University); NKA, [β-Ala^8]NKA(4–10) (Peninsula Lab. Inc.); cimetidine; mepyramine maleate and heparin (Sigma) were used. SR 48968 ((−)-N-methyl-N[4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide) was synthesized (Hale *et al.*, 1992) by Merck Research Laboratories, Rahway, NJ, U.S.A.

Data analysis

Specific leakage was calculated by subtracting non-specific from the total plasma leakage occurring after the administration of agonists. The dose of agonist causing 50% of the maximum plasma leakage is expressed in terms of ED_{50} (nmol kg^{-1}). The dose of SR48968 inhibiting 50% of agonist (3 nmol kg^{-1})-induced plasma leakage is defined as ID_{50} (mg kg^{-1}). Each animal received only one dose of agonist, or antagonist. ED_{50} and ID_{50} values were calculated from pooled data. The statistical significance of the data was evaluated with Student's t test for independent samples. P values lower than $P<0.05$ were considered significant.

Results

The results presented in Figure 1 show that NKA induced a dose-dependent increase in plasma [^{125}I]-fibrinogen extravasation in the trachea (a), mean bronchi (b), secondary bronchi (c) and intraparenchymal airways (d); ED_{50} values were 0.4, 0.4, 0.9 and 0.9 nmol kg^{-1} respectively (Figure 1). In stark contrast, [β-Ala^8]NKA(4–10) consistently failed to induce any [^{125}I]-fibrinogen leakage in either the trachea (a) or main bronchi (b). A dose-dependent relationship for [β-Ala^8]NKA(4–10)-induced leakage was observed only in the secondary bronchi (c) and intraparenchymal airways (d) (Figure 1). The maximal responses to both the NK_2 selective agonist and NKA were obtained after 3 nmol kg^{-1}. At this dose, [β-Ala^8]NKA(4–10) increased plasma [^{125}I]-fibrinogen extravasation in the seconday bronchi by 307% and in the intraparenchymal airways by 380%. Mean values for plasma extravasation were 4.3 ± 1.3 and 4.2 ± 0.7 μl plasma 100 mg^{-1} tissue in these respective areas. Doses causing 50% of the maximum plasma extravasation were approximately 0.6 nmol kg^{-1} in secondary bonchi and 1.5 nmol kg^{-1} in the intraparenchymal airways (Figure 1). NKA and [β-Ala^8]NKA(4–10) produced closely similar maximum effects and were approximately equipotent in inducing extravasation (Figure 1).

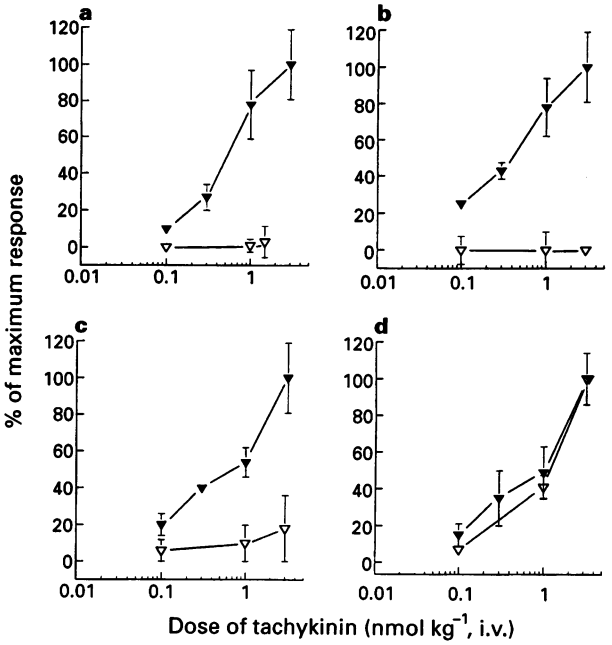

Figure 1 Dose-response curves for intravenous neurokinin A (NKA, ▼)- or [β-Ala^8]NKA(4–10) (▽)-induced plasma [^{125}I]-fibrinogen extravasation (% of control) in guinea-pig trachea (a), main bronchi (b), secondary bronchi (c) and intraparenchymal airways (d). Each data point represents the mean ± s.e.mean (vertical bars) of 4 observations.

The results presented in Figure 2 illustrate the time course for [^{125}I]-fibrinogen extravasation after either saline (control) or [β-Ala^8]NKA(4–10). The mean background (control) levels for plasma [^{125}I]-fibrinogen extravasation obtained following 15 min saline exposure were 12.5 ± 2.7 (trachea, a), 4.7 ± 0.3 (main bronchi, b), 1.4 ± 0.3 (secondary bronchi, c) and 1.1 ± 0.1 μl plasma 100 mg^{-1} tissue (intraparenchymal airways, d). Induction of plasma extravasation in the lower airways was rapid, maximal responses being obtained within 5 min following the administration of the NK_2 selective agonist. This effect was well sustained over a 30 min measurement period (Figure 2). Similar results were obtained with NKA (data not shown). Figure 2 also illustrates that the basal (control) leakage of [^{125}I]-fibrinogen is substantially greater in the tracheal region compared with the main bronchi and lower airways.

[^{125}I]-fibrinogen extravasation induced by NKA or [β-Ala^8]NKA(4–10) was examined after pretreatment with the selective NK_2 antagonist, SR 48968. The results (Figure 3) show that SR 48968 failed to inhibit NKA-induced leakage in either the trachea (a) or main bronchi (b). In contrast, dose-dependent inhibition was observed in both the secondary bronchi (ID_{50} 0.4 mg kg^{-1}; c) and intraparenchymal airways (ID_{50} 0.2 mg kg^{-1}; d). Plasma [^{125}I]-fibrinogen extravasation induced by [β-Ala^8]NKA(4–10) (3 nmol kg^{-1}) in the secondary bronchi (c) and intraparenchymal airways (d) was inhibited by SR 48968 in a dose-dependent manner. The ID_{50} values for SR 48968 against [β-Ala^8]NKA(4–10)-induced leakage in the secondary bronchi and intraparenchymal airways were 0.2 and 0.08 mg kg^{-1} respectively. SR 48968 was consistently 2–4 times more potent in inhibiting NKA-and

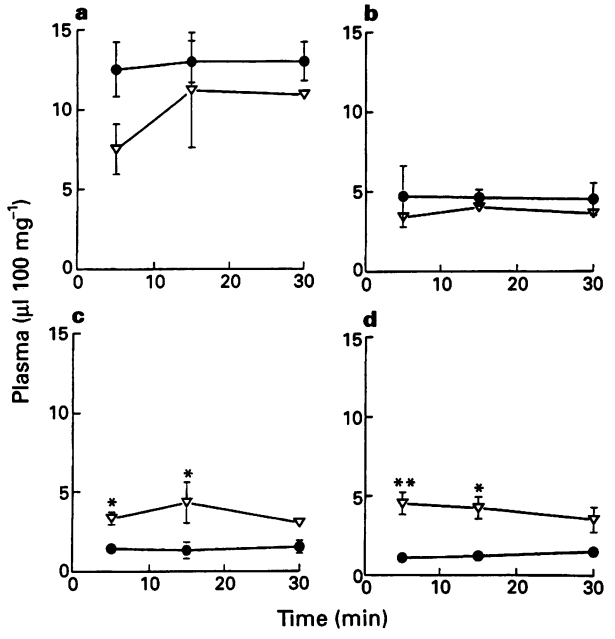

Figure 2 Time course (min) of intravenous [β-Ala^8]NKA(4–10) (3 nmol kg^{-1}) (▽) or saline (●)-induced plasma [^{125}I]-fibrinogen extra-vasation (µl plasma 100 mg^{-1}) in guinea-pig trachea (a), main bronchi (b), secondary bronchi (c) and intraparenchymal airways (d). Vertical bars represent s.e. mean of results of 4 observations. *$P<0.05$, **$P<0.01$.

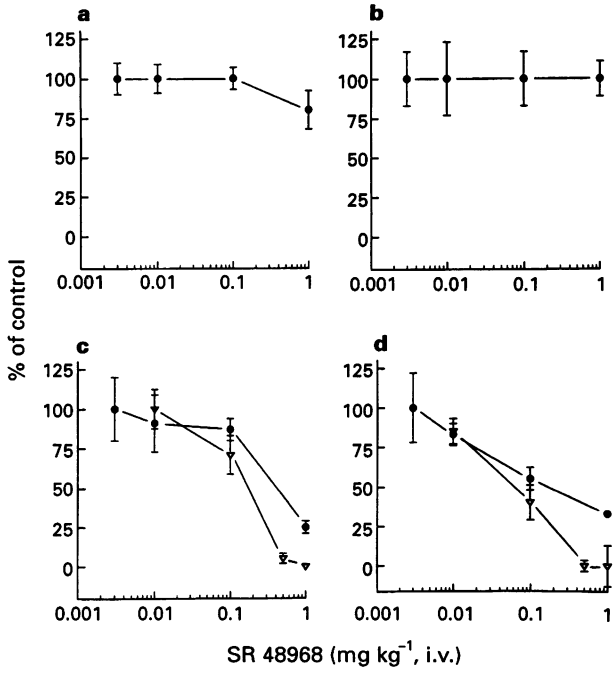

Figure 3 Dose-dependent inhibitions of intravenous [β-Ala^8]NKA(4–10) (3 nmol kg^{-1}) (▽) or neurokinin A (3 nmol kg^{-1}) (●)-induced plasma [^{125}I]-fibrinogen extravasation (% of control) by the NK_2 selective antagonist, SR 48968 (mg kg^{-1}, i.v.) in guinea-pig trachea (a), main bronchi (b), secondary bronchi (c) and intraparenchymal airways (d). Each data point represents the mean ± s.e.mean (vertical bars) of 4 observations.

[β-Ala^8]NKA(4–10)-induced leakage in the intraparenchymal airways than in the secondary bronchi. Furthermore, whereas SR 48968 completely inhibited [β-Ala^8]NKA(4–10)-induced leakage in the intraparenchymal airways and secondary bronchi, it failed to inhibit fully the NKA-induced leakage in doses up to 1 mg kg^{-1}. SR 48968 was devoid of any agonist activity in doses up to 10 mg kg^{-1}.

NKA- and [β-Ala^8]NKA(4–10)-induced plasma [^{125}I]-fibrinogen extravasation was unaffected by pretreatment with either mepyramine or cimetidine in doses up to 0.5 mg kg^{-1}.

Discussion

The major finding of this study is the demonstration of NK_2 receptor-mediated plasma extravasation in guinea-pig lower airways (secondary bronchi and intraparenchymal airways). To our knowledge, this is the first demonstration of NK_2-induced extravasation in the lung of any mammalian species.

In this study we used [^{125}I]-fibrinogen, a recently described marker of protein extravasation (Pedersen *et al.*, 1991) in place of albumin which is more commonly used (Seale *et al.*, 1991; Lötvall *et al.*, 1991). Fibrinogen is a large protein molecule found in the blood (400 kDa compared to albumin 69 kDa) (Harper, 1975). [^{125}I]-fibrinogen is rapidly cleaved during activation of the coagulation system in the extravascular space precipitating as non-diffusable [^{125}I]-fibrin (Saldeen, 1969). This property permits, therefore, the accurate measurement of microvascular leakage since the [^{125}I]-fibrin is not cleared from the extravascular space by lymphatic drainage unlike albumin. Consistent with published data (Pedersen *et al.*, 1991) the magnitude of the endogenous [^{125}I]-fibrinogen extravasation was greatest in the large airways and least in the most distal airways: trachea 12.5 ± 2.7> main bronchi 4.7 ± 0.3> secondary bronchi 1.4 ± 0.3> intraparenchymal airways 1.1 ± 0.1 µl plasma 100 mg^{-1} tissue.

NKA induced a dose-dependent leakage of plasma [^{125}I]-fibrinogen extravasation at all levels of the airway tree examined. In contrast, [β-Ala^8]NKA(4–10)-induced a selective dose-dependent leakage only in the secondary bronchi and intraparenchymal airways. Both NKA- and [β-Ala^8]NKA(4–10)-induced plasma [^{125}I]-fibrinogen extravasation was inhibited dose-dependently by SR 48968, a potent nonpeptide NK_2 antagonist. These results suggest, therefore, that the [β-Ala^8]NKA(4–10) responses, and in large part those to NKA, in the guinea-pig secondary bronchi and intraparenchymal airways are mediated via NK_2 receptors. Our findings are also consistent with those of Abelli *et al.* (1991) who showed that [β-Ala^8]NKA(4–10) failed to increase Evans blue extravasation in the guinea-pig tracheobronchial region.

The NKA-induced microvascular leakage in guinea-pig trachea and main bronchi can be explained via an action on NK_1 receptors. Whilst NKA is preferentially active at the NK_2 receptor it retains substantial activity at NK_1 receptors, especially in tissues which are devoid of NK_2 receptors (Regoli *et al.*, 1988). In recent studies we have also shown (unpublished data) that NKA cross reacts substantially with substance P/NK_1 receptor binding sites in guinea-pig lung tissue. NK_1 receptor-mediated Evans blue extravasation in guinea-pig trachea and main bronchi has been convincingly confirmed by use of the nonpeptide NK_1 antagonist, CP 96,345 (Lei *et al.*, 1992). It is wholly conceivable, therefore, that NKA-induced leakage in the large airways of the guinea-pig is NK_1-receptor-mediated.

Several studies have suggested that tachykinin-induced histamine release from mast cells occurs via both NK receptor-dependent (Erjavec *et al.*, 1981; Devillier *et al.*, 1986) and NK-independent pathways (Mousli *et al.*, 1990). It has also been demonstrated recently that NKA-induced histamine release is primarily mediated via NK_2 receptors in a murine mast cell line (Krumins & Broomfield, 1992). Given that histamine is a potent inducer of plasma protein extravasation in guinea-pig airways (Saria *et al.*, 1983) it was important to rule it out as a mediator of NK-induced leakage in the small airways. Since both mepyramine and cimetidine were without effect on NK-induced leakage (at doses that inhibited

histamine-induced extravasation) the results argue for an effect mediated directly via NK_2 receptors.

In conclusion, by using [^{125}I]-fibrinogen as a marker for plasma protein extravasation, we have shown that NK_2 receptors mediate plasma extravasation in the small airways of guinea-pigs. This effect is direct and does not depend upon histamine released from mast cells. Thus, it appears that both NK_1 (Lei *et al.*, 1991) and NK_2 receptors are involved in the microvascular protein leakage that occurs in the airways of guinea-pigs in response to neurokinins.

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α-Sialyl cholesterol reverses AF64A-induced deficit in passive avoidance response and depletion of hippocampal acetylcholine in mice

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1 The effect of α-sialyl cholesterol (α-SC; α-D-N-acethylneuraminyl cholesterol) on disturbances of the central cholinergic system induced by ethylcholine mustard aziridinium ion (AF64A) and by scopolamine were studied by means of a step-down passive avoidance response and locomotor activities in mice. The levels of acetylcholine (ACh) in certain regions of the brain were measured to assess the neurochemical recovery promoted by α-SC.

2 Treatment with AF64A (2.5, 5 and 10 nmol, i.c.v.) impaired the 24 h retention latencies of animals in a dose-dependent manner, and scopolamine (0.5 mg kg^{-1}, i.p.) also impaired the retention performance. Administration of α-SC (1 and 4 mg kg^{-1}, p.o.) once daily for 13 days improved the retention performance in AF64A-treated animals in a dose-dependent manner, but not in the scopolamine-treated animals.

3 Treatment with AF64A (2.5, 5 and 10 nmol, i.c.v.) and scopolamine (0.5 mg kg^{-1}, i.p.) increased vertical and horizontal locomotor activities. α-SC dose-dependently attenuated the increase in locomotor activies induced by 2.5 nmol of AF64A, but not the locomotor activities caused by 5 or 10 nmol of AF64A, or scopolamine (0.5 mg kg^{-1}, i.p.).

4 The deficit retention performance of AF64A-treated animals was associated with depletion of ACh levels in the hippocampus, but not in the septum or cerebral cortex. Administration of α-SC to AF64A-treated animals dose-dependently reversed the depletion of ACh levels in the hippocampus.

5 The results indicate that α-SC had significant effects after oral administration of AF64A-treated animals. The behavioural recovery promoted by α-SC may be based on the reversal of ACh depletion in the hippocampus.

Keywords: α-Sialyl cholesterol; ethylcholine mustard aziridinium ion (AF64A); scopolamine; acetylcholine; hippocampus; passive avoidance response; locomotor activity

Introduction

α-Sialyl cholesterol (α-SC; α-D-N-acetylneuraminyl cholesterol) currently appears to be a neurotrophic factor. Recent neurobiological studies have shown that α-SC induces neuritogenesis in a mouse neuroblastoma cell line (Tsuji *et al.*, 1988). This effect may be necessary for the survival of cells and for neurite growth, and also for the maintenance of functions related to neurotransmitter production (Di Patre *et al.*, 1989). Other sialic acid-containing glycosphingolipids, gangliosides, especially GM_1 ganglioside, stimulate neurite outgrowth *in vitro* (Roisen *et al.*, 1981; Tsuji *et al.*, 1988), and facilitate recovery of high affinity choline uptake, choline acetyltransferase activity in the cortex and active and passive avoidance response after lesion of the nucleus basalis of rats (Pedata *et al.*, 1984; Casamenti *et al.*, 1985). Thus, it is of interest to study the effects of α-SC on animal models of neurodegenerative disorders in the brain.

Ethylcholine mustard aziridinium ion (AF64A) has been proposed as a specific cholinergic neurotoxin, because intracerebroventricular (i.c.v.) administration of AF64A to rats selectively destroys the central cholinergic system and reduces the number of presynaptic cholinergic markers, such as high-affinity choline uptake, choline acetyltransferase activity, acetylcholine (ACh) release and ACh level (Leventer *et al.*, 1987; Hörtnagl *et al.*, 1987). It also causes impairment of memory performances including a deficit of the avoidance response in mice and rats (Pope *et al.*, 1985; Gower *et al.*, 1989). The reductions in cholinergic markers are long-lasting (Leventer *et al.*, 1987). However, the density of brain muscarinic recognition sites was unchanged (Vickroy *et al.*, 1985). The muscarinic receptor antagonist, scopolamine, blocks neurotransmission at central cholinergic muscarinic receptors and transiently impairs the passive avoidance response in rats and mice (Flood & Cherkin, 1986; Verloes *et al.*, 1988). These drugs have been used as a model of amnesia and are useful for drug screening in animal models of cholinergic dysfunction.

In the present study, we used a passive avoidance task to investigate whether α-SC can reverse the effect of AF64A and protect against the effect of scopolamine. Vertical and horizontal locomotor activities were examined to determine if locomotor dysfunction influenced the passive avoidance response. In parallel with behavioural studies, regional brain levels of ACh were measured.

Methods

Animals

Male ddY mice (Nihon SLC, Hamamatsu) weighing between 27–29 g at the beginning of the experiment were housed in groups of 10–12 under conditions of controlled temperature (22 ± 1°C), humidity (55 ± 5%) and a 12 h light-dark cycle (lights on 07h 00min). The animals were allowed free access to water and standard laboratory chow.

Drug administration

The experimental design and number of animals in each group are summarized in Table 1 and Figures 1–6. In the AF64A-treatment experiments, mice were anaesthetized with

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pentobarbitone sodium (Nembutal; 50 mg kg^{-1}, i.p.) and were mounted on a stereotaxic instrument (ST-7, Narishige, Japan). The animals were infused with either isotonic saline (pH 7.40) or AF64A (2.5, 5 or 10 nmol per ventricle) to a total volume of 4.0 μl in 4 min into the left cerebral ventricle. After the infusion, the needle was held in place for an additional 3 min to allow for diffusion of the solution. The solution of AF64A diffused bilaterally into the ventricle and bilaterally reduced the ACh levels in the hippocampus. Stereotaxic coordinates were: P. 1 mm, L. − 0.1 mm from bregma and V. 2.5 mm below dura; the height of the incisor bar was − 13 mm. In the groups receiving 5 and 10 nmol of AF64A, 3 mice in each group died within 3 days of the surgery. Five days after the surgery, the groups of AF64A-treated animals and sham-treated animals were given either vehicle (distilled water, 10 ml kg^{-1}, p.o.) or α-SC (1 or 4 mg kg^{-1}, p.o.) once daily for 13 days. On day 13, 2 h after the last dose of α-SC, animals were trained in an acquisition trial in a step-down passive avoidance test or locomotor activity test.

In the scopolamine experiments, mice received either vehicle or α-SC (1 or 4 mg kg^{-1}, p.o.) once daily for 13 days. On day 13, 30 min after the last dose of vehicle or α-SC, the animals were treated with either saline (0.1 ml kg^{-1}, i.p.) or scopolamine (0.5 mg kg^{-1}, i.p.) and 90 min later they were trained in a passive avoidance acquisition trial or locomotor activity test. On the testing day, all doses of α-SC or vehicle were randomly administered. The administration period and doses of α-SC were based on the results of pilot studies, since a single dose of α-SC (1 or 4 mg kg^{-1}, p.o.) was ineffective on the passive avoidance response and ACh levels in AF64A-treated animals. The time course of α-SC was 2 h after oral administration, which was the time of the peak brain concentration (unpublished data supplied by MECT Co., Ltd.). The dose, route of administration and pre-administration time of scopolamine were according to the methods of Kameyama *et al.* (1986) and Verloes *et al.* (1988).

Passive avoidance response task

The apparatus consisted of a clear acrylic compartment (15 × 15 × 17 cm high) with a lid and an electrifiable grid of stainless steel rods (4 mm in diameter, 8 mm between bars). A wooden platform (5 × 5 × 4 cm high) was situated at a corner of the grid floor. An electronic stimulator (SEN-310, Nihon Koden, Japan) and an isolator (SS-302J, Nihon Koden) were used to deliver electric shocks.

On day 13, each group of animals were trained in an acquisition trial in a step-down passive avoidance task. One animal was placed on the platform, and as soon as it had all four feet on the grid floor, it received a foot-shock (1 mA, 1 s) and was then immediately removed from the apparatus. The latency of the animal's descent from the platform was recorded as the baseline latency (acquisition latency). The retention trial was carried out 24 h after the acquisition trial on day 14 (without drug administration). Each animal was again placed on the platform and the latency until it stepped down to the floor was recorded, with a cut-off time of 300 s (retention latency). Any retention latencies statistically shorter than those of the respective control group were taken as indications of drug-induced impairment of retention of memory. All tests were performed between 09 h 00 min and 13 h 00 min. A completely randomized design was used for the allocation of treatment within the groups.

Locomotor activities

Vertical and horizontal locomotor activities were measured according to the method of Itoh *et al.* (1987). The apparatus consisted of clear acrylic walls and a lid. An infrared photocell was mounted at a height of 1.8 cm to measure horizontal locomotor activity and 9 infrared photocells were mounted at a height of 6.5 cm to measure vertical locomotor activity. The output from the photocells was amplified and entered directly into an EPSON PC 386M computer. The locomotor measurements were carried out at equivalent times after appropriate drug administration as described in the acquisition trial.

On day 13, the sham and AF64A groups were tested 2 h after the last doses of vehicle or α-SC (1 or 4 mg kg^{-1}, p.o.) were administered. In the scopolamine group, 30 min after the last doses of vehicle or α-SC, scopolamine (0.5 mg kg^{-1}, i.p.) was given and 90 min later the locomotor recordings were started. Testing was performed in blocks, using 10 sets of cages, with treatments randomized between all animals within the experiment. Each mouse was placed singly in the cage and, 1 min later, the locomotor activities were automatically recorded by the number of light beam interruptions due to the animal's movements for 30 min. For purpose of analysis this was divided into 5 min periods. The apparatus was thoroughly cleaned after each mouse was tested. All animals were used only once, and locomotor activities were always investigated between 09 h 00 min and 13 h 00 min.

Acetylcholine assay

On day 14, animals which had undergone the passive avoidance test were analyzed neurochemically. Since the effect of scopolamine is transient, only the AF64A-treated animals underwent neurochemical analysis.

After the retention test, each animal was killed by a microwave beam focused on the head (0.7 s, 5 kW, TMW-6402C, Toshiba). Each brain was removed and dissected bilaterally to obtain the cerebral cortex and hippocampus, and the region of the septum was dissected as the septal complex according to the brain atlas of Slotnick & Leonard (1975). The individual tissues were rapidly frozen on dry ice, weighed and homogenized in 0.1 M perchloric acid (200 μl per 10 mg tissue) containing 0.1 mM EDTA and 100 mM ethylhomocholine (an internal standard) with an ultrasonic cell disruptor (Model 200, Branson) and centrifuged (12,000 *g* for 20 min at 4°C). The supernatant was filtered (0.45 μm) and stored at − 80°C until analysis. The level of ACh was measured by high performance liquid chromatography with electrochemical detection as previously described (Murai *et al.*, 1989).

Drugs

AF64A was freshly prepared by dissolving AF64-picrate (Mitsubishi Kasei, Yokohama) in physiological saline according to the technique of Fisher *et al.* (1982). The pH was adjusted to 7.4 with solid $NaHCO_3$ and the solution was maintained at room temperature for 1 h. The solution of AF64A was then maintained on ice and used within 5 h of pH adjustment. Scopolamine hydrobromide (Sigma, U.S.A.) was dissolved in physiological saline. α-Sialyl cholesterol (NM-711; MECT Co., Ltd, Tokyo) was dissolved in distilled water. Pentobarbitone sodium (Nembutal; Abbott Lab., U.S.A.) was administered in a volume of 0.01 ml per 10 g body weight.

Data analysis

Results are expressed as means ± s.e.mean. Statistical analysis was performed with computer software (SuperANOVA, Abacus Concepts, Inc., U.S.A.) for either Dunnett's multiple comparison test (two-tailed) or Duncan's new multiple range test (one-tailed). Probability (*P*) values less than 0.05 were considered significant.

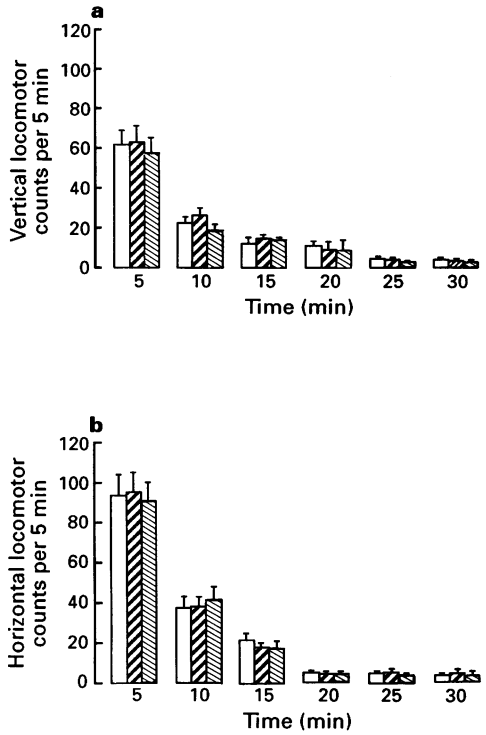

Figure 1 The effects of α-sialyl cholesterol (α-SC, 1 or 4 mg kg^{-1}, p.o., once daily for 13 days) on (a) vertical and (b) horizontal locomotor activities in sham-treated mice. Each column represents the mean movement counts accumulated in 5 min periods over 30 min with vertical lines indicating the s.e.mean ($n = 12$ in each group). Groups are: sham + vehicle (□); sham + α-SC 1 mg kg^{-1} (▨); sham + α-SC 4 mg kg^{-1} (▨). There were no significant differences (Dunnett's test).

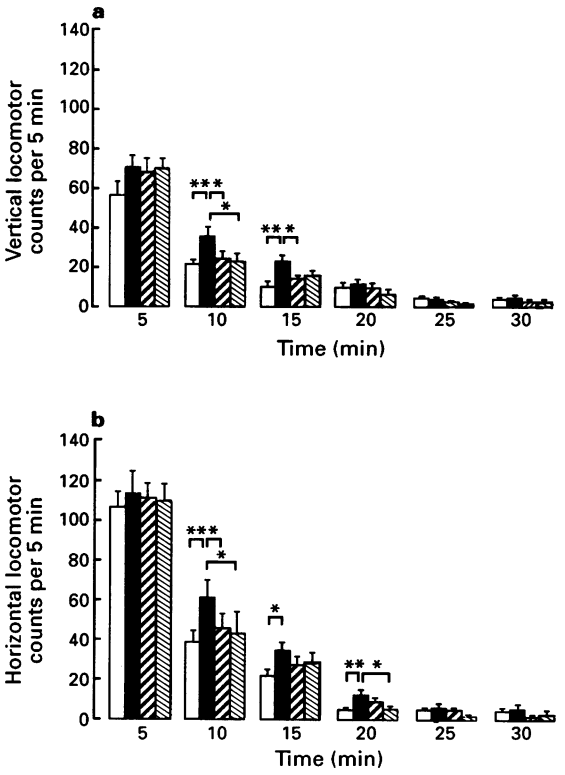

Figure 2 The effects of α-sialyl cholesterol (α-SC, 1 or 4 mg kg^{-1}, p.o., once daily for 13 days) on (a) vertical and (b) horizontal locomotor activities in AF64A (2.5 nmol, i.c.v.)-treated mice. Each column represents the mean movement counts accumulated in 5 min periods over 30 min with vertical lines indicating the s.e.mean ($n = 12$ in each group). Groups are: sham + vehicle (□); AF64A + vehicle (■); AF64A + α-SC 1 mg kg^{-1} (▨) and AF64A + α-SC 4 mg kg^{-1} (▨). $^{*}P<0.05$; $^{**}P<0.01$ relative to appropriate groups (Duncan's test).

Results

Passive avoidance response

There were no significant differences in acquisition latency among the groups. The retention latencies of AF64A-treated (5 and 10 nmol, i.c.v.) animals were significantly lower than those of the respective control sham-treated animals. Scopolamine-treated animals similarly showed a significantly lower retention latency. The decrease in retention latencies in the AF64A-treated animals were reversed by α-SC (1 and 4 mg kg^{-1}) in a dose-dependent manner. There was a trend

Table 1 Step-down passive avoidance response in mice: effect of repeated administration of α-sialyl cholesterol (α-SC, 1 or 4 mg kg^{-1}, p.o., once daily for 13 days) to sham-, AF64A (2.5, 5, 10 nmol, i.c.v.)- and scopolamine (0.5 mg kg^{-1}, i.p.)-treated mice

Treatments (dose)			n	*Acquisition latency* (s)	*Retention latency* (s)
Sham	+	Vehicle	8	12.6 ± 2.5	203.1 ± 32.6
Sham	+	α-SC (1 mg kg^{-1})	9	13.2 ± 3.6	191.7 ± 32.1
Sham	+	α-SC (4 mg kg^{-1})	9	11.8 ± 4.7	186.8 ± 38.0
AF64A (10 nmol)	+	Vehicle	9	8.5 ± 2.6	84.0 ± 27.9††
AF64A (10 nmol)	+	α-SC (1 mg kg^{-1})	9	9.8 ± 6.7	102.2 ± 44.6†
AF64A (10 nmol)	+	α-SC (4 mg kg^{-1})	9	13.3 ± 5.2	155.0 ± 25.8*
Sham	+	Vehicle	12	12.6 ± 2.5	280.0 ± 12.5
AF64A (5 nmol)	+	Vehicle	11	12.4 ± 3.1	79.0 ± 35.2††
AF64A (5 nmol)	+	α-SC (1 mg kg^{-1})	11	9.2 ± 7.6	138.6 ± 31.0†
AF64A (5 nmol)	+	α-SC (4 mg kg^{-1})	11	7.8 ± 8.2	175.1 ± 22.4*
Sham	+	Vehicle	12	19.8 ± 2.5	285.5 ± 21.8
AF64A (2.5 nmol)	+	Vehicle	11	15.5 ± 3.6	202.5 ± 28.7
AF64A (2.5 nmol)	+	α-SC (1 mg kg^{-1})	12	15.3 ± 5.9	264.0 ± 15.2
AF64A (2.5 nmol)	+	α-SC (4 mg kg^{-1})	12	13.2 ± 7.3	270.3 ± 25.8
Vehicle	+	Vehicle	9	10.4 ± 3.4	252.9 ± 22.6
Scopolamine (0.5 mg kg^{-1})	+	Vehicle	9	10.7 ± 2.7	65.8 ± 20.9††
Scopolamine (0.5 mg kg^{-1})	+	α-SC (1 mg kg^{-1})	9	19.8 ± 2.1	63.5 ± 24.2††
Scopolamine (0.5 mg kg^{-1})	+	α-SC (4 mg kg^{-1})	9	13.3 ± 5.2	57.3 ± 35.2††

Each value represents the mean ± s.e.mean. *n*: numbers of animals.
†$P<0.05$; ††$P<0.01$ compared with corresponding sham + vehicle group.
*$P<0.05$ compared with corresponding AF64A + vehicle group (Duncan's test).

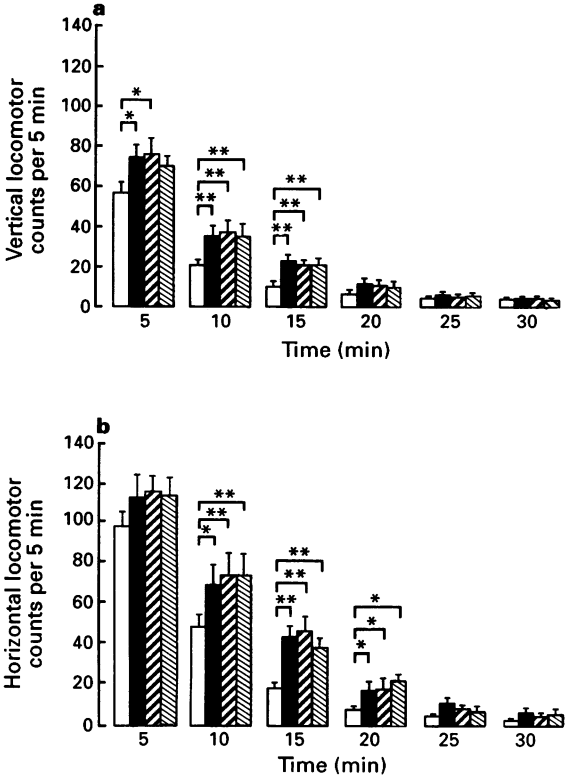

Figure 3 The effects of α-sialyl cholesterol (α-SC, 1 or 4 mg kg^{-1}, p.o., once daily for 13 days) on (a) vertical and (b) horizontal locomotor activities in AF64A (5 nmol, i.c.v.)-treated mice. Each column represents the mean movement counts accumulated in 5 min periods over 30 min with vertical lines indicating the s.e.mean ($n = 12$ in each group). For key to column shading see Figure 2 legend. *$P < 0.05$; **$P < 0.01$ relative to appropriate groups (Duncan's test).

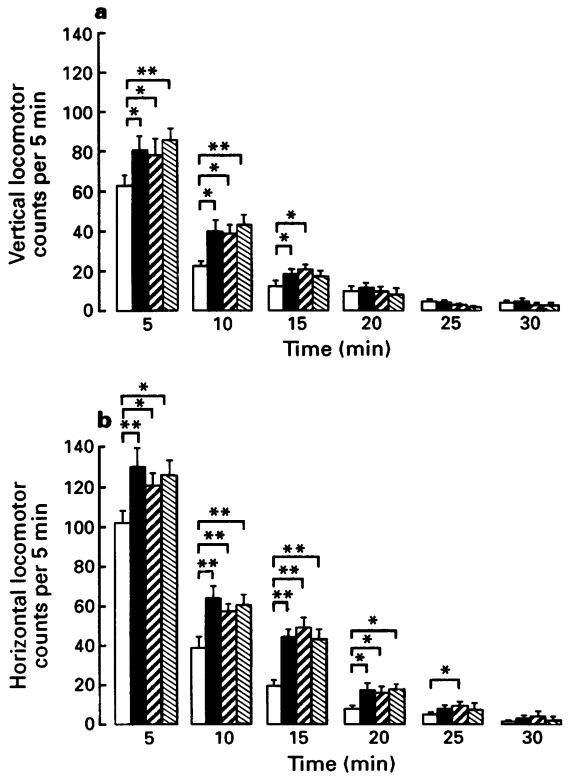

Figure 4 The effects of α-sialyl cholesterol (α-SC, 1 or 4 mg kg^{-1}, p.o., once daily for 13 days) on (a) vertical and (b) horizontal locomotor activities in AF64A (10 nmol, i.c.v.)-treated mice. Each column represents the mean movement counts accumulated in 5 min periods over 30 min with vertical lines indicating the s.e.mean ($n = 12$ in each group). For key to shading of columns see Figure 2 legend. *$P < 0.05$; **$P < 0.01$ relative to appropriate groups (Duncan's test).

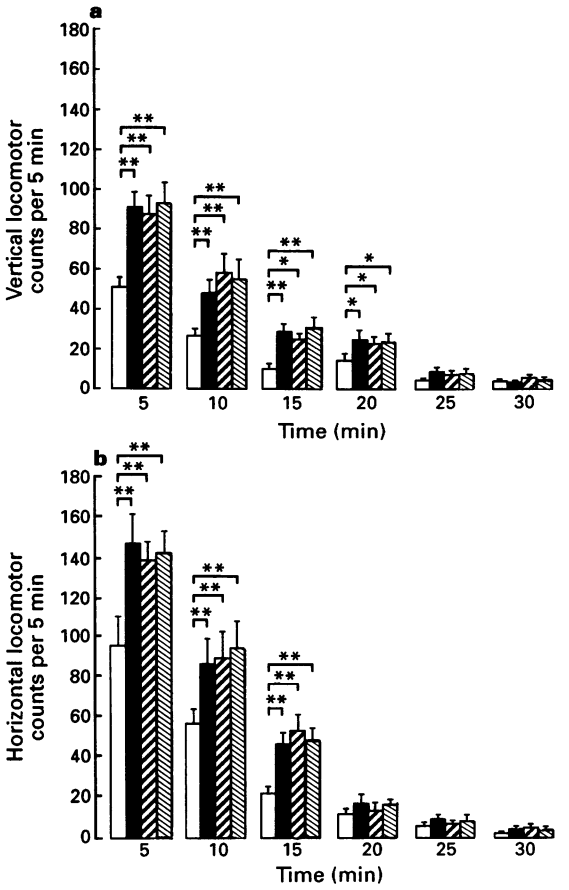

Figure 5 The effects of α-sialyl cholesterol (α-SC, 1 or 4 mg kg^{-1}, p.o., once daily for 13 days) on (a) vertical and (b) horizontal locomotor activities in scopolamine (0.5 mg kg^{-1}, i.p.)-treated mice. Each column represents the mean movement counts accumulated in 5 min periods over 30 min with vertical lines indicating the s.e.mean ($n = 9$ in each group). Groups are: vehicle + vehicle (□); scopolamine + vehicle (■); scopolamine + α-SC 1 mg kg^{-1} (▨) and scopolamine + α-SC 4 mg kg^{-1} (▨). *$P < 0.05$; **$P < 0.01$ relative to appropriate groups (Duncan's test).

towards a decreased retention latency in the group that received 2.5 nmol of AF64A. α-SC (1 of 4 mg kg^{-1}) did not attenuate the scopolamine-induced deficit of retention performance, however (Table 1).

Locomotor activities

Administration of α-SC (1 or 4 mg kg^{-1}, p.o.) to sham-treated mice did not significantly change either vertical and horizontal locomotor activities during the test (Figure 1). The mice treated with AF64A (2.5, 5 and 10 nmol, i.c.v.) showed a significant increase in both vertical and horizontal locomotor activities and increasing doses of AF64A prolonged the duration of its action. α-SC dose-dependently attenuated the increase in locomotor activities induced by AF64A at a dose of 2.5 nmol, but not at doses of 5 or 10 nmol (Figures 2–4). Scopolamine (0.5 mg kg^{-1}, i.p.) similarly increased both vertical and horizontal locomotor activities, but α-SC did not attenuate the increase in locomotor activities (Figure 5).

Regional acetylcholine levels

AF64A significantly decreased ACh levels in the hippocampus in a dose-dependent manner, but not in the septum and cerebral cortex. The corresponding ACh levels (mean ± s.e.mean) in the sham-treated group were: 38.2 ± 1.1 in the septum and 25.5 ± 0.8 in the cerebral cortex. The administration of α-SC (4 mg kg^{-1}, p.o.) to AF64A-treated animals

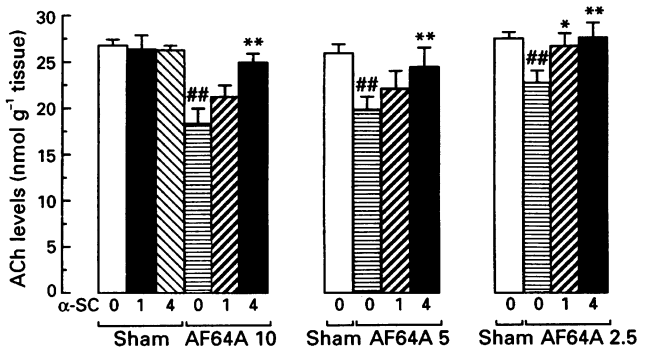

Figure 6 Acetylcholine (ACh) levels in the hippocampus: effect of repeated administration of α-sialyl cholesterol (α-SC, 1 or 4 mg kg^{-1}, p.o., one daily for 13 days) to sham- and AF64A (2.5, 5 or 10 nmol, i.c.v.)-treated mice. Each column represents the mean with s.e.mean indicated by the vertical lines ($n = 8-12$, as indicated in Table 1). The doses of drugs are indicated under the columns. Groups are: sham + vehicle (□); sham + α-SC 1 mg kg^{-1} (■); sham + α-SC 4 mg kg^{-1} (▨); AF64A + vehicle (▤); AF64A + α-SC 1 mg kg^{-1} (▨) and AF64A + α-SC 4 mg kg^{-1} (▨). ††$P<0.01$ compared with corresponding sham + vehicle group. *$P<0.05$; **$P<0.01$ compared with corresponding AF64A + vehicle group.

significantly reversed the depletion of hippocampal ACh. In contrast, administration of α-SC (1 or 4 mg kg^{-1}) to sham-treated animals did not change the level of ACh in the hippocampus (Figure 6).

Discussion

The present study provides the first evidence that the deficit in behavioural performance and depletion of ACh induced by AF64A can be reversed by α-SC.

AF64A and scopolamine significantly decreased retention latency in the passive avoidance response. These results are consistent with earlier studies of AF64A (Pope *et al.*, 1985; Yamazaki *et al.*, 1991) and scopolamine (Kameyama *et al.*, 1986; Verloes *et al.*, 1988) reported using rats and mice. AF64A (Yamazaki *et al.*, 1991), scopolamine (Kameyama *et al.*, 1986) or α-SC (unpublished observation) did not change the threshold footshock intensity, suggesting that changes in sensitivity to the electric shock does not contribute to changes in the passive avoidance response. Increased locomotor activities were observed in both AF64A- and scopolamine-treated animals. These effects possibly contributed to the decrease in acquisition and retention latencies. Acquisition latencies in the groups given AF64A at higher doses (5 and 10 nmol, i.c.v.) were considerably lower than in the sham group. α-SC was ineffective in reversing the increase in locomotor activities of the AF64A (5 and 10 nmol, i.c.v.)-treated group, and there was no trend of decreasing acquisition latency in the scopolamine group. Thus, it seems that these nonspecific drug effects did not contribute to the passive avoidance response, and the reversal of decreased retention latencies by α-SC was related to improving the deficit memory capacity of AF64A-treated animals.

In parallel with the deficit in passive avoidance response, AF64A significantly decreased ACh levels in the hippocampus, but not in the other regions examined, which is in agreement with other reports (Leventer *et al.*, 1985; Yamazaki *et al.*, 1991). The reason for selectivity in the hippocampus after i.c.v. administration of AF64A is not well understood. It has been shown that intrastriatal (Sandberg *et al.*, 1984) and intracortical (Mouton *et al.*, 1989) injection of AF64A decreased the levels of ACh in the area of the injection site. Accordingly, the selectivity of AF64A for the hippocampus may be due to the infusion site, since the hippocampus borders upon the ventricle (Vickroy *et al.*, 1985).

Although AF64A at a dose of 2.5 nmol significantly decreased the ACh level in the hippocampus and increased locomotor activities, it did not impair the passive avoidance response. These results suggest that the minimum effective dose of AF64A which impairs the passive avoidance response is higher than that causing ACh depletion. Supporting this hypothesis, impairment of the working memory in the radial maze and T-water maze tasks was observed in rats receiving AF64A at doses ranging from 2 to 6 nmol, i.c.v., but ACh levels in the hippocampus decreased at a dose from 0.6 nmol, i.c.v. (Gower *et al.*, 1989). The minumum effective dose of AF64A to impair 24 h retention in the passive avoidance response was 3.75 nmol, i.c.v. in rats (Yamazaki *et al.*, 1991).

Decreases in noradrenaline, dopamine and glutamate levels accompanied by a reduction in ACh levels have been observed in the rat hippocampus after i.c.v. administration of AF64A (Hörtnagl *et al.*, 1987; 1991). However, these non-cholinergic alterations are secondary to functional changes in the cholinergic system (Hörtnagl *et al.*, 1987; 1991), and AF64A disrupts only presynaptic cholinergic substrates *in vitro* (Sandberg *et al.*, 1985; Vickroy *et al.*, 1985). Therefore, a key factor for the improvement of the deficit in retention latencies and the increase in locomotor activities of the AF64A-treated animals by α-SC involves reversal of ACh depletion in the hippocampus. The precise actions of α-SC on the cholinergic system are unknown. However, α-SC may act through repair of the dysfunction of cholinergic system and/or through subsequent stimulation of sprouting and of other reparative effects by neurones that were not damaged by AF64A.

The scopolamine-induced deficit in behavioural performance was not effectively attenuated by α-SC. The effects of scopolamine in the behavioural performance (amnesia and locomotor stimulation) are due to the blockade of muscarinic receptors in the brain (Verloes *et al.*, 1988; Pepeu *et al.*, 1989; Toide, 1989). These observations provide the additional suggestion that α-SC does not affect muscarinic receptors, either directly or indirectly.

Sialic acid-containing glycosphingolipids, gangliosides, also act as neurotrophic factors (Tsuji *et al.*, 1988). GM_1 ganglioside alleviates memory impairment and prevents decreases in choline acetyltransferase activity and in high affinity choline uptake in the cerebral cortex after electrolytic or ibotenic acid lesions of the nucleus basalis in rats (Casamenti *et al.*, 1985; Di Patre *et al.*, 1989). These observations indicate that the neurotrophic action of gangliosides can act against neuronal dysfunction in the brain and against memory impairments. However, gangliosides have a limited therapeutic benefit in patients with neurodegenerative brain disorders, because only low concentrations of these compounds can cross the blood-brain barrier (Orland *et al.*, 1979). In man, less than 0.05% of peripherally administered GM_1 ganglioside is taken up into the brain (Svennerholm, 1991). Unlike GM_1 ganglioside, orally administered α-SC easily crosses the blood-brain barrier (unpublished observations). It may prove to be suitable for the treatment of human disorders requiring central activity after peripheral administration. Hence, the pharmacological availability of α-SC may offer a therapeutic potential for drugs aimed at alleviating cognitive disorders associated with brain cholinergic abnormalities.

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Interaction of selective compounds with muscarinic receptors at dispersed intestinal smooth muscle cells

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1 The characterization of muscarinic receptors on single cells of the guinea-pig ileum longitudinal smooth muscle, devoid of neuronal elements, was functionally studied by estimating the affinities of muscarinic antagonists on acetylcholine-induced contractions.

2 Atropine (5×10^{-11} to 5×10^{-6} M), 4-diphenylacetoxy-N-methyl-piperidine methiodide (4-DAMP, 5×10^{-8} to 5×10^{-6} M), cyclohexyl(4-fluoro-phenyl) (3-piperidinopropyl) silanol (pFHHSiD, 5×10^{-7} to 5×10^{-5} M) as well as pirenzepine (5×10^{-7} to 5×10^{-5} M) competitively antagonized the acetylcholine-dependent contractions with different affinities (atropine > 4-DAMP > pFHHSiD > pirenzepine).

3 Methoctramine (5×10^{-7} to 5×10^{-5} M), and AF-DX 116 (5×10^{-6} and 5×10^{-5} M) also showed antagonist properties but these deviated from simple competition. These compounds, which discriminate between M_2 and M_3 receptors, showed a potency lower than that of pirenzepine, the rank order of potencies being pirenzepine > methoctramine > AF-DX 116. When concentrations of AF-DX 116, methoctramine and pirenzepine were increased an unspecific contractile effect occurred.

4 McN-A-343, a partial agonist on intact guinea-pig longitudinal smooth muscle strips, on this preparation induced a weak contraction (about 7% in comparison to control) that was not reversed by antimuscarinic agents.

5 These data indicate that M_3 rather than M_2 receptor sites are present on this tissue.

Keywords: Muscarinic receptors; selective M_1, M_2 and M_3 antagonists; selective M_1 agonist; dispersed intestinal cells

Introduction

Muscarinic receptors have been divided, on a pharmacological basis, into at least three subtypes which have been termed M_1, M_2 and M_3 (Doods *et al.*, 1987; Hulme *et al.*, 1990).

M_1-receptors, are predominantly found in neuronal tissue, but are also present in effector organs (e.g. canine vascular tissue; O'Rourke & Vanhoutte, 1987), and are most sensitive to the antagonist, pirenzepine (Hammer & Giachetti, 1984; Birdsall & Hulme, 1983; Gilbert *et al.*, 1984).

M_2-receptors, identified prejunctionally in many tissues (Bognar *et al.*, 1990; Kilbinger *et al.*, 1991; Costa & Majewski, 1991) and mainly present in cardiac and smooth muscle (Doods *et al.*, 1987; Michel & Whiting, 1988), are most sensitive to methoctramine, AF-DX 116 and himbacine (Giachetti *et al.*, 1986; Melchiorre *et al.*, 1987; Eglen *et al.*, 1988; Lazareno & Roberts, 1989).

The M_3-receptor subtype, so far described in peripheral glandular and muscular tissues (Delmendo *et al.*, 1989; Eglen *et al.*, 1989a; Verspohl *et al.*, 1990; Pfeiffer *et al.*, 1990), exhibits a high affinity for 4-diphenylacetoxy-N-methyl-piperidine methiodide (4-DAMP) (Barlow *et al.*, 1976; Brown *et al.*, 1980; Eltze & Figala, 1988) and cyclohexyl(4-fluoro-phenyl) (3-piperidinopropyl) silanol (pFHHSiD), which, unlike 4-DAMP, discriminates M_3 from M_1 receptors (Lambrecht *et al.*, 1988).

More recently, by comparing the affinities of the selective antimuscarinic compounds, Waelbroeck *et al.* (1991) and Dorje *et al.* (1990) have suggested the existence of an M_4 muscarinic receptor subtype at the level of the corpus striatum and in guinea-pig uterus. However, Eglen *et al.* (1989b) and Bognar *et al.* (1992) have argued that the latter tissue contains M_2- rather than M_4-receptors. At present, based on functional findings carried out on rabbit iris sphincter, Bognar *et al.* (1992) hypothesized the existence of a novel muscarinic receptor-type differing from M_1-M_4 receptors (M_5?).

So far, attempts to develop muscarinic agonists selective for the individual receptor subtypes have met with little success. McN-A-343, considered to be a selective stimulant of M_1-receptors in ganglia (Hammer & Giachetti, 1982), has been proved to interact also with other muscarinic receptors in peripheral tissues (Mitchelson, 1984; Eglen *et al.*, 1987; personal observations).

Accordingly, a pirenzepine-sensitive inhibitory neuronal effect of McN-A-343 has been described on electrically-induced contractions of guinea-pig ileum (Schuurkes *et al.*, 1988). A simultaneous direct motor activity in response to McN-A-343, non competitively antagonized by antimuscarinic drugs (atropine, 4-DAMP, methoctramine and pirenzepine), has been observed on guinea-pig ileum longitudinal muscle strips (Impicciatore *et al.*, unpublished data).

The suggested heterogeneity of muscarinic receptors has been supported by radioligand binding studies with membranes from rat brain and peripheral tissues (Mitchelson, 1988; Nathanson, 1987). In addition, muscarinic receptor subtypes have been, more recently, defined genetically through the identification of discrete (albeit closely related) cDNA and RNA species (Kerlavage *et al.*, 1987; Hulme *et al.*, 1990).

The functional studies are mostly carried out on *in vivo* or *in vitro* preparations (Nathanson, 1987). In these cases, the interpretation of the results is complicated by a sequence of events which occur between the interaction of the drugs with the receptors and neuronal elements.

Dispersed intestinal smooth muscle cells are functional units completely devoid of neuronal elements (Bitar & Makhlouf, 1982; Makhlouf, 1987). However, contractile or relaxant responses can be seen. Studies carried out on human and guinea-pig dispersed intestinal muscle cells exhibited a higher affinity for atropine (1900 and 3290 fold) in comparison to pirenzepine in antagonizing the contractile response to acetylcholine. Grider *et al.* (1987) suggested that muscarinic receptors mediating contraction were of the M_2/M_3 subtype.

The availability of new selective antimuscarinic compounds led us to characterize further the muscarinic receptors present

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in these cells. The muscarinic receptor subtypes have been distinguished by examining the effects of these compounds on the contractile response to agonists in this system.

Methods

Preparation of dispersed cells

Smooth muscle cells were isolated from the longitudinal muscle layer of guinea-pig ileum cut into strips (Souquet *et al.*, 1985). The layers were incubated for two successive 45 min periods at 31°C in 10 ml of HEPES medium containing 0.1% collagenase and 0.01% soybean trypsin inhibitor and aerated with 95% O_2 and 5% CO_2. The composition of the solution was (in mM): NaCl 115, KCl 5.8, KH_2PO_4 2.1, $CaCl_2$ 2.0, $MgCl_2$ 0.6, HEPES 25, glucose 11 and Eagle's essential amino acid mixture 2.1%.

At the end of the second incubation period, the medium was filtered out using 500 μm Nitex mesh and the partly digested strips washed with 50 ml of collagenase-free medium.

The strips were reincubated in 15 ml of enzyme-free buffer solution and the muscle cells allowed to dissociate spontaneously. The cells were harvested by filtration and the experiment started within 30 min.

Aliquots (0.5 ml) of cell suspension were added to 0.2 ml of HEPES medium containing the agents to be tested and the reaction was terminated after 30 s by adding acrolein to a final concentration of 1%.

In control experiments 0.2 ml of collagenase-free HEPES medium was substituted for the test agent. In the control state and after the addition of test agent, the length of the first 50 cells randomly encountered in sequential microscopic fields (microscope Leitz Dialux 20 EB) was measured by an image-analysing system (Leitz ASM 68K).

The contractile response was expressed as percentage decrease in cell length from control.

Concentration-response curves were constructed for acetylcholine (1×10^{-12} to 1×10^{-4} M) in the absence and presence of different concentrations of each antagonist, atropine (5×10^{-11} to 5×10^{-8} M), 4-DAMP (5×10^{-8} to 5×10^{-6} M), pFHHSiD (5×10^{-7} to 5×10^{-5} M), AF-DX 116 (5×10^{-6} to 5×10^{-4} M), methoctramine (5×10^{-7} to 5×10^{-4} M) and pirenzepine (5×10^{-7} to 5×10^{-4} M).

The compound McN-A-343 has been tested as agonist (1×10^{-10} to 1×10^{-4} M) to characterize further its contractile postjunctional action observed on longitudinal strips of guinea-pig ileum (Mitchelson, 1984; personal observations).

Statistical and data analysis

Each value is presented as mean ± s.e.mean of $n = 6-8$ preparations. Differences between mean values were assessed by Student's t test. Values with $P < 0.05$ were considered significant.

The values of contractile responses were plotted against the logarithm of the agonist concentration and a regression line was fitted to the linear segment of the curve using the least squares method to calculate the EC_{50} of the agonist. The concentration-ratio was estimated when, in the presence of the antagonists, the maximum response evoked by the agonist did not change. Schild plots were constructed by plotting the logarithm of the concentration ratio − 1 against the logarithm of the molar concentration of the antagonists and linear regression analysis was used to obtain both the slope and pA_2 for each antagonist (Arunlakshana & Schild, 1959).

Drugs

The following drugs were used: acetylcholine chloride, atropine sulphate (Fluka Chemical, Switzerland); pFHHSiD (cyclohexyl (4-fluoro-phenyl) (3-piperidinopropyl)sinanol) (gift from Dr Lambrecht, University of Frankfurt, Germany); pirenzepine, 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methi- odide), McN-A-343 (4-(*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride) (gift from Dr Sagrada De Angeli, Milano); AF-DX 116 ((11//2-/(diethylamino)methyl/-1-piperidinyl/acetyl/-5,11-dihydro-6H-pyrido/2,3-b//1,4/benzodiazepine 6-)one) (gift from Dr Karl Thomae, Boehringer Ingelheim, Germany); methoctramine (N,N'-*bis*-(6-/(2-methoxybenzyl)amino/-hexyl)-1,8-octadiamine tretrahydrochloride) (gift from Prof. Melchiorre, Dep. Pharmaceutical Science, University of Bologna).

Results

Acetylcholine elicited a concentration-dependent contraction of dispersed smooth muscle cells isolated from guinea-pig longitudinal muscle layers, in the range of 1×10^{-12}–1×-10^{-8} M (Figure 1). In control experiments, mean cell length was similar in the different treatment groups (117.6 ± 1.9 μm in $n = 25$ experiments).

The maximally effective concentration of acetylcholine (1×10^{-8} M), induced a 23.0 ± 0.7% reduction in cell length (Figure 1). High sensitivity to acetylcholine ($pD_2 = 10.00 \pm 0.07$) as well as the tendency for the response to decrease at supramaximal doses are typical features of the response of isolated muscle cells according to the findings previously reported (Makhlouf, 1987).

McN-A-343 caused a negligible contraction in the very narrow range of concentrations from 1×10^{-9} to 4.6×10^{-9} M, the maximum response being approximately 7% in comparison to control (Figure 1). This effect was antagonized by none of the selective antimuscarinics employed.

Atropine, 4-DAMP and pFHHSiD, which are antagonists with high affinities for M_3-receptors, each produced parallel rightward shifts in the concentration-response curve to acetylcholine with no change in the maximal response (Figure 2a, b and c respectively). Competitive antagonism was exhibited by the three compounds, the slopes of their Schild plots being not significantly different from unity (1.13 ± 0.14, 1.01 ± 0.06 and 0.96 ± 0.12 respectively) (Table 1).

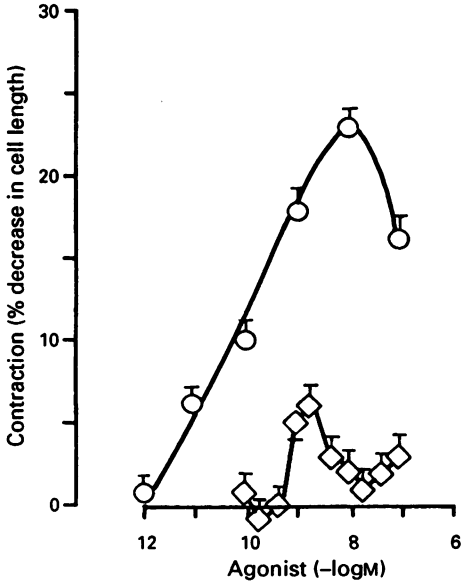

Figure 1 Concentration-response curves for the contractile effect of acetylcholine (○) ($n = 25$) and McN-A-343 (◇) ($n = 8$) on dispersed longitudinal muscle cells of guinea-pig ileum. Each point represents the mean and vertical lines show s.e.mean.

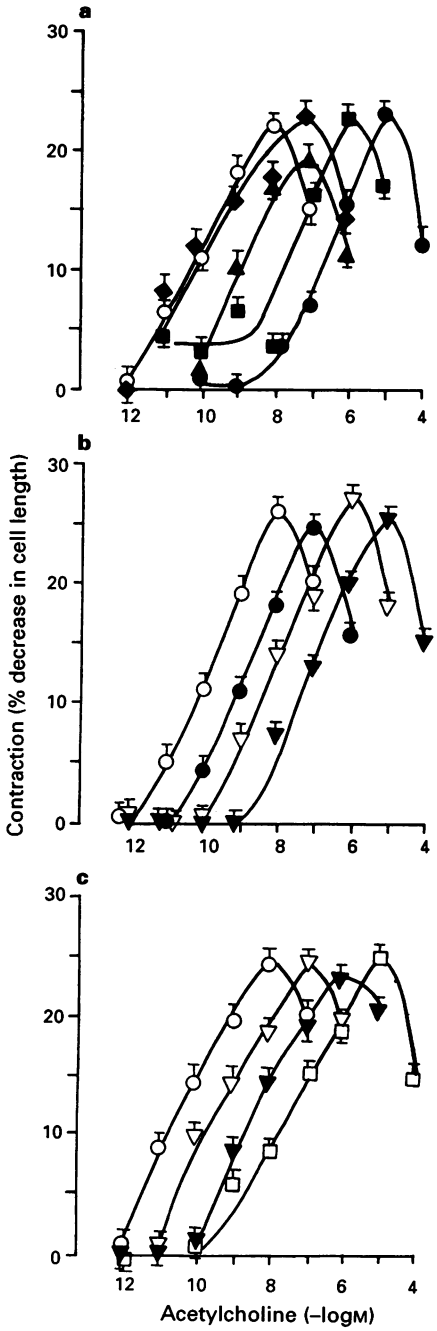

Figure 2 Concentration-response curves for the contractile effect of acetylcholine in the absence (○) and in the presence of the antagonists (a) atropine; (b) 4-diphenylacetoxy-N-methyl-piperidine methiodide (4-DAMP); and (c) cyclohexyl(4-fluoro-phenyl)(3-piperidinopropyl)silanol (pFHHSiD): 0.05 nM (◆), 0.5 nM (▲), 5 nM (■), 50 nM (●), 500 nM (▽), 5 μM (▼), 50 μM (□) on dispersed longitudinal muscle cells of guinea-pig ileum. Each point represents the mean ($n = 6$) and vertical lines show s.e.mean.

Nevertheless, quite different affinities were shown by these compounds, the potency of atropine being about 100 times greater than that of 4-DAMP which, in its turn, was about 10 times more potent than pFHHSiD (Table 1).

Pirenzepine caused a competitive antagonism at concentrations of 5×10^{-7}–5×10^{-5} M (Figure 3a).

The selective M_2-receptor antagonists, methoctramine and AF-DX 116, were effective in antagonizing the acetylcholine contractile responses (Figure 3b, 3c). However, the Schild plot slope for methoctramine was significantly different from unity (1.88 ± 0.14) (Table 1).

Table 1 pA_2 and Schild plot values for selective muscarinic antagonists against acetylcholine contractile effect on guinea-pig ileal dispersed longitudinal smooth muscle cells

Antagonist	*pA_2*	*Slope*	*Correlation coefficient*
Atropine	10.29 ± 0.27	1.13 ± 0.14	0.9686
4-DAMP	8.29 ± 0.40	1.01 ± 0.06	0.9921
pFHHSiD	7.31 ± 0.26	0.96 ± 0.12	0.9720
Pirenzepine	6.51 ± 0.19	1.06 ± 0.13	0.9789
Methoctramine	6.10 ± 0.53	1.88 ± 0.25*	0.9921

Each value represents the mean ± s.e.mean of $n = 6$–8 preparations.
*Slope significantly different from unity ($P < 0.05$).
For abbreviations, see text.

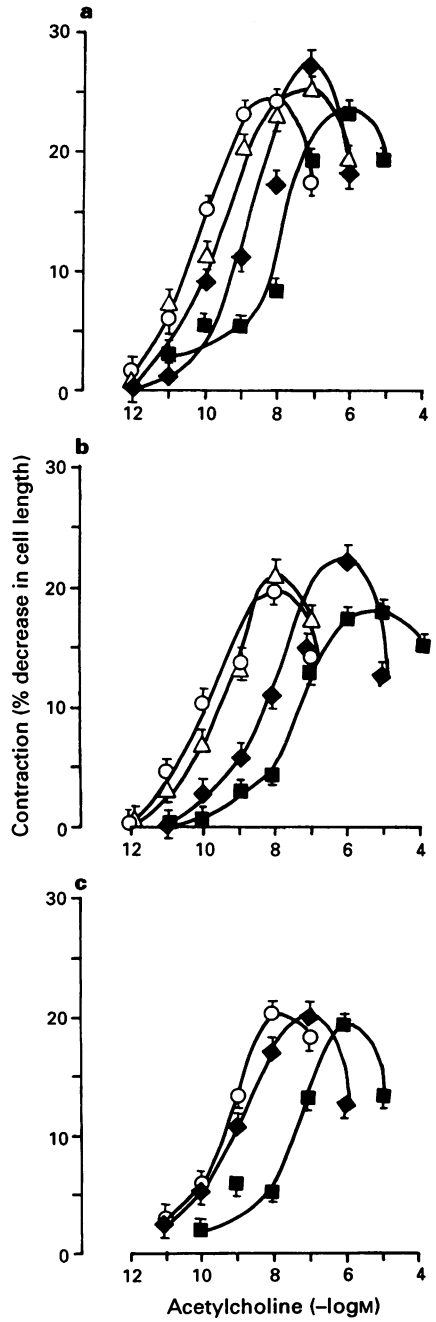

Figure 3 Concentration-response curves for the contractile effect of acetylcholine in the absence (○) and in the presence of the antagonists (a) pirenzepine, (b) methoctramine, (c) AF-DX 116: 500 nM (△), 5 μM (◆), 50 μM (■) on dispersed longitudinal muscle cells of guinea-pig ileum. Each point represents the mean ($n = 6$) and vertical lines show s.e.mean.

Methoctramine and AF-DX 116, like pirenzepine, at 5×10^{-4} M, displayed an unspecific contractile effect probably due to the very high concentration employed.

Discussion

The results of the present study show that acetylcholine stimulates smooth muscle cells of the longitudinal guinea-pig ileum in a concentration-dependent manner by predominantly interacting with postjunctional M_3-receptor sites which mediate contraction.

Supermaximal doses of acetylcholine constantly produced a fall in the contractile response. This effect does not seem to be peculiar to acetylcholine since it is shared by other stimulants such as histamine, 2-aminothiazole, dopamine, cholecystokinin octapeptide (8-CCK) and gastrin-like peptides when tested at supramaximal concentrations on the same preparation (Makhlouf, 1987; Morini *et al.*, 1990; personal observations). Since the bell-shaped curves produced by acetylcholine were shifted rightwards in their entirety by antagonists, the depressive property would appear to be associated with receptor occupancy by acetylcholine. This depressive effect may be due to receptor desensitization.

The high sensitivity to acetylcholine of this preparation in comparison to that demonstrated by longitudinal smooth muscle intact strips, is consistent with the results obtained by other authors who hypothesized the presence of high-affinity receptors (Grider *et al.*, 1987; Makhlouf, 1987). The high concentration of the drug attainable at the receptor compartment, owing to the absence of diffusional barriers (Kenakin, 1987a) as well as the absence of acetylcholinesterase, could account for the high acetylcholine potency on dispersed cells. Another explanation could be that the process of cell dispersion eliminates neurohumoral agents which, when present in smooth muscle strips, would tend to counteract the contractile action of acetylcholine.

The fact that the receptors activated by acetylcholine could be of the M_3 subtype is supported by the observed competitive inhibition displayed by 4-DAMP and pFHHSiD and by their high affinities. The rank potency order was atropine > 4-DAMP > pFHHSiD. However, atropine possessed a very high affinity for such cholinoceptor sites ($pA_2 = 10.29$). The different affinity between atropine and 4-DAMP is in agreement with that reported by Grider *et al.* (1987) in their studies on single muscle cells of human and guinea-pig intestine. At variance, Kilbinger *et al.* (1984), Clague *et al.* (1985), Eltze & Figala (1988), and Michel & Whiting (1988) found no or little difference in functional studies carried out on the guinea-pig isolated ileum, neither did Barocelli *et al.* (1990) on guinea-pig oesophageal muscularis mucosae. No difference between the relative affinities of atropine and 4-DAMP was reported by De Jonge *et al.* (1986) and Lazareno & Roberts (1989) in binding studies on submandibular glands. The reason for these differences is still not clear and requires further study.

The observed greater potency of 4-DAMP and pFHHSiD relative to pirenzepine is in accordance with the results obtained by other authors concerning the interaction of the antagonists with M_3-receptors (Collins & Crankshaw, 1986; Grider *et al.*, 1987; Hulme *et al.*, 1990; Barocelli *et al.*, 1990).

The affinity for pirenzepine ($pA_2 = 6.51 \pm 0.19$) in antagonizing the contractile action of acetylcholine, in the concentration range from 5×10^{-7} to 5×10^{-5} M, suggests, as expected, the absence of neuronal M_1-receptors. The affinity value for pirenzepine could be mostly attributable to the involvement of M_3 rather than M_1 receptor sites. This supposition is supported by the fact that both methoctramine and AF-DX 116, which discriminate between M_2 and M_3 muscarinic receptors (Giachetti *et al.*, 1986; Eltze & Figala, 1988; Lazareno & Roberts, 1989; Hulme *et al.*, 1990), exhibited lower affinities than that displayed by pirenzepine (Table 1).

However, AF-DX 116 exerted such a weak antimuscarinic activity in this experimental model that it was not possible to calculate its pA_2 value (Figure 3c). This unusual response to AF-DX 116 in comparison to methoctramine is hard to explain on the basis of the experiments described here and requires further investigation.

The high Schild slope for methoctramine (1.88 ± 0.25) could be due to inadequate equilibration time for the antagonist as suggested by Kenakin (1987b) who demonstrated that Schild regression slopes greater than unity occur in non-equilibrium steady-state.

It is known that McN-A-343 inhibits electrically-stimulated contractions of guinea-pig ileum (Schuurkes *et al.*, 1988) by selectively interacting with neuronal M_1-receptors. McN-A-343 has been reported to act as full agonist on taenia caeci muscarinic receptors (Eglen *et al.*, 1987) and as a partial agonist on guinea-pig ileum (Van Rossum, 1962). On dispersed intestinal smooth muscle cells, McN-A-343 hardly exerted any direct contractile action (Figure 1), which was insensitive to antimuscarinic agents, thus suggesting an involvement of receptor sites different from M_3.

Based upon these results, it is reasonable to postulate that muscarinic receptors present on dispersed longitudinal smooth muscle cells predominantly belong to the M_3 subtype. The rank order of affinity found for all the antagonists studied resembles that reported for M_3-receptors. The pA_2 values for atropine (higher than expected from other test system) or for 4-DAMP (lower than that reported by Grider *et al.* (1987) and by other authors on intestinal dispersed cells) requires further study.

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A comparison of the inhibitory effects of sodium nitroprusside, pinacidil and nifedipine on pressor response to N^G-nitro-L-arginine

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1 The inhibitory effects of sodium nitroprusside (SNP), a nitric oxide (NO) donor, on mean arterial pressure (MAP) responses to N^G-nitro-L-arginine (L-NNA) (NO synthase inhibitor), angiotensin II (AII) and noradrenaline (NA) were compared with those of pinacidil (K_{ATP} channel opener) and nifedipine (L-type calcium antagonist) in conscious, unrestrained rats.

2 Intravenous bolus injections of L-NNA (1–64 mg kg^{-1}), AII (0.02–1.28 μg kg^{-1}) and NA (0.25–16 μg kg^{-1}) dose-dependently increased MAP to similar maxima. Intravenous infusions of SNP (1, 4 and 16 μg kg^{-1} min^{-1}) dose-dependently increased ED_{50}s of L-NNA, AII and NA. However, the maximum response evoked by L-NNA, but not by AII nor NA, was dose-dependently reduced by SNP. Moreover, the inhibitory effect of SNP on the pressor response to L-NNA ceased when the infusion of SNP was terminated.

3 Pinacidil (80 μg kg^{-1} min^{-1} for 30 min followed by 5 μg kg^{-1} min^{-1}) increased ED_{50}s of L-NNA, AII and NA but did not decrease the maximum responses to any of these agents.

4 Nifedipine (1 mg kg^{-1} min^{-1}) non-selectively reduced maximum responses to L-NNA, AII and NA to similar levels and increased ED_{50}s of AII and NA but not L-NNA.

5 The results show that SNP causes a selective, non-competitive and reversible inhibition of the pressor response to L-NNA. This inhibition by SNP is unlikely to be related to hypotension, the opening of ATP-sensitive potassium channels or blockade of L-type calcium channels.

Keywords: N^G-nitro-L-arginine; sodium nitroprusside; nitric oxide; pinacidil; ATP-sensitive potassium channel; nifedipine; calcium channel; angiotensin II; noradrenaline; blood pressure

Introduction

N^G-nitro-L-arginine (L-NNA) is a potent inhibitor of nitric oxide (NO) synthase in endothelial cells (Ishii *et al.*, 1990; Mülsch & Busse, 1990). L-NNA suppressed endothelium-dependent relaxations of isolated blood vessels (Kobayashi & Hattori, 1990; Moore *et al.*, 1990; Mülsch & Busse, 1990). The systemic administration of L-NNA or its methyl ester, N^G-nitro-L-arginine methyl ester (L-NAME), caused pressor responses in rats (Gardiner *et al.*, 1990; 1991a,b; Wang & Pang, 1990; Lacolley *et al.*, 1991a,b; Wang *et al.*, 1991b), rabbits (Humphries *et al.*, 1991), cats (Bellan & Minkes, 1991), dogs (Woodman & Dusting, 1991) and sheep (Fineman *et al.*, 1991). The pressor effect of L-NNA or L-NAME is selectively antagonized by L-arginine (L-Arg) but not by D-Arg (Gardiner *et al.*, 1990; Fineman *et al.*, 1991; Lacolley *et al.*, 1991a; Wang *et al.*, 1991a) nor by impairments of the central nervous system (Wang & Pang, 1992), autonomic nervous system or renin-angiotensin system (Wang & Pang, 1991). The results suggest that endogenous NO regulates blood pressure (see Moncada *et al.*, 1991).

In our preliminary studies, we found that sodium nitroprusside (SNP) attenuated pressor response to L-NNA. SNP has been shown to release NO in vascular smooth muscle cells (Feelish & Noack, 1987a; Brien *et al.*, 1991; Bates *et al.*, 1991; Marks *et al.*, 1991; see Moncada *et al.*, 1991). NO, on the other hand, was reported to block calcium influx via the activation of guanylate cyclase and increase of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels (Collins *et al.*, 1986; Ratz *et al.*, 1987; Tohse & Sperelakis, 1991), and/or opening of ATP-sensitive potassium (K_{ATP}) channels (Standen *et al.*, 1989). It is not clear if the inhibitory effect of SNP on L-NNA is selective. It is also not known if the inhibition of L-NNA by SNP is related to the blockade of calcium channels, the opening of potassium channels or the hypotensive action of SNP. To resolve these problems, the effect of SNP on pressor response of L-NNA was compared with those of angiotensin II (AII) and noradrenaline (NA). The effects of SNP on pressor responses evoked by L-NNA, AII and NA were also compared with those of nifedipine, an L-type calcium channel antagonist, and pinacidil, a K_{ATP} channel opener (Cook *et al.*, 1989; Garrino *et al.*, 1989; Lebrun *et al.*, 1990).

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Methods

Surgical preparation

Twenty one groups ($n = 6$ each group) of Sprague-Dawley rats (300–380 g) were anaesthetized with halothane (4% in air for induction and 1.25% in air for surgery). A polyethylene cannula (PE50) was inserted into and remained at the left iliac artery for the recording of mean arterial pressure (MAP) by a pressure transducer (P23DB, Gould Statham, CA, U.S.A.). Heart rate was determined electronically from the upstroke of the arterial pulse pressure by a tachograph (Grass, Model 7P4G). PE50 cannulae were also inserted into both iliac veins for the administrations of drugs. The cannulae were filled with heparinized normal saline (25 iu ml^{-1}), tunnelled subcutaneously along the back and exteriorized at the back of the neck. The rats were given >6 h for recovery from surgery and the effects of halothane without administering analgesics. Afterwards, the conscious rats were allowed to wander freely in a small cage but not given access to food or water. The rats were killed after the experiments were finished and were not used repeatedly.

Experimental protocol

(1) *Effects of SNP, pinacidil and nifedipine on MAP and HR* Groups of rats were continuously infused with normal saline (0.06 ml kg^{-1} min^{-1}), SNP (1, 4 and 16 μg kg^{-1} min^{-1}), ethanol (20% v/v in 5% glucose solution, 8 mg kg^{-1} min^{-1}), pinacidil (80 μg kg^{-1} min^{-1} for 30 min followed by 5 μg kg^{-1} min^{-1} for maintenance) or nifedipine (1 mg kg^{-1} min^{-1}). MAP and HR were continuously monitored for 2 h.

(2) *Effects of SNP, pinacidil and nifedipine on MAP and HR response to L-NNA* Groups of rats were continuously infused with normal saline, SNP, ethanol, pinacidil and nifedipine, at the same doses as those given in Protocol 1. Twenty min (40 min for pinacidil) after the start of each infusion, cumulative dose-response curves for MAP and HR to i.v. bolus injections of L-NNA (1–64 mg kg^{-1} at volumes of 0.25–3.2 ml kg^{-1}) were constructed, at dose-intervals of 10–15 min, the time required to obtain stable responses to L-NNA. Ten min after the last dose of L-NNA, the infusion of SNP was discontinued. MAP and HR were continuously recorded for another 20 min. The total duration of the study was approximately 2 h.

(3) *Effects of SNP, pinacidil and nifedipine on MAP and HR responses to AII and NA* Groups of rats were continuously infused with normal saline, SNP, ethanol, pinacidil or nifedipine. The doses given were the same as described in Protocol 1. Twenty min (40 min for pinacidil) after the start of each infusion, dose-response curves for MAP and HR to i.v. bolus injections of AII (0.02–1.28 μg kg^{-1}, 0.12–3.2 ml kg^{-1}) and NA (0.25–16 μg kg^{-1}, 0.12–3.2 ml kg^{-1}) were constructed at dose-intervals of 3–6 min to allow complete recovery of the responses to the previous dose. The sequence of AII and NA administered was reversed in half of the experiments. The total duration of the study was approximately 2 h.

Drugs

N^G-nitro-L-arginine (L-NNA), angiotensin II (AII) acetate, noradrenaline (NA) hydrochloride and nifedipine were obtained from Sigma Chemical Co. (MO, U.S.A.). Sodium nitroprusside (SNP) was obtained from Fisher Scientific Co. (NJ, U.S.A.). Pinacidil was a gift from Eli Lilly & Co. (IN, U.S.A.). L-NNA (solubilized by 20 min sonication), AII, NA and SNP were dissolved in normal saline (0.9% NaCl). Pinacidil and nifedipine were dissolved in anhydrous ethyl alcohol and diluted with 5% glucose solution to given a final ethanol concentration of 20%.

Calculation and statistical analysis

ED_{50} and maximum effect (E_{max}) were calculated from individual dose-response curves. All results were expressed as mean ± standard error (s.e.mean) and analyzed by analysis of variance followed by Duncan's multiple range test with $P<0.05$ as the criterion for statistical significance.

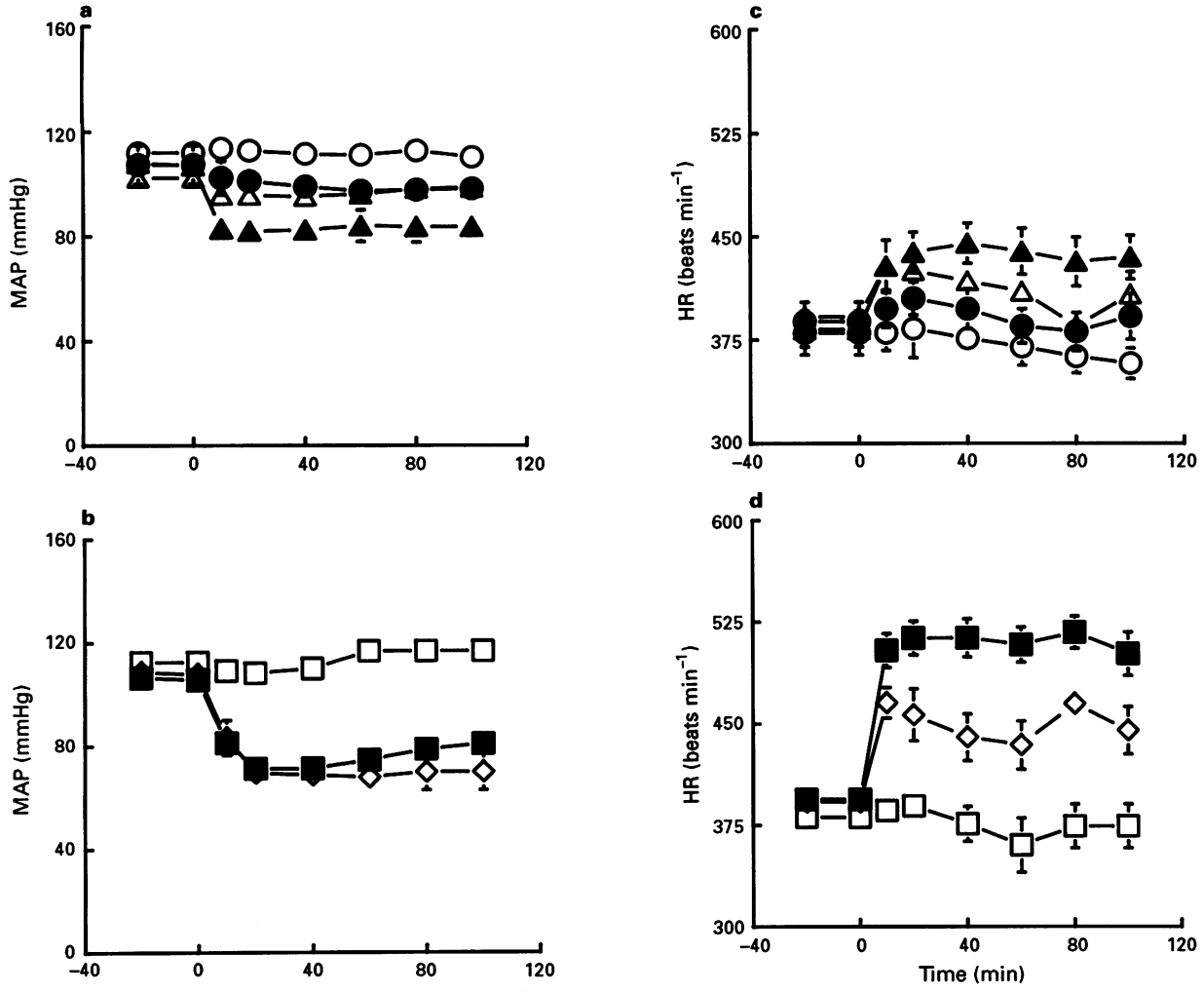

Figure 1 Effects (mean ± s.e.mean) of i.v. infusion of normal saline (0.9% NaCl, ○, a, c), sodium nitroprusside (1 μg kg^{-1} min^{-1}, ●; 4 μg kg^{-1} min^{-1}, △; 16 μg kg^{-1} min^{-1}, ▲; a, c), ethanol (8 mg kg^{-1} min^{-1}, □; b, d), pinacidil (80 μg kg^{-1} min^{-1} for 30 min followed by 5 μg kg^{-1} min^{-1}, ■; b, d) or nifedipine (1 mg kg^{-1} min^{-1}, ◇ b, d) on mean arterial pressure (MAP) and heart rate (HR) in conscious rats ($n = 6$ each group).

Table 1 Baseline values of mean arterial pressure (MAP) and heart rate (HR) prior to and 20 min (40 min for pinacidil) after i.v. infusions of normal saline, sodium nitroprusside (SNP), ethanol, pinacidil or nifedipine in conscious rats

Treatment	Dose (μg or mg kg^{-1} min^{-1})	MAP (mmHg)		HR (beats min^{-1})	
		Before	After	Before	After
Saline	0.06 ml	108 ± 2	106 ± 1	391 ± 9	393 ± 9
SNP	1 μg	108 ± 5	98 ± 3*	370 ± 8	398 ± 8*
SNP	4 μg	106 ± 4	90 ± 3*	383 ± 4	415 ± 6*
SNP	16 μg	106 ± 2	78 ± 3*	389 ± 9	434 ± 12*
Ethanol	8 mg	112 ± 2	115 ± 2	381 ± 7	389 ± 8
Pinacidil	80 μg[a]	106 ± 1	78 ± 2**	378 ± 6	510 ± 11**
Nifedipine	1 mg	105 ± 2	68 ± 2**	380 ± 10	472 ± 8**

Values are mean ± s.e.mean; $n = 18$ per group *Denotes significant difference fron normal saline-treated control group ($P<0.05$); **Denotes significant from ethanol-treated control group ($P<0.05$).
[a]Pinacidil (80 μg kg^{-1} min^{-1} for 30 min followed by 5 μg kg^{-1} min^{-1}).

Results

Effects of sodium nitroprusside, pinacidil and nifedipine on MAP and HR

Table 1 shows the baseline values of MAP and HR prior to and 20 min (40 min for pinacidil) after the infusions of vehicle or drugs in protocols (1) to (3). The values from the three protocols were pooled since they were not significantly different from each other.

In protocol (1), i.v. infusions of normal saline did not significantly alter MAP or HR in conscious rats for 100 min (Figure 1a,c). Intravenous infusions of SNP dose-dependently reduced MAP which reached steady-state levels at 10 min after the start of each infusion, and increased HR which is

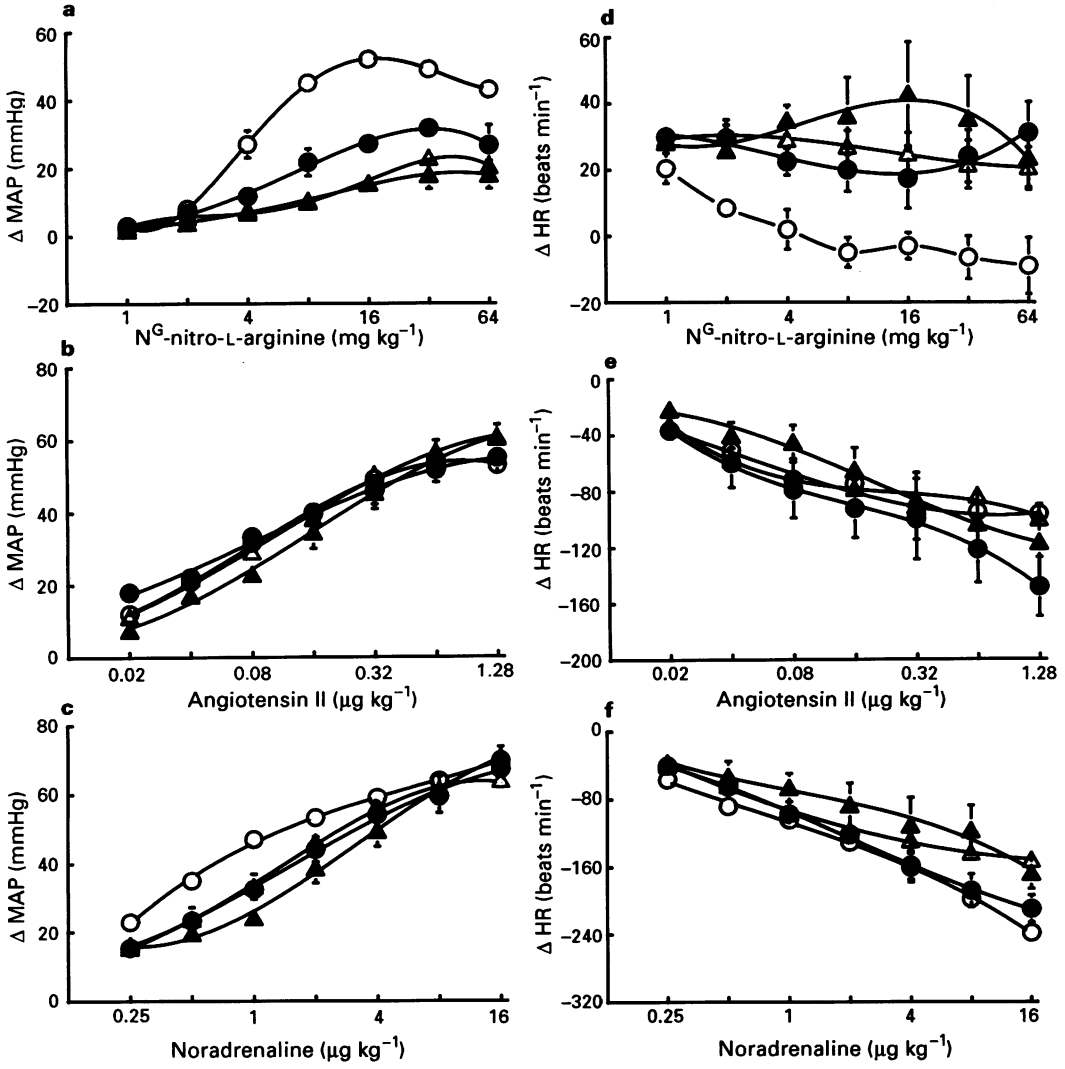

Figure 2 Effects (mean ± s.e.mean) of i.v. infusions of sodium nitroprusside (SNP) on mean arterial pressure (MAP) and heart rate (HR) responses to i.v. bolus injections of N^G-nitro-L-arginine (a, d), angiotensin II (b, c) and noradrenaline (c, f) in conscious rats ($n = 6$ each group). (○) Normal saline; and SNP (●) 1, (△) and (▲) 16 μg kg^{-1} min^{-1}-treated rats.

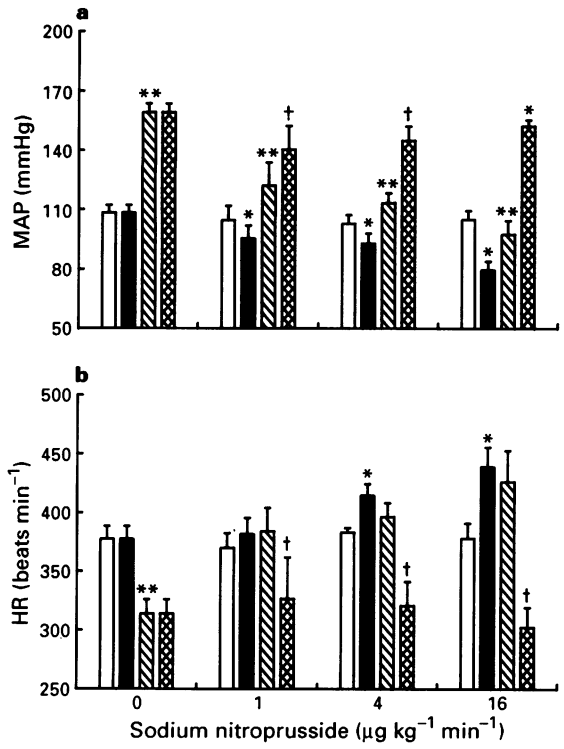

Figure 3 Recovery from the inhibition induced by sodium nitroprusside (SNP) on mean arterial pressure (MAP, a) and heart rate (HR, b) responses to N^G-nitro-L-arginine (64 mg kg^{-1}) in conscious rats ($n = 6$ each group). Open and closed columns represent MAP and HR prior to and 20 min after i.v. infusions of normal saline or doses of SNP, respectively; left diagonal and cross-hatched columns represent MAP and HR responses to N^G-nitro-L-arginine during i.v. infusion of normal saline or SNP, and 10 min after the cessation of the infusions of normal saline or SNP. *, **, and † denote significant difference from open columns, filled columns and left diagonal columns within the same group.

well-known to be mediated via reflex (Figure 1a,c). While i.v. infusion of the vehicle, ethanol did not significantly change MAP or HR, pinacidil and nifedipine caused similar decreases in MAP 20 min after the start of each infusion. However, pinacidil caused more reflex tachycardia than did nifedipine (Figure 1b,d).

Effects of SNP on MAP and HR responses to L-NNA, AII and NA

In normal saline-treated rats, i.v. bolus injections of L-NNA dose-dependently increased MAP (Figure 2a), with an ED_{50} of 3.20 ± 0.4 mg kg^{-1} and E_{max} of 54 ± 6 mmHg (Table 2). L-NNA also dose-dependently reduced HR (Figure 2d). Treatments of SNP (1, 4 and 16 μg kg^{-1} min^{-1}) increased ED_{50} of L-NNA and dose-dependently reduced the maximum MAP responses (Figure 2a) by 41%, 57% and 70%, respectively (Table 2). SNP inhibited bradycardia induced by L-NNA (Figure 2d). Upon termination of the infusion of the highest dose of SNP, MAP immediately rose while HR decreased and 10 min later, MAP and HR reached similar levels attained in L-NNA-treated rats given normal saline instead of SNP (Figure 3).

In normal saline-pretreated rats, i.v. bolus injections of AII and NA caused similar increases in MAP (Figure 2b, c) but AII (Figure 2e) caused less bradycardia than did NA (Figure 2f). Intravenous infusions of SNP dose-dependently increased ED_{50}s of AII and NA but did not alter the maximal effects (Figure 2b,c).

The E_{max} of AII and NA in the presence of SNP (1, 4 and 16 μg kg^{-1} min^{-1}) were 109%, 109%, 120%, and 107%, 103%, 113%, respectively, of the values reached in the respective control rats given normal saline (Table 2). AII or NA caused similar bradycardia in the absence as well as in the presence of various doses of SNP (Figure 2e,f).

Effects of pinacidil and nifedipine on MAP and HR responses to L-NNA, AII and NA

Intravenous bolus injections of L-NNA, AII and NA dose-dependently increased MAP and decreased HR in ethanol-treated rats (Figure 4), with the respective values of E_{max} and ED_{50} of the MAP effects of each agent not significantly different from those in normal saline-treated rats (Table 2).

Intravenous infusion of pinacidil did not reduce maximum MAP effects of L-NNA, AII or NA (Figure 4a,b,c). In fact, both the ED_{50} and E_{max} of the pressor response to L-NNA were increased by pinacidil, however, only the increase in E_{max} was statistically significant (Table 2). Since maximum MAP responses to AII and NA in the presence of pinacidil were not attained, the values of ED_{50} and E_{max} were not calculated (Table 2). Pinacidil potentiated bradycardic responses to L-NNA (Figure 4d) but not NA. With the exception of the highest dose (1.28 μg kg^{-1}) of AII pinacidil also did not potentiate the bradycardic response of AII (Figure 4e).

Nifedipine reduced maximum MAP responses to L-NNA, AII and NA by similar amounts, 62%, 64% and 69%, respectively (Figure 4a,b,c; Table 2) but did not significantly affect ED_{50} of L-NNA. ED_{50}s of AII and NA were, however, increased by nifedipine (Table 2). Nifedipine also almost completely inhibited the bradycardic responses induced by L-NNA (Figure 4d), AII (Figure 4e) and NA (Figure 4f).

Table 2 Values of ED_{50} and E_{max} of the pressor responses to i.v. bolus injections of N^G-nitro-L-arginine (L-NNA), angiotensin II (AII) and noradrenaline (NA) in conscious rats infused i.v. with normal saline, sodium nitroprusside (SNP), ethanol, pinacidil or nifedipine

Treatment	*Dose* (μg or mg kg^{-1} min^{-1})	*L-NNA*		*AII*		*NA*	
		ED_{50} (mg kg^{-1})	E_{max} (mmHg)	ED_{50} (ng kg^{-1})	E_{max} (mmHg)	ED_{50} (μg kg^{-1})	E_{max} (mmHg)
Saline	0.06 ml	3.2 ± 0.4	54 ± 6	62 ± 5	54 ± 2	0.8 ± 0.1	68 ± 3
SNP	1 μg	5.9 ± 1.2*	32 ± 3*	73 ± 12	59 ± 4	1.0 ± 0.2	73 ± 4
SNP	4 μg	8.9 ± 2.2*	23 ± 3*	91 ± 6*	59 ± 3	1.2 ± 0.3	70 ± 4
SNP	16 μg	6.8 ± 1.5*	16 ± 4*	125 ± 23*	65 ± 6	1.6 ± 0.3*	77 ± 12
Ethanol	8 mg	4.9 ± 1.0	42 ± 5	66 ± 11	50 ± 2	1.1 ± 0.2	67 ± 3
Pinacidil	80 μg[a]	8.6 ± 2.2	62 ± 8**	–	–	–	–
Nifedipine	1 mg	5.6 ± 1.3	16 ± 3**	240 ± 43**	18 ± 1**	3.2 ± 0.6**	21 ± 3**

Values are mean ± s.e.mean; $n = 6$ per group. *Denotes significant difference from normal saline-treated control group ($P < 0.05$); **denotes significant difference from ethanol-treated control group ($P < 0.05$).
[a]Pinacidil (80 μg kg^{-1} min^{-1} for 30 min followed by 5 μg kg^{-1} min^{-1}).

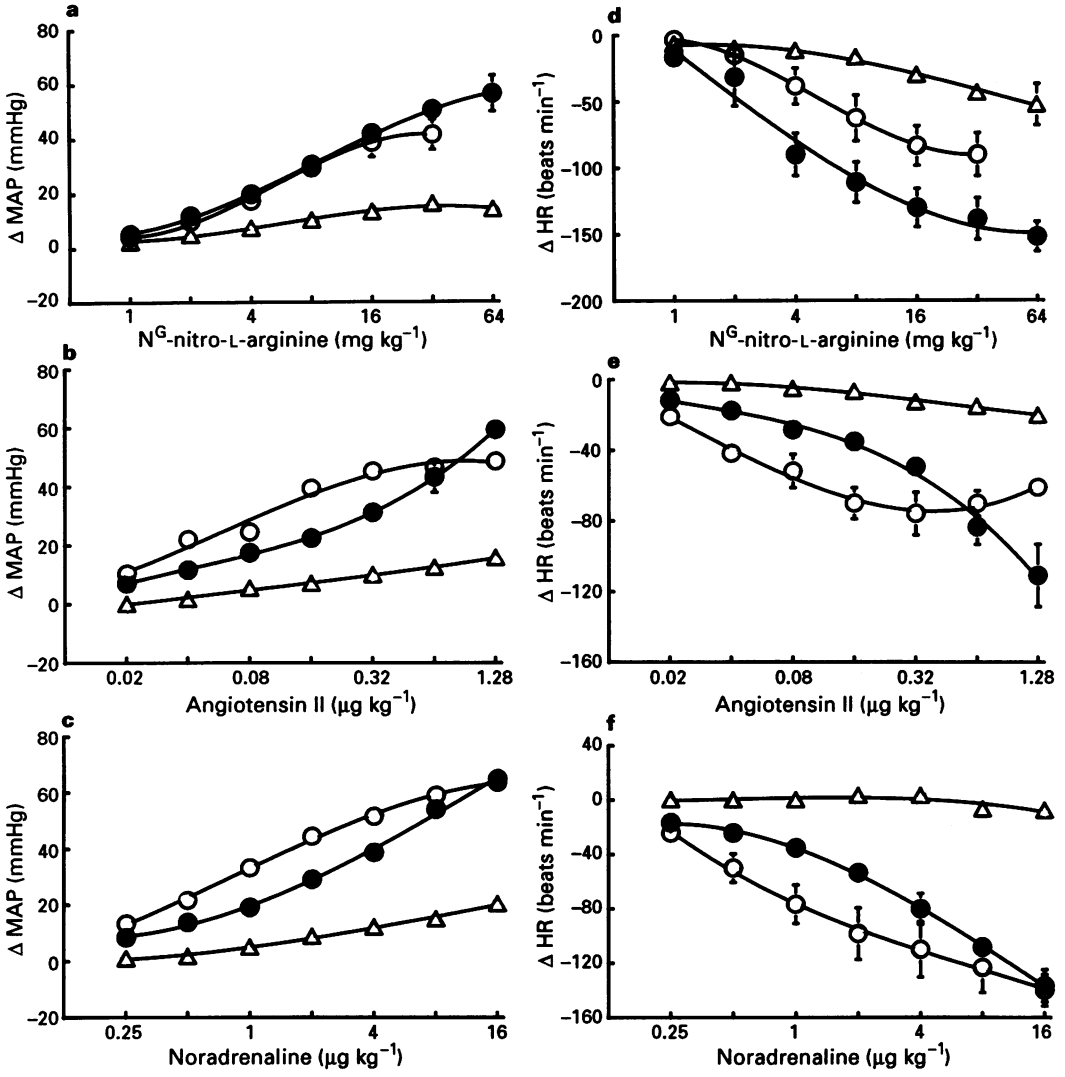

Figure 4 Effects (mean ± s.e.mean) of i.v. infusions of pinacidil and nifedipine on mean arterial pressure (MAP) and heart rate (HR) responses to i.v. bolus injections of N^G-nitro-L-arginine (a, d), angiotensin II (b, e) and noradrenaline (c, f) in conscious rats ($n = 6$ each group). (○) Vehicle (ethanol, 8 mg kg^{-1} min^{-1}), pinacidil, (●, 80 μg kg^{-1} min^{-1} for 30 min followed by 5 μg kg^{-1} min^{-1}) and nifedipine (△, 1 mg kg^{-1} min^{-1})-treated rats.

Discussion

The results from the present study show the characteristics of the inhibition by SNP of the pressor response to L-NNA. SNP dose-dependently reduces maximum pressor and bradycardic effects of L-NNA suggesting that it acts as a non-competitive antagonist. The inhibition by SNP is reversible since MAP and HR responses to L-NNA almost immediately return to levels attained in rats not treated with SNP, upon the termination of SNP infusion. The transient inhibitory effect of SNP is consistent with the reported short half-life of NO (see Moncada *et al.*, 1991). The inhibition is relatively selective for L-NNA since maximum pressor responses to AII and NA are not reduced by the same doses of SNP. Thus, the inhibitory effect of SNP is unlike that of nifedipine which similarly reduced maximum MAP and HR responses to L-NNA, AII and NA (see below).

The mechanism by which SNP selectively inhibits the pressor response to L-NNA is unclear but is most likely related to SNP-induced NO production. Nitrovasodilators such as SNP and nitroglycerin are known to react with hemoprotein- and sulphydryl-components in vascular smooth muscle cells to release NO which subsequently activates guanylate cyclase to cause vasodilatation (Feelish & Noack, 1987a; Marks *et al.*, 1991; Brien *et al.*, 1991; Bates *et al.*, 1991). It has been reported that L-NAME and other NO synthase inhibitors potentiated the vasodilator effects of SNP and other nitrovasodilators *in vitro* (see Lüscher & Vanhoutte, 1990) and *in vivo* (Gardiner *et al.*, 1991a; Wang *et al.*, 1992). The responses were explained as due to increased sensitivity of guanylate cyclase to NO (from SNP) as a result of the administration of NO synthase inhibitors which lowered endogenous production of NO (Gardiner *et al.*, 1991a). Consistent with this hypothesis, in the presence of high levels of NO (released by SNP), the sensitivity of guanylate cyclase for NO might have been decreased causing reduced pressor response to the NO synthase inhibitor L-NNA. In addition, high levels of SNP-derived NO might also have caused negative feed-back inhibition of endogenous NO biosynthesis thereby diminishing the effect of L-NNA. A more likely and simpler hypothesis for the selective inhibition by SNP of pressor response to L-NNA may be that SNP generates large quantities of NO which overcomes the inhibitory effect of L-NNA on endogenous NO biosynthesis.

It is generally accepted that NO activates guanylate cyclase resulting in the production of cyclic GMP and vascular relaxation (Kukovetz *et al.*, 1979; Feelisch & Noack, 1987b; Brien *et al.*, 1991). However, the mechanism by which cyclic

GMP causes vascular relaxation is unclear. The relaxation of the rabbit aorta by acetylcholine, SNP and 8-bromo-cyclic GMP was associated with reduced Ca^{2+} influx (Collins *et al.*, 1986). It has also been reported that 8-bromo-cyclic GMP inhibited the L-type calcium channels in cultured chick embryonic cardiomyocytes (Tohse & Sperelakis, 1991). To find out if SNP suppresses the effects of L-NNA via the blockage of L-type calcium channels, the effects of SNP were compared with those of nifedipine on pressor responses of L-NNA, NA and AII. Our results show that nifedipine similarly reduced maximum MAP responses to L-NNA, AII and NA. Therefore, the selective inhibition by SNP of the pressor response to L-NNA is unlikely to be due to non-selective blockage of calcium channels which should have similarly suppressed responses to L-NNA, AII and NA.

It has been suggested that the vasodilator effects of nitrovasodilators involve the opening of K_{ATP}-channels. The relaxant effect of NO was shown to involve partially the hyperpolarization of smooth muscles (Tare *et al.*, 1990). SNP and nitroglycerin were shown to reduce membrane resistance of isolated vascular smooth muscles and hyperpolarization (Ito *et al.*, 1978; 1980). NO, acetylcholine, SNP as well as nitroglycerin caused hyperpolarization and relaxation of guinea-pig uterine arteries (Tare *et al.*, 1991). Moreover, the hyperpolarizing effects of acetylcholine and SNP in rat and rabbit mesenteric arteries antagonized the K-channel blocker, glibenclamide, suggesting that K_{ATP} channels were involved (Standen *et al.*, 1989). In contrast to the above reports, the relaxant effects of SNP and 8-bromo-cyclic GMP on rat tail arteries were not associated with membrane hyperpolarization (Cheung & Mackay, 1985). The relaxant effects of the K_{ATP}-channel openers pinacidil and BRL 34915 but not that of SNP on rat aorta were blocked by glibenclamide (Lebrun *et al.*, 1990). Pinacidil and BRL 34915 but not SNP increased ^{86}Rb outflow (Lebrun *et al.*, 1990). Therefore, the role of K_{ATP}-channels on the relaxant effect of SNP remains unclear. In the present study, the effects of SNP were compared with those of pinacidil in an attempt to find out if the two agents produce similar suppression of pressor responses to L-NNA, AII and NA. Our results show that pinacidil, at a dose which lowered MAP to a similar level as that caused by the highest dose of SNP, did not attenuate pressor responses to L-NNA, AII or NA suggesting that the activation of K_{ATP} channels and hypotension are not involved in the inhibitory action of SNP on L-NNA-induced vasoconstriction.

It has been reported that K_{ATP}-channel opener BRL 38227 (the active (−)-enantiomer of BRL 34915) lowered MAP and increased renal, hindquarter and mesenteric vascular conductances; these responses were not affected by L-NAME. The results suggest that the haemodynamic effects of BRL 38227 were independent of NO-mediated mechanisms (Gardiner *et al.*, 1991b). In the present study, pinacidil non-selectively displaced the dose response-curves of L-NNA, AII and NA to the right. This is in accordance with results of others which show that K_{ATP}-channel opener, BRL 34915, shifted the dose-response curves of AII (Buckingham, 1988; Cook *et al.*, 1989), NA, phenylephrine, methoxamine and vasopressin (Buckingham, 1988) to the right in anaesthetized rats. It was suggested that this displacement by BRL 34915 was due to the hyperpolarization of smooth muscle cells or short-circuiting of depolarizing stimuli (Buckingham, 1988).

In summary, SNP caused a selective, non-competitive and reversible inhibition of the pressor response to L-NNA. This inhibitory effect of SNP is not due to hypotension, the blockade of calcium channels or opening of K_{ATP} channels.

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Pathological events in experimental acute pancreatitis prevented by the bradykinin antagonist, Hoe 140

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1 In a previous investigation, Hoe 140, a specific and potent bradykinin B_2 receptor antagonist, prevented the pancreatic oedema and the hypotension observed during acute experimental pancreatitis; however, it augmented the associated rises in the serum activities of pancreatic enzymes. Therefore, we have now investigated the consequences of the pancreatic oedema for the fate of activated enzymes released into the tissue during the course of acute pancreatitis.

2 Acute oedematous pancreatitis was induced in rats, pretreated with captopril (50 μmol kg^{-1}, i.p.), by hyperstimulation of the exocrine function of the pancreas with the cholecystokinin analogue, caerulein (4 nmol kg^{-1} h^{-1}, i.v.), for up to 120 min.

3 Pancreatic oedema began to develop 10 min after the start of the caerulein infusion, reached a maximum within about 45 min, and then declined slightly. The development of the oedema parallelled the second phase of the caerulein-induced fall in blood pressure found in earlier experiments. No further extravasation of plasma proteins occurred during the 2nd hour of the caerulein infusion. The oedema formation was completely blocked in animals pretreated with the bradykinin receptor antagonist, Hoe 140 (100 nmol kg^{-1}, s.c.). Pretreatment with aprotinin or soy bean trypsin inhibitor did not result in a significant inhibition of the oedema.

4 The haematocrit of animals with experimental pancreatitis showed a pronounced increase which started 10 min after the start of the caerulein infusion and reached maximal values at 60 min. The changes in haematocrit showed a reduction in total blood volume of 28% due to a 48% loss of plasma. This effect was completely blocked by Hoe 140.

5 In rats with caerulein-induced pancreatitis, there was a time-dependent increase in the activities of amylase and lipase in blood serum as well as in the pancreas. Pretreatment with Hoe 140 greatly augmented the caerulein-induced rise in enzyme activities in blood serum but potently attenuated it in the pancreas. The activities of trypsin in both the blood serum and the pancreas were below or near the limit of detection in all experimental groups.

6 It is concluded that the second phase of hypotension in this model of acute pancreatitis is due to the liberation of kinins which cause a massive loss of blood plasma into the pancreas and into the retroperitoneal space. Activated enzymes are trapped in the pancreas, at least in part, by the oedema of the gland. Treatment with Hoe 140 prevents the oedema formation and greatly facilitates the egress of activated enzymes from the pancreas.

Keywords: Bradykinin antagonists; Hoe 140; pancreatitis (experimental); caerulein; hypovolaemia; plasma protein extravasation; amylase; lipase; trypsin; protease inhibitors

Introduction

Hyperstimulation of the exocrine function of the rat pancreas with the cholecystokinin (CCK) analogue, caerulein, leads to morphological changes in the pancreas which resemble those found in human acute interstitial oedematous pancreatitis (Willemer *et al.*, 1990). CCK antagonists have been shown to inhibit the occurrence of pathological events in several models of experimental pancreatitis thought to mimic certain forms of pancreatitis in man (see Woodruff & Hughes, 1991). Thus, a stimulation of CCK receptors is thought to play a causative role in the pathogenesis of the disease.

Since during the course of acute pancreatitis enzymes potentially capable of releasing kinins are released into the interstitial space of the pancreas (Watanabe *et al.*, 1984; Saluja *et al.*, 1987), we have previously (Griesbacher & Lembeck, 1992a) used the potent bradykinin (BK) B_2 receptor antagonist, Hoe 140 (Lembeck *et al.*, 1991; Hock *et al.*, 1991; Wirth *et al.*, 1991; Griesbacher & Lembeck, 1992b) in this model. It was demonstrated that pretreatment of rats with Hoe 140 prevented both the formation of the pancreatic oedema and the second phase of the characteristic, biphasic fall in blood pressure induced by an infusion of caerulein. The fall in blood pressure was not reversed by Hoe 140 if the antagonist was administered at a time when the hypotension had already fully developed. Therefore, it appeared that kinin-mediated plasma extravasation is likely to be the cause of the observed hypotension rather than kinin-mediated vasodilatation. In contrast to the effects of Hoe 140 on blood pressure and oedema formation, this BK antagonist did not inhibit the caerulein-induced increases in the activities of amylase and lipase in serum, but augmented them. It was hypothesized that prevention of the development of pancreatic oedema would improve the egress of activated enzymes from the gland.

The purpose of the present investigation was: (1) to determine, in detail, the role of the pancreatic oedema in the development of the hypotension during acute pancreatitis, (2) to elucidate why Hoe 140 enhances the rise in enzyme activities in serum, and (3) to compare the effects of the BK antagonist with those of protease inhibitors. A preliminary account of these results has been presented to the British Pharmacological Society (Griesbacher & Lembeck, 1992c).

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Methods

Experimental procedure

Female Sprague-Dawley rats (200–280 g) were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}, i.p.) and phenobarbitone sodium (160 mg kg^{-1}, i.p.). In order to inhibit the catabolism of endogenously released kinins, captopril (50 μmol kg^{-1}, i.p.), an inhibitor of kininase II, was injected at the same time. Hoe 140 (100 nmol kg^{-1}, s.c.), or a corresponding volume (0.5 ml kg^{-1}) of a 154 mM solution of sodium chloride (saline), was injected 10 min later. Caerulein was infused into a jugular vein at a rate of 67 pmol kg^{-1} min^{-1} for periods of 10, 45 or 120 min. Control animals received an infusion of saline (0.034 ml min^{-1}).

In similar experiments, the pretreatment with Hoe 140 was replaced by i.v. injections of aprotinin (20,000 u kg^{-1}) or of soybean trypsin inhibitor (SBTI, 16 mg kg^{-1}). These injections were made either 10 min before or 10 min after the start of the infusion of caerulein.

In all experiments, one group of animals was treated with Hoe 140 alone in order to exclude any possible intrinsic effects of the antagonist. No such effects were observed. The results from this group are not shown in the figures for the sake of clarity.

Quantification of pancreatic oedema

Water content A portion of the pancreas of about 2 g wet weight was taken, weighed, dried in a vacuum centrifuge and reweighed. The difference between wet weight and dry weight, expressed as a fraction of the dry weight, was taken as an estimate of the water content of the tissue.

Plasma protein extravasation In another set of experiments, the azo dye, Evans blue (5 mg kg^{-1}, i.v.), which binds quantitatively to plasma proteins, was injected 5 min before the infusion of caerulein. At the end of the experiment, the animals were perfused via the thoracic aorta with 40 ml of saline to remove intravascular Evans blue. After determining the water content of the pancreas as described above, the dried tissue was immersed in formamide for 24 h at 55°C to extract the dye (Gamse *et al.*, 1980). Evans blue was measured photometrically at 620 nm (Saria & Lundberg, 1983). The amount of Evans blue found in the tissue was taken as a measure of plasma protein extravasation.

Measurement of enzyme activities

For the measurements of the activities of amylase and lipase in serum, the rats were decapitated at the end of the experiment and the trunk blood was collected. The blood was allowed to coagulate and was then centrifuged for 15 min at 2×10^4 m s^{-2}. The serum was removed and stored at −20°C.

Part of the pancreas adjacent to the duodenum was excised, cut into small pieces and immersed in 2 ml of ice-cold saline. These samples were centrifuged at 2×10^5 m s^{-2} for 25 min in order to extract the tissue fluid. The supernatant was removed and stored at −20°C until the determinants of the activities of amylase and lipase were carried out. For the measurement of trypsin activity, fresh samples of the supernatant were used. The pellet of pancreatic tissue was dried, as described above, to determine the dry weight.

Measurements of the activities of amylase and lipase were carried out on an Ektachem 700 Analyser. The enzymic amylase and lipase methods (Eastman Kodak, New York, U.S.A.) have a dynamic range of 5–1200 u l^{-1} and 10–2000 u l^{-1}, respectively. Trypsin activity in the samples was determined by a colorimetric method (Test Combination Trypsin, Boehringer Mannheim, Germany). The limit of detection was 1 u l^{-1}.

Total protein in serum and pancreas

The total protein concentration in serum was determined by the Biuret method. The Coomassie Brilliant Blue G-250 method (Total Protein Test, Bio Rad, Hemel Hempstead, U.K.) was used to measure the total protein concentration in the tissue fluid samples.

Determinations of haematocrit

In addition to the surgical procedure described above, a carotid artery was cannulated with a short piece (1.5 cm) of polyethylene tubing (Intramedic PE-20, Clay Adams/Beckton Dickinson, Parsippany, N.J., U.S.A.). A small blood sample (40–50 μl) was withdrawn immediately before the i.v. infusion of caerulein to determine the basal haematocrit (Ht_0). Further determinations of haematocrit (Ht) were made at 10, 25 and 45 min, and subsequently at regular intervals of 15 min until the end of the 2 h infusion of caerulein. Since no haemorrhagic lesions were observed, the % change in total blood volume (ΔV_B) was estimated from the equation:

$$\Delta V_B = 100 \left(\frac{Ht_0}{Ht} - 1 \right)$$

and the % change in plasma volume (ΔP_P) was estimated as

$$\Delta V_P = 100 \left[\frac{\left(\frac{Ht_0}{Ht} - Ht_o \right)}{\left(1 - Ht_0 \right)} - 1 \right]$$

Substances used

Hoe 140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin) was a gift from Hoechst (Frankfurt/Main, Germany). Sulphated caerulein was obtained from Sigma (St. Louis, M.O., U.S.A.). Stock solutions of both peptides were prepared in a 154 mM solution of NaCl (saline). Pentobarbitone sodium (Nembutal) was obtained from Ceva (Germany). Phenobarbitone sodium (Apoka, Vienna, Austria) was dissolved in saline at a concentration of 80 mg ml^{-1}. Captopril was obtained from Squibb-von Heyden (Vienna, Austria). Evans blue was purchased from Sigma (U.S.A.). Aprotinin (Trasylol) was a gift from Bayer (Leverkusen, Germany). Aprotinin activity is expressed as kallikrein inactivator units; one unit will inhibit approximately 500 ng trypsin. SBTI was purchased from Sigma; 1 mg SBTI will inhibit approximately 1.6 mg trypsin.

Statistical analysis

Multiple nonparametric comparisons (Zar, 1984) were used to compare the effects of caerulein at the different time points and to evaluate the inhibition or potentiation of these effects by Hoe 140. All values are presented as means ± s.e.mean except those values obtained for the activities of trypsin in serum and in the pancreas. These values are given as median and range.

Results

Pancreatic oedema

Ten minutes after the start of the infusion of caerulein no increase in the water content of the pancreas was found. At the time of the maximum fall in blood pressure described earlier (Griesbacher & Lembeck, 1992a), i.e. at 45 min, the water content had increased by over fourfold (Figure 1a). At

the end of the infusion of caerulein (120 min), the mean value of the water content was slightly, but not significantly, lower.

A separate group of rats was injected with Evans blue, 5 min prior to the start of the caerulein infusion, in order to stain the plasma proteins. No Evans blue could be found in the pancreas after infusing caerulein for 10 min duration. However, 45 min after the start of the caerulein infusion there was over 500 μg Evans blue g^{-1} dry weight present in the pancreas (Figure 1b). Similar values were found at 120 min indicating that no further extravasation of plasma proteins was taking place during the second hour of the caerulein infusion. The total protein concentration in the oedema fluid (Figure 1c) showed a continuous increase during the 120 min of the caerulein infusion.

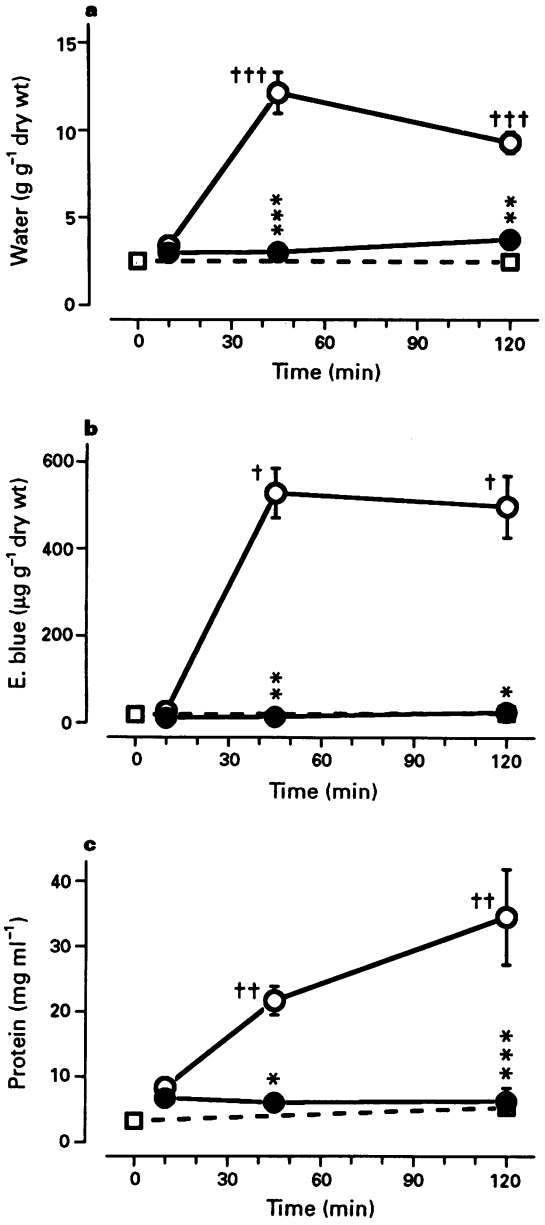

Figure 1 Oedema of the pancreas during caerulin-induced acute pancreatitis in rats: caerulein (4 nmol kg^{-1} h^{-1}) was infused i.v. for 10, 45 or 120 min. Thirty minutes before the start of the caerulein infusion, the rats were injected either with saline (0.5 ml kg^{-1}, ○) or with Hoe 140 (100 nmol kg^{-1}, ●). Control animals received saline for both the s.c. injection and the i.v. infusion (□). The oedema was quantified as the water content of the pancreas (in g g^{-1} dry weight, $n = 14-21$, a). For the quantification of the extravasation of plasma proteins, rats were additionally injected with the dye, Evans blue (5 mg kg^{-1}, i.v.), 5 min prior to the i.v. infusion, and the tissue content of Evans blue (in μg g^{-1} dry weight, $n = 5-7$, b) was determined. The total protein content of the oedema fluid (in g ml^{-1}, $n = 7-14$, c) was measured in rats not injected with Evans blue. Symbols are mean values, vertical lines give s.e.mean; where no s.e.mean is given it was smaller than the symbol. Significance of inhibition by Hoe 140: $*P<0.05$; $**P<0.01$; $***P<0.001$. Significance of difference from controls: $\dagger P<0.05$; $\dagger\dagger P<0.01$; $\dagger\dagger\dagger P< 0.001$.

In order to clarify these results, the effect of a 120 min infusion of caerulein on oedema formation and plasma protein extravasation was compared in 2 groups of 8 rats: the first group was pretreated with Evans blue 5 min before the caerulein infusion, the second was injected with the dye 60 min after the start of the infusion. In both groups, the water content of the pancreas at the end of the experiment was similar (7.73 ± 0.53 g g^{-1} dry weight and 6.99 ± 0.66 g g^{-1} dry weight, respectively). However, Evans blue in the pancreas was increased only in the first group (501 ± 71 μg g^{-1} dry weight) whereas the amount of Evans blue determined in the pancreas of the second group (20 ± 6 μg g^{-1} dry weight) was not different from that in the tissue blanks (15 ± 8 μg g^{-1} dry weight, $n = 4$).

Pretreatment with Hoe 140 completely prevented the formation of the pancreatic oedema. The water and Evans blue contents of the pancreas, as well as the protein content of the interstitial oedema fluid remained normal throughout the infusion of caerulein in these animals (Figure 1).

Since caerulein caused a significant extravasation of water and of proteins into the pancreas, the total protein concentration in the serum was also determined. During the 120 min of infusion of caerulein, no changes in serum protein levels were observed. All experimental groups showed protein concentrations of 45–55 mg ml^{-1} in the trunk blood collected after decapitation.

Haematocrit

The basal haematocrit values of the rats in all experimental groups were 0.42–0.46. Two hours i.v. infusion of saline (0.034 ml min^{-1}) caused a slight reduction of these values (Figure 2a) due to the increase in blood and plasma volume (Figure 2b and c). Ten minutes after the start of the i.v. infusion of caerulein (4 nmol kg^{-1} h^{-1}), the haematocrit was not different from controls; subsequently, a progressive rise in haematocrit was observed which reached maximum values of 0.59 ± 0.01 at 60 min (Figure 2a). The rise in haematocrit values at this time point indicated a reduction in total blood volume of $28 \pm 3\%$ (Figure 2b) and a reduction in plasma volume of $48 \pm 4\%$ (Figure 2c). During the remaining course of the experiment, the haematocrit values declined slowly.

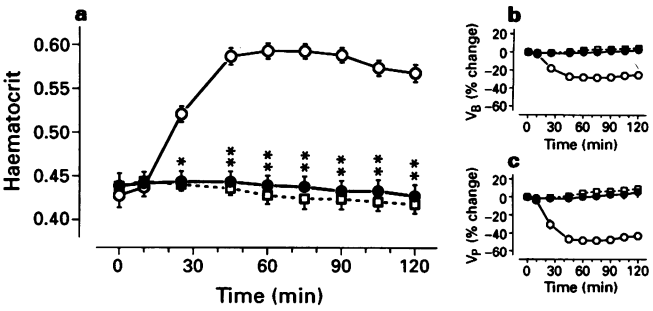

Figure 2 Haemoconcentration during experimental acute pancreatitis in rats: Haematocrit (a) of blood samples, drawn from a carotid artery, was measured repeatedly during a 2 h infusion of either caerulein (4 nmol kg^{-1} h^{-1}, circles) or of a corresponding volume of saline (0.034 ml min^{-1}, squares); 30 min prior to the beginning of this infusion, the animals were injected s.c. either with saline (0.5 mg kg^{-1}, open symbols), or with Hoe 140 (100 nmol kg^{-1}, closed symbols). From the changes in haematocrit, the % changes in total blood volume (V_B, b) and in plasma volume (V_P, c) were calculated. Symbols represent mean values; vertical lines show s.e.mean. Where no s.e.mean is given it was smaller than the symbol; $n = 5-6$ in all experimental groups. Significance of difference from rats receiving caerulein only: $*P<0.05$; $**P<0.01$.

The effect of caerulein was completely abolished in rats which had been pretreated with Hoe 140 (100 nmol kg^{-1}, s.c.). Hoe 140, administered to animals not receiving caerulein, did not have any effect by itself (not shown in Figure 2).

Enzyme activities in serum and in the pancreas

The activities of amylase and lipase in blood serum were not increased 10 min after the start of the infusion of caerulein, but showed a continuous rise thereafter (Figure 3a and c). In animals which had been pretreated with Hoe 140 (100 nmol kg^{-1}, s.c.), this rise in the serum activities of amylase and lipase was significantly augmented ($P<0.05$) both at 45 min and at 120 min. The values for lipase activity obtained at 120 min were somewhat higher than those determined in our previous investigation (Griesbacher & Lembeck, 1992a). This is probably due to the differences in the methods used for the measurement of lipase activity.

Ten minutes after the start of the caerulein infusion, the activities of amylase and lipase in the pancreas were not increased. When caerulein was infused for periods of up to 120 min, the enzyme activities rose progressively to values that were about 30 times higher that the basal values (Figure 3b and d). In contrast to the effect of Hoe 140 on caerulein-induced changes in enzyme activities in the serum (see above), the caerulein-induced increases in enzyme activities in the pancreas were significantly attenuated by Hoe 140 both at 45 min ($P<0.05$) and at 120 min ($P<0.01$). At 120 min, the amylase and lipase activities in the pancreas were increased only by about five fold ($P<0.05$).

Trypsin activity was below the limit of detection in the serum of control animals. After a 2 h infusion of caerulein, a small, but significant ($P<0.05$), trypsin activity was found (median 0.005 u ml^{-1}, range 0.002–0.011 u ml^{-1}, $n=8$). The trypsin activities found in the serum of rats pretreated with Hoe 140 (median 0.008 u ml^{-1}, range 0–0.012 u ml^{-1}, $n=7$) were not significantly different from those obtained from rats treated with caerulein alone. In the pancreas of control animals, trypsin activity was found in only 1 animal (0.301 u g^{-1} dry weight), whereas no trypsin activity was detectable in the other 4 controls. The infusion of caerulein did not induce a significant elevation of active trypsin in the pancreas (median 0.049 u g^{-1} dry weight, range 0–0.672 u g^{-1} dry weight). Hoe 140 had no influence on these values (median 0 u g^{-1} dry weight, range 0–0.277 u g^{-1} dry weight).

Effects of protease inhibitors

To investigate the effects of protease inhibitors on the pancreatic oedema in caerulein-induced pancreatitis, aprotinin (20,000 u kg^{-1}), or SBTI (16 mg kg^{-1}), was injected i.v. either 10 min before, or 10 min after, the start of the caerulein infusion. All animals were given an i.v. injection of Evans blue (5 mg kg^{-1}) 5 min before caerulein in order to stain the plasma proteins. The effect of aprotinin was inconsistent when it was injected 10 min after the beginning of the infusion of caerulein. In 3 out of the 8 animals in this experimental group both the water content and the Evans blue content of the pancreas were almost normal, whereas in the other 5 animals caerulein-induced oedema was as pronounced as in animals that had not been given a protease inhibitor. In this group, the water content of the pancreas (Figure 4a) was not statistically different from both the basal values and from the values found in the group treated with caerulein alone. Pretreatment with aprotinin 10 min prior to the caerulein infusion had no effect on the caerulein-induced extravasation of water (Figure 4a) or on extravasation of plasma proteins (Figure 4b). SBTI also had no effect on the caerulein-induced extravasation of water or plasma proteins into the pancreas.

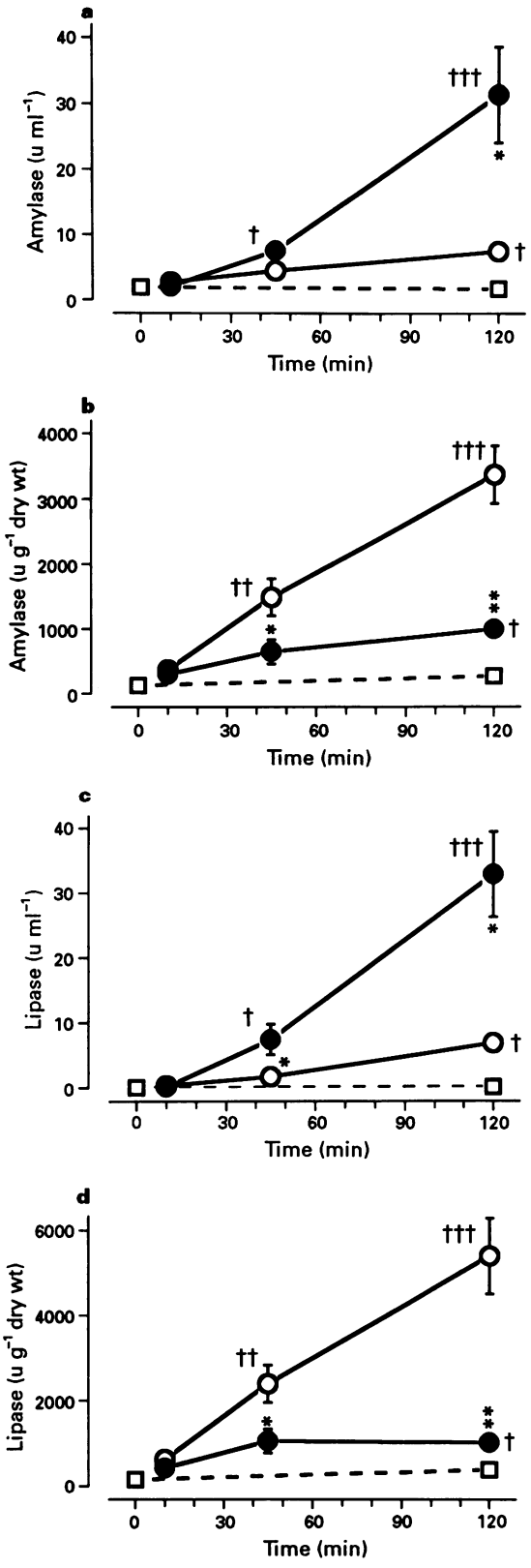

Figure 3 Activities of amylase (a and b) and of lipase (c and d) in serum (in u ml^{-1}, a and c) and in the pancreas (in u g^{-1} dry weight, b and d): acute pancreatitis was induced in rats by an i.v. infusion of caerulein (4 nmol kg^{-1} h^{-1}) for periods of 10, 45 and 120 min; 30 min prior to this infusion, the rats were injected s.c. either with saline (0.5 ml kg^{-1}, ○) or with Hoe 140 (100 nmol kg^{-1}, ●). Control animals received saline as s.c. injection and as i.v. infusion (□). Symbols represent mean values, vertical lines show s.e.mean; where no s.e.mean is given it was smaller than the symbol. $n=7$–15. Significance of difference between the effects of caerulein in rats injected with Hoe 140 and in rats injected with saline: $*P<0.05$; $**P<0.01$. Significance of difference from results in control animals infused with saline: †$P<0.05$; ††$P<0.01$; †††$P<0.001$.

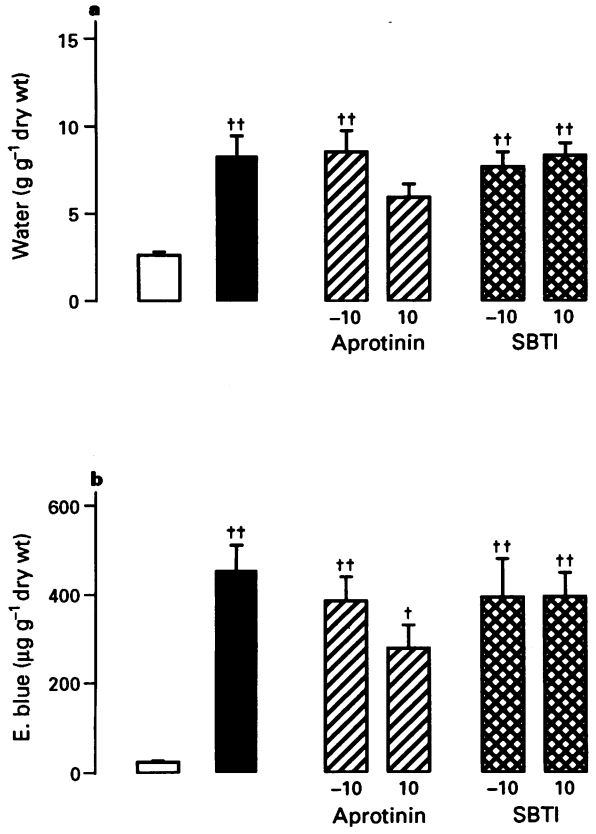

Figure 4 Effects of aprotinin and soybean trypsin inhibitor (SBTI) on the formation of the pancreatic oedema in caerulein-induced acute pancreatitis in rats: caerulein (8 nmol kg^{-1} administered over 120 min) was given as an i.v. infusion. The pancreatic oedema was measured as the water content of the tissue (in g g^{-1} dry weight of the pancreas, a) and the extravasation of plasma proteins was quantified as the tissue content of Evans blue (in μg g^{-1} dry weight, b). Aprotinin (20,000 u kg^{-1}, hatched columns), or SBTI (16 mg kg^{-1}, cross-hatched columns), was injected i.v. either 10 min before, or 10 min after, the start of the caerulein infusion. Solid columns show the effects of caerulein alone; open columns give control values determined in animals given an i.v. infusion of saline (0.034 ml min^{-1}) instead of caerulein. Column heights represent mean values, vertical lines show s.e.mean; $n = 8-14$. Values obtained after treatment with aprotinin or SBTI were not significantly different from those obtained with caerulein alone. Significance of difference from control values: †$P<0.05$; ††$P<0.01$.

Discussion

In man, acute pancreatitis occurs in two forms, the interstitial oedematous form and the necrotizing haemorrhagic. The majority of the patients show the former course of the disease (Büchler, 1991). Experimental models exist for both types of acute pancreatitis (see Adler *et al.*, 1986). Hyperstimulation of the exocrine function of the pancreas with caerulein, a decapeptide analogue of cholecystokinin, is a widely used method of inducing morphological and biochemical changes in the gland, that are similar to those found in oedematous pancreatitis in man (Willemer *et al.*, 1990). The involvement of endogenous kinins in clinical pancreatitis was already suspected soon after the discovery of the kallikrein-kinin system (Werle *et al.*, 1958). Several enzymes found in the pancreas are able to release kinins from their precursors, the kininogens. Kallikrein, which is present in the pancreas in large amounts, is a specific kininogenase. Trypsin and cathepsin B are also capable of releasing kinins. Both enzymes are activated in the pancreas during experimental pancreatitis (Yamaguchi *et al.*, 1989). The intracellular fusion of zymogen granules with lysosomes, and subsequent release of their contents into the interstitial space, observed after hyperstimulation of the exocrine pancreas (Watanabe *et al.*, 1984; Saluja *et al.*, 1987), suggests a mechanism for the activation of such enzymes. Previously, we have shown that Hoe 140, a recently developed, potent and long-acting BK antagonist (Lembeck *et al.*, 1991; Hock *et al.*, 1991; Wirth *et al.*, 1991; Griesbacher & Lembeck, 1992b), is effective in preventing the formation of the pancreatic oedema and of the second phase of hypotension observed during experimental acute pancreatitis (Griesbacher & Lembeck, 1992a). However, it was observed that the caerulein-induced increases in the activities of amylase and lipase in serum were augmented by Hoe 140. These results suggested that the kinin-mediated pancreatic oedema partly traps activated enzymes, such as amylase or lipase, within the pancreas.

Pancreatic oedema, hypovolaemia and haemoconcentration

The concept that endogenous kinins are responsible for some of the symptoms of pancreatitis, in particular the pronounced hypotension which can extend to circulatory shock, was based mainly on the fact that the blood or ascitic fluid of patients or experimental animals with pancreatitis contained a factor that lowered blood pressure when injected into recipient animals (Nugent & Atendido, 1966), had a BK-like effect on smooth muscles (Amundsen *et al.*, 1968; Ofstad, 1970), and had physicochemical properties similar to BK (Amundsen *et al.*, 1968; Ofstad, 1970; Ellison *et al.*, 1981). Intravenous injection of BK lowers blood pressure mainly by vasodilatation. This might lead to the assumption that the kinins, which are released endogenously during acute pancreatitis, produce hypotension by this mechanism. However, kinins are extremely shortlived *in vivo* (Ward, 1991) and a continuous release of kinins over a prolonged period of time would be necessary to produce the hypotension which occurs during acute pancreatitis by this mechanism. On the other hand, it has also been demonstrated that a considerable amount of fluid, presumably blood plasma, accumulates in the pancreas and in the peritoneal cavity (Ryan *et al.*, 1964). This suggests that hypovolaemia, due to plasma extravasation, may be the main cause for the circulatory shock in acute pancreatitis.

In our previous study with Hoe 140 (Griesbacher & Lembeck, 1992a) we showed that this compound prevented or reduced the development of pancreatic oedema in rats when it was injected before, or up to 25 min after, the start of an infusion of caerulein. Hoe 140, injected s.c. in the dose used (100 nmol kg^{-1}), effectively blocks the actions of i.v. BK within 5 min (personal observations). Therefore, it was concluded that endogenous kinins increase vascular permeability in the pancreas only during the first 30–45 min of the infusion of caerulein. The time course of the development of the pancreatic oedema shows that the oedema formation reaches a maximum within about 45 min (see Figure 1). Plasma proteins no longer leave the circulation during the 2nd h of the caerulein infusion. This indicates that vascular permeability apparently has returned to a normal value by this time. In addition, reduced blood flow through the pancreas, due to the collapse of a considerable proportion of the capillaries (Gress *et al.*, 1990), might also be partially responsible for the fact that no further extravasation takes place. The water content of the pancreas showed a slight decline during this period (Figure 1a); the amount of Evans blue-stained plasma proteins in the pancreas remained constant (Figure 1b). The continuous increase in the total protein concentration in the oedema fluid (Figure 1c) could, thus, be explained by a concentrating effect due to the onset of reabsorption of fluid, but not of proteins, from the tissue to the vasculature.

The results described above show that the vascular permeability in the pancreas is increased only during the first 30–45 min of the caerulein infusion. This increase in vascular permeability can be entirely attributed to the actions of endogenous kinins because Hoe 140 completely prevented the

formation of the oedema. On the other hand, the pronounced hypotension is present throughout the 2 h of the experiment (see Griesbacher & Lembeck, 1992a). Thus, a kinin-mediated hypovolaemia seems to be the most likely explanation for the fall in blood pressure. Provided that the total volume of the corpuscular elements of the blood remains constant, changes in the haematocrit allow the estimation of changes in total blood volume and plasma volume. By measuring the haematocrit repeatedly during the experiment, we were able to demonstrate a 48% fall in plasma volume and a reduction of total blood volume by 28%. Thus, the kinin-mediated pancreatic and retroperitoneal oedema can be seen as the main, if not the sole, cause of the hypotension. The fact that the time-course of the changes in the haematocrit (Figure 2) runs in parallel with the time-course of the hypotension (compare Figure 2 in Griesbacher & Lembeck, 1992a) and with the time-course of the oedema formation in the pancreas (see Figure 1) further supports this view. Whether there are additional factors contributing to hypotension is currently being investigated. The haemoconcentration observed during acute pancreatitis, together with the pronounced hypotension, will certainly have a severe effect on the microcirculation of the pancreas. Hoe 140 completely prevents haemoconcentration and hypotension and, therefore, might prevent tissue damage due to impaired tissue oxygenation.

Egress of activated enzymes from the pancreas

It was noted previously, that pretreatment of rats with the BK antagonist, Hoe 140, despite its inhibitory actions on oedema formation and hypotension, augmented the increases in the serum activities of amylase and lipase at the end of the 2 h infusion of caerulein (Griesbacher & Lembeck, 1992a). It was hypothesized that the oedema of the pancreas partially traps activated enzymes in the tissue and that prevention of the oedema formation would improve the egress of the enzymes from the gland. In order to check this explanation we have now determined the enzyme activities in serum as well as in the pancreas. The augmentation by Hoe 140 of the enzyme activities in serum was again seen when the time-courses of the rises in amylase and lipase activities in serum were determined. In the pancreas, the activities of amylase and lipase increased throughout the course of the infusion of caerulein. If our hypothesis were correct, then the enzyme activities in the pancreas ought to be reduced by treatment with Hoe 140. As can be seen from Figure 3, pretreatment with Hoe 140 potently attenuated the caerulein-induced rises in enzyme activities in the pancreas.

The present results verify the proposition that prevention by Hoe 140 of increased vascular permeability would greatly facilitate the egress of activated enzymes from the pancreas during acute pancreatitis. Since accumulation of active lipase in the tissue, in conjunction with other factors frequently implicated in the pathogenesis of acute pancreatitis, such as bile salts, is thought to be important for the development of pancreatic necroses (Creutzfeldt & Schmidt, 1970), the effects of Hoe 140 described here would appear to be of great importance for the treatment of acute pancreatitis in man.

The augmentation by Hoe 140 of the serum activities of amylase and lipase during caerulein-induced acute pancreatitis also indicated that Hoe 140 does not inhibit the caerulein-induced stimulation of the exocrine pancreas. *In vitro* experiments confirmed that Hoe 140, in concentrations which effectively abolish the effects of BK, do not affect caerulein-induced contractions of the guinea-pig ileum and, thus, has no effect on CCK receptors (data not shown).

Trypsin has been implicated in the pathogenesis of acute pancreatitis by Forrell (1955). Trypsin and other proteases have also been found to be increased in a model of experimental pancreatitis similar to the one used in the present investigation (Yamaguchi *et al.*, 1989). In the present investigation, we have found that active trypsin might be slightly increased in serum and in the pancreas. However, the measured trypsin activities were near the limits of detection so that a definite statement as to the effects of Hoe 140 on trypsin activity cannot be made.

Comparison of the effects of Hoe 140 and protease inhibitors

In caerulein-induced pancreatitis oedema was found to be wholly due to the actions of endogenously released kinins since Hoe 140 prevented the oedema formation. In addition to kallikrein, the specific kininogenase, trypsin has the ability to release kinins from their precursor peptides (Rocha e Silva *et al.*, 1949). Both trypsin and kallikrein can be inhibited by protease inhibitors such as aprotinin or SBTI (see Vogel, 1979). Aprotinin has been used for the treatment of acute pancreatitis in experimental and in clinical trials. Neither aprotinin nor SBTI had a significant inhibitory action on oedema formation in our experiments regardless of whether they were administered before or after the start of the caerulein infusion (compare Figure 4). In contrast, our previous results with Hoe 140 (Griesbacher & Lembeck, 1992a) showed that it was effective even when injected up to 25 min after the induction of pancreatitis by caerulein.

All clinical studies with aprotinin, with one exception (Trapnell *et al.*, 1974), have failed to show a positive effect which contrasts with the results obtained in experimental investigations (see Steinberg & Schlesselman, 1987). This discrepancy has been explained by the fact that, in experimental studies, aprotinin was usually administered before the induction of pancreatitis, whereas this of course does not occur in clinical situations. On the other hand, recent clinical investigations have shown that positive results can be obtained when therapy with protease inhibitors is introduced within the first 24 h after the onset of the disease (Harada *et al.*, 1991). Bearing in mind that the time course of pathological events is much more rapid in experimental studies than in the clinical course of the human disease (see Steinberg & Schlesselman, 1987), the ability of Hoe 140 to reduce pathological events in the pancreas when administered after the induction of pancreatitis (Griesbacher & Lembeck, 1992a) adds to the possible therapeutic value of Hoe 140.

In summary, it has been demonstrated that in experimental acute pancreatitis the release of endogenous kinins in the pancreas leads to a massive extravasation of plasma into the pancreatic and adjacent retroperitoneal tissues. The loss of plasma from the circulation results in severe hypotension and haemoconcentration. These pathological events could be prevented by timely intervention with the potent BK antagonist, Hoe 140. Treatment with Hoe 140 also reduced the accumulation of enzymes which are released and activated in the pancreas during the course of the pancreatitis. The concomitant augmentation of the enzyme activities in serum necessitates further assessment of the prognostic consequences of treatment with Hoe 140. Thus, other factors, which, when released into the blood stream, cause tissue or organ damage, have to be studied. Since the severity of pancreatitis does not correlate with the serum levels of amylase and lipase, but rather with the extent of pancreatic oedema and its sequelae (Büchler, 1991), it is conceivable that prevention of oedema formation will also prevent the formation of such factors.

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Pharmacological differentiation by pertussis toxin of the *in vivo* acute responses to fMLP and PAF in guinea-pig lungs

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1 The effects of pertussis toxin on the N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) and platelet-activating factor (PAF)-induced variations in pulmonary capillary albumin exchanges, blood volume, leucocyte or platelet sequestration were studied in the guinea-pig, by use of radioactive tracers. The effects of pertussis toxin on pulmonary insuflation pressure were studied in parallel.

2 The i.v. administration of fMLP and PAF to the guinea-pig was followed by bronchoconstriction, increased lung capillary albumin exchanges (vasopermeability) sequestration of leucocytes, leucopenia and reduction of blood volume (vasoconstriction). PAF also induced platelet sequestration in lungs and thrombocytopenia.

3 Pertussis toxin (10 $\mu g\ kg^{-1}$, i.v., 72 h before the experiment) prevented all the studied fMLP-induced effects, but failed to modify PAF-induced bronchoconstriction, lung vasoconstriction, platelet sequestration, thrombocytopenia and the increased capillary vasopermeability. In the same conditions the lung leucocyte sequestration was not significantly affected when leucopenia was partially reduced.

4 It is suggested that the effects of fMLP, but not those of PAF, involve a G_i-like protein.

Keywords: G protein; leucocyte; lung; N-formyl-L-methionyl-L-leucyl-L-phenylalanine; pertussis toxin; platelet activating factor; platelet

Introduction

The systemic injection of the inflammatory agonist and cell secretagogue N-formyl-methionyl-leucyl-phenylalanine (fMLP) into the guinea-pig leads to a well characterized response, including bronchoconstriction (Boukili *et al.*, 1986; Hamel *et al.*, 1984), systemic hypotension, pulmonary hypertension, increased vascular permeability, transient thrombocytopenia (Bureau *et al.*, 1992; Boukili *et al.*, 1986), prolonged leucopenia and the recruitment of platelets and leucocytes into the lungs (Bureau *et al.*, 1989). Similarly, platelet-activating factor (PAF), a mediator released during inflammatory and immunological process (Braquet *et al.*, 1987) also induces bronchoconstriction, hypotension, thrombocytopenia (Vargaftig *et al.*, 1980), leucopenia, increased vasopermeability (Bolin *et al.*, 1987) and the trapping of circulating cells into the lungs (Bureau *et al.*, 1989; Hultkvist-Bengtsson *et al.*, 1991). It has been previously shown that the bronchopulmonary effects of systemic fMLP are cyclo-oxygenase-dependent and independent from the activation of circulating neutrophils (Boukili *et al.*, 1986; 1989), whereas those of PAF are cyclo-oxygenase-independent and require the presence of intact circulating platelets (Vargaftig *et al.*, 1980). In addition, fMLP contracts respiratory smooth muscle *in vitro* (Boukili *et al.*, 1986; Hamel *et al.*, 1984), whereas PAF is ineffective and may even induce its relaxation (Prancan *et al.*, 1982).

Pertussis toxin, an exotoxin derived from *Bordetella pertussis*, inactivates some G proteins, including the regulatory guanine nucleotide protein G_i (Birnbaumer *et al.*, 1991). Those proteins transduce the signal between activated membrane receptors and their intracellular effectors and pertussis toxin has indeed been shown to suppress the *in vitro* activation of alveolar macrophages induced by PAF and by fMLP. In this work, we took advantage of our recent demonstration that pertussis toxin suppresses bronchoconstriction induced by fMLP (Imaizumi *et al.*, 1992), to study whether other effects of fMLP, and of PAF as a comparison, are also inhibited by its *in vivo* administration. Our results indicate that PAF and fMLP operate via pathways involving different transduction mechanisms.

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Methods

Equipment

A γ detector (DM1, Nuclear Enterprise) connected to a pulse-height-analyser (PHA1, Numelec) was used to measure simultaneously the radioactivities of different isotopes in the lung region. The probe was collimated with lead in order to observe a limited field (the diameter of the hole was 1 cm). From the measured γ spectrum of the three radioactive tracers used (^{99m}Tc, ^{111}In and ^{131}I), three energy windows for counting the γ emissions were set. A well-type counter (Compugamma CS, LKB Wallac) was used to measure blood samples radioactivities. Data from external detection and blood samples were collected on diskettes for further calculations.

A Palmer miniature pump (Bioscience, U.K.) allowed guinea-pig ventilation. Physiological pressure transducers P23XL (Gould Electronics, U.S.A.), connected to a Beckman Dynograph R 511, were used to record the carotid arterial pressure and the pulmonary insufflation pressure using a T cannula placed between the pump and the animal. Variations of pulmonary insufflation pressure (PIP), recorded on a Beckman Dynograph R 511, were expressed in cmH_2O. The PIP value expresses the increase of the pulmonary resistance to inflation, i.e. bronchocontriction (BC).

Isolation, radiolabelling and in vivo *functional study of the cells*

Albumin and erythrocytes were labelled with ^{131}I and ^{99m}Tc respectively, and leucocytes and platelets with ^{111}In. Autologous leucocytes were isolated from 4 ml of guinea-pig blood according to Sweatmann *et al.* (1987). An equivalent volume of Plasmagel was reinjected to the animal. Following their isolation, leucocytes were suspended in Dulbecco's buffer without calcium and magnesium, containing PGE_2 (10^{-6} M) to insure cytoprotection (Takenawa *et al.*, 1986). The cell suspension ($2-10 \times 10^6$ leucocytes with 60–80% of neutrophils) was incubated with [^{111}In]-tropolonate, obtained by mixing [^{111}In]-chloride with tropolonate (10–20 μCi ^{111}In for $5-40 \times 10^6$ cells ml^{-1}, final concentration of tropolone

$3-5 \times 10^{-4}$ M) for 5 min, according to the procedure of Dampure *et al.* (1982). The cells were then washed with buffer, resuspended in autologous plasma for 10–12 min in presence of PGE_2 (10^{-6} M final), and injected i.v. into the animal ($2-5 \times 10^6$ cells).

Platelets were isolated from 3 ml of guinea-pig blood and radiolabelled with [^{111}In]-oxinate, according to Page *et al.* (1982) and then injected i.v. to the experimental animal (400×10^6 cells).

Erythrocytes were labelled with ^{99m}Tc, according to the procedure described in the kit from CEA-ORIS (France). Briefly, 2 ml of blood were collected on sodium citrate ($3.8 \times$ mg.ml^{-1}), and 0.5 ml of stannous pyrophosphate solution (in saline) was added. Five minutes later, the cells were washed with saline containing 1,000 iu heparin l^{-1} and then incubated with ^{99m}Tc for 5 min. The cells were then washed twice in heparinazed saline (5,000 iu l^{-1}). Lastly, ^{99m}Tc-erythrocytes were mixed with ^{131}I serum albumin before i.v. injection.

In some experiments, leucocyte viability was checked with the trypan blue exclusion test (more than 95% of leucocytes were viable). In addition, the fMLP-induced superoxide generation of non-radiolabelled leucocyte was also checked from the reduction of cytochrome c in the presence of cytochalasin B (Borregaard & Tauber, 1984). fMLP 10^{-7} M ($n = 7$) induced the generation of 3.5 nM O_2^- for 10^6 cells.

In vivo *procedure*

Pertussis toxin (10 μg kg^{-1}) was administered 72 h before the experiment via the saphenous vein to Hartley guinea-pigs of either sex (400–700 g). This schedule of administration derived from the study of Imaizumi *et al.* (1992), who used 20 μg kg^{-1} of pertussis toxin, but had the same results with 10 μg kg^{-1}. Saline (control) or pertussis toxin-treated animals were anaesthetized (sodium pentobarbitone, 40 mg kg^{-1}, i.p.) and ventilated with a Palmer miniature pump through the cannulated trachea (tidal volume, 10 ml kg^{-1}; frequency, 60 cycles min^{-1}), and spontaneous breathing was suppressed with 2 mg kg^{-1}, i.p. pancuronium. When needed, a supplement of pentobarbitone of 5 mg kg^{-1} was administered i.p., 0.5–1 h before the first injection of the agonists. Baseline PIP did not vary significantly during the interval before the agonist injection. Catheters were inserted into the jugular vein for injection and into both carotid arteries, one for blood sampling and the other for monitoring arterial blood pressure.

For external detection of the radioactivity, a γ probe was placed in front of the right lung region in contact with the chest: 20–40 μCi of ^{111}In-labelled leucocytes or platelets in separate experiments were injected into the jugular vein and were allowed to equilibrate for 3 h. Then, the cell suspension (see above) containing erythrocytes and albumin, radiolabelled respectively with ^{99m}Tc (100–200 μCi) and ^{131}I (5–10 μCi), was injected into the jugular catheter (0.3–1 ml). Blood samples (200 μl) were collected from the carotid artery on citrate (3.8 mg ml^{-1}) at defined time intervals before and after the injection of the agonist, the first sample being collected 30 min after the injection of ^{99m}Tc-erythrocytes and ^{131}I-labelled albumin. Radioactivities of blood and plasma were counted in a γ well-type counter.

PAF (100 ng kg^{-1}), fMLP (10 μg kg^{-1}) and acetylcholine (20 μg kg^{-1}) were injected i.v. as a bolus in a volume of 0.2–0.5 ml.

Significance of the different parameters

Calculations were described in detail by Bureau *et al.* (1989). Briefly, the substraction of blood radioactivity from the external measurement allowed the evaluation of the variations of the two parameters: (1) The lung content in non-circulating radiolabelled leucocytes or platelets, which accounts for their sequestration. (2) The lung content in extra-vascular radiolabelled albumin (Ev alb), which is dependent of the trans-endothelial albumin exchanges and which was named vasopermeability.

In addition, the lung content in radiolabelled erythrocytes (RBC) was used as an index of blood volume.

Results are expressed as a percentage of the mean basal blood radioactivity value before injection of the tested agent.

Chemicals

Pertussis toxin was a gift of Dr A. Ginnaga (The Chemo-sero-therapeutic Research Institute, Japan and Seikagaku Kogyo Co., Japan). N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), stored at −20°C as a 1 mg ml^{-1} stock solution in dimethyl sulphoxide (<1%); acetylcholine, stored at −20°C as a 10 mg ml^{-1} stock solution; dextran (average molecular weight: 298,000), stored at −20°C and prepared as a 6% stock solution in saline; prostaglandins E_2 (PGE_2) and I_2 (PGI_2), stored at −20°C as a 10^{-3} M stock solution in ethanol; cytochrome c and cytochalasin B stored at −20°C as a 250 mg ml^{-1} stock solution were from Sigma Chemical Co. (U.S.A.). PAF, stored at −20°C as a 1 mg ml^{-1} stock solution in saline with bovine serum albumin (0.01%), was from Bachem (Switzerland); sodium citrate (3.8%, wt/vol) from Labo Express Service (France); Acid-Citrate-Dextrose (ACD) (Aster & Jandl, 1964) and sodium pentobarbitone were from Sanofi (France); pancuronium bromide (Pavulon) was from Organon Tecknika (France) and Plasmagel from Roger Bellon (France); heparin (25,000 iu 5 ml^{-1}) was from Choay (France).

Reagents for radiolabelling

Tropolone from Sigma Chemical Co., (U.S.A.) was stored at −20°C as a 4.4×10^{-3} M stock solution in saline. [^{131}I]-albumin, [^{111}In]-oxynate, sodium pyrophosphate, decahydrate and stannous chloride were from Cis Biointernational (France). ^{99m}Tc pertechnetate was a gift from the 'Service central des Radio-isotopes', Hôpital Necker, Paris, France.

Statistical evaluation

Statistical significance of the difference between the different groups was evaluated by unpaired Student's t test at the level of $P \leqslant 0.05$.

Results

Effect of pertussis toxin on pulmonary entrapment of leucocytes induced by fMLP

The i.v. administration of 10 μg kg^{-1} of fMLP to saline-treated (control) animals induced lung sequestration of radiolabelled leucocytes and a corresponding fall in the radioactivity bound to the circulating leucocytes (leucopenia), which persisted throughout the duration of the experiment, i.e. at least for 1 h. When fMLP was injected to guinea-pigs pretreated with 10 μg kg^{-1}, i.v. of pertussis toxin 72 h before the experiment, both lung leucocyte sequestration and leucopenia were significantly inhibited from 10 min on (Figure 1). Comparisons between control animals injected with fMLP ($n = 7$) or with saline (not shown, $n = 8$) at the peak effect at $t = 10$ min were significant at $P \leqslant 0.01$.

Effect of pertussis toxin on fMLP-induced lung increased vasopermeability and blood volume decrease

Lung vasopermeability was increased by fMLP, as shown by an increased lung extra-vascular radiolabelled albumin content (Figure 2). This was accompanied by a reduction in the pulmonary content of radiolabelled erythrocytes, indicating a reduction of lung blood volume. Both effects peaked at

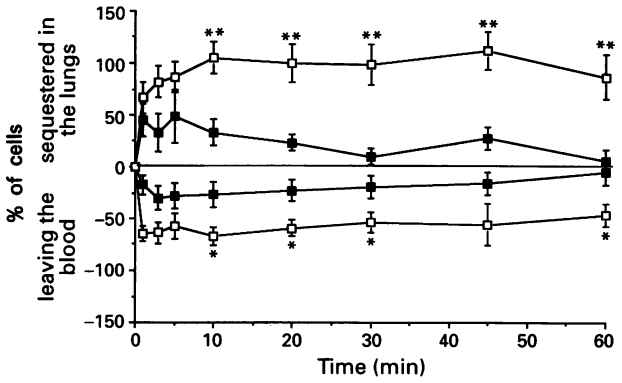

Figure 1 Effect of pertussis toxin treatment on fMLP-induced lung leucocyte sequestration and leucopenia. Lung and blood contents in radiolabelled leucocytes are expressed as the percentage of basal blood leucocyte radioactivity before the i.v. injection of 10 $\mu g\,kg^{-1}$ fMLP to pertussis-treated (10 $\mu g\,kg^{-1}$, 72 h before the experiment) (■) and control (□) guinea-pigs. All values are mean ± s.e.mean (vertical bars) of 7 experiments for each group; comparison between pertussis-treated and control animals: $*P \leqslant 0.05$ and $**P \leqslant 0.01$.

10 min and plateaued for the subsequent hour. Pertussis toxin inhibited significantly the increased vasopermeability and the reduction of the lung blood volume. Comparisons between control animals injected with fMLP ($n = 7$) or with saline (not shown, $n = 8$), performed at the peak effect at $t = 10$ min, were significant at $P \leqslant 0.001$.

Interference of pertussis toxin with fMLP, PAF and acetylcholine-induced bronchoconstriction

As seen in Figure 3, fMLP-induced bronchoconstriction was also inhibited by pertussis toxin, under conditions where bronchoconstriction induced by both acetylcholine and PAF were unaffected.

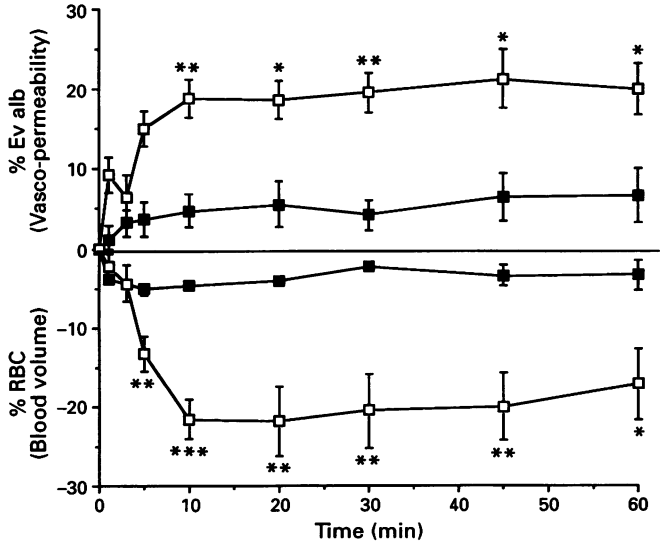

Figure 2 Effect of pertussis toxin treatment on fMLP-induced variations of lung extravascular albumin and erythrocyte content. The lung content in extravascular radiolabelled albumin (Ev alb) and in radiolabelled erythrocytes (RBC) are expressed as a percentage of basal blood radioactivity before the i.v. injection of 10 $\mu g\,kg^{-1}$ fMLP to pertussis-treated (10 $\mu g\,kg^{-1}$, 72 h before the experiment) (■) and control (□) guinea-pigs. All values are mean ± s.e.mean (vertical bars). Comparison between pertussis-treated and control animals: $***P \leqslant 0.001$, $**P \leqslant 0.01$ and $*P \leqslant 0.05$ ($n = 7$ for each group).

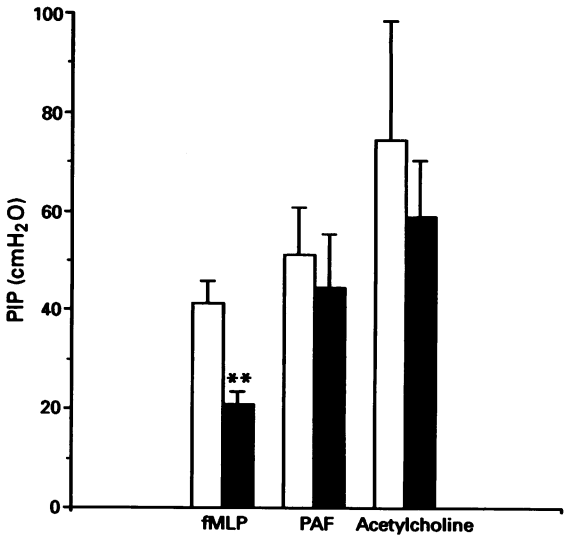

Figure 3 Effect of pertussis toxin treatment on fMLP-, PAF- and acetylcholine-induced bronchoconstriction. Bronchoconstriction was evaluated according to the increase of insuflation pressure given in cmH_2O. Values are mean ± s.e.mean (vertical bars) of 7 experiments for each group. Comparison between pertussis-treated (10 $\mu g\,kg^{-1}$, 72 h before the experiment) (solid column) and control (open column) animals: $**P \leqslant 0.01$.

Interference of pertussis toxin with leucocyte pulmonary recruitment and leucopenia following PAF

Sequestration of radiolabelled leucocytes in the lungs following the i.v. injection of 100 ng kg^{-1} of PAF started at 1 min, peaked at 5 min, and returned slowly to basal levels during the subsequent hour (Figure 4). Leucopenia accompanied lung sequestration. After pertussis toxin administration, the PAF-induced lung leucocyte sequestration was marginally but not significantly reduced, whereas leucopenia was significantly reduced for all time intervals studied, except at 1 h. Comparison between control animals injected with PAF ($n = 6$) or saline (not shown, $n = 8$) was performed at the peak effect at $t = 10$ min and was significant at $P \leqslant 0.01$.

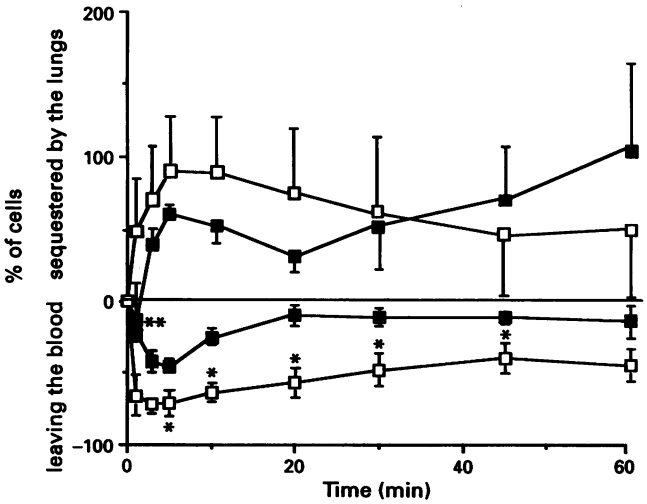

Figure 4 Effect of pertussis toxin treatment on PAF-induced lung leucocyte sequestration and leucopenia. Lung and blood contents in radiolabelled leucocytes are expressed as the percentage of basal blood leucocyte radioactivity before the i.v. injection of 100 ng kg^{-1} PAF to pertussis-treated (10 $\mu g\,kg^{-1}$, 72 h before the experiment) (■) and control (□) guinea-pigs. All values are mean ± s.e.mean (vertical bars) of 6 experiments for each group. Comparison between pertussis-treated and control animals: $**P \leqslant 0.01$ and $*P \leqslant 0.05$.

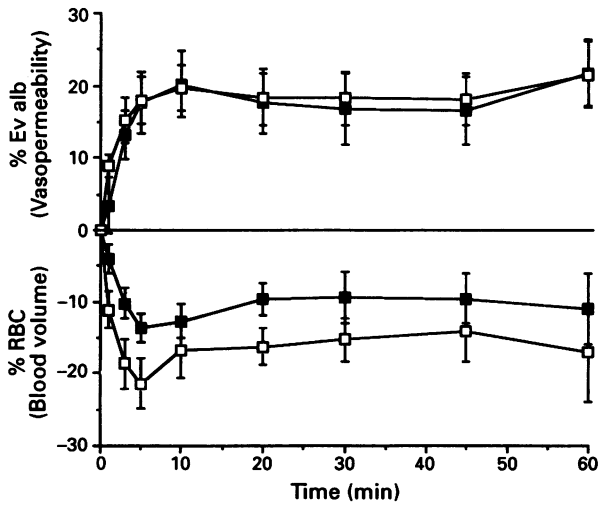

Figure 5 Effect of pertussis toxin treatment on PAF-induced variation of lung extravascular albumin and erythrocyte contents. The lung content in extravascular radiolabelled albumin (Ev alb) and in radiolabelled erythrocytes (RBC) are expressed as a percentage of the basal blood radioactivity before the i.v. injection of 100 ng kg^{-1} PAF to pertussis-treated (10 μg kg^{-1}, 72 h before the experiment) (■) and control (□) guinea-pigs. All values are mean ± s.e.mean (vertical bars) of 6 experiments for each group. Comparison between pertussis-treated and control animals showed no significant difference.

Interference of pertussis toxin with the increased lung vasopermeability and with the decreased lung blood volume induced by PAF

Figure 5 shows that PAF induced an early increase of extravascular albumin radioactivity peaking at 10 min and then persisting elevated in a quasi-steady state. It also induced an early decrease of lung erythrocyte radioactivity, which peaked at 5 min and stabilized at approximately −15% of the basal erythrocyte radioactivity indicating a blood volume reduction. Pertussis toxin failed to interfere with either effect of PAF. Comparison between control animals injected with PAF ($n = 6$) or saline (not shown, $n = 8$) was performed at the peak effect at time $t = 10$ min and was significant at $P \leqslant 0.01$.

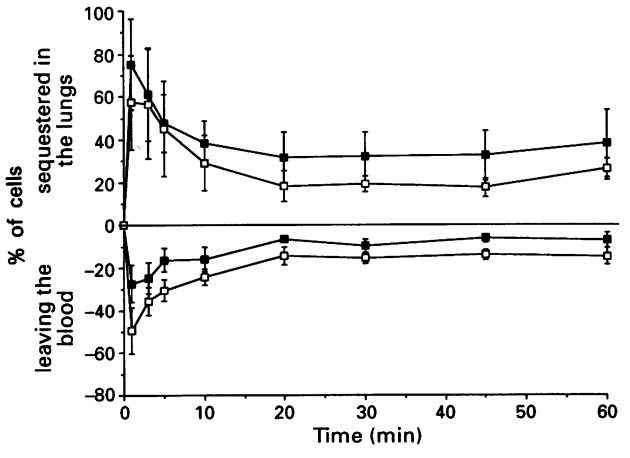

Figure 6 Effect of pertussis toxin treatment on PAF-induced lung platelet sequestration and thrombocytopenia. Lung and blood contents in radiolabelled platelets are expressed as the percentage of basal blood platelet radioactivity before the i.v. injection of 100 ng kg^{-1} PAF for pertussis-treated (10 μg kg^{-1}, 72 h before the experiment) (■) and control (□) guinea-pigs. All values are mean ± s.e.mean (vertical bars) of 6 experiments for each group. Comparison between pertussis-treated and control animals showed no significant difference.

Interference of pertussis toxin with lung platelet recruitment and thrombocytopenia induced by PAF

The i.v. administration of 100 ng kg^{-1} PAF induced lung radiolabelled platelet recruitment and concomitantly, a transient thrombocytopenia (Figure 6). Both were significant during the first 5 min as compared to saline-injected animals. Pertussis toxin failed to interfere with the pulmonary platelet recruitment and with thrombocytopenia induced by PAF. Comparison between control animals injected with PAF ($n = 6$) or saline (not shown, $n = 8$) was performed at the peak effect at 3 min and was statistically significant ($P \leqslant 0.01$).

Discussion

Inflammatory pulmonary diseases are associated with the recruitment and activation of leucocytes in the lungs (Sibille & Reynolds, 1990), an important contributing factor for subsequent tissue damage. The mechanisms accounting for this recruitment involve the coordinate expression of adhesive proteins at the endothelial cell surface and the activation of specific leucocyte receptors, leading to the secretion of toxic products. Transduction systems containing guanosine 5'-triphosphate (GTP)-binding proteins (G proteins) bridge the gap between receptor activation and generation of second messengers and cell activation (Birnbaumer *et al.*, 1990). Pertussis toxin, which induces the ADP-ribosylation of G_i and other G proteins (G_o and transducin) is used to suppress signal transduction and thus to inhibit receptor-mediated events, such as *in vitro* chemoattraction by fMLP of human (Lad *et al.*, 1985) or guinea-pig neutrophils (Okajima *et al.*, 1985), or of human neutrophils by PAF (Lad *et al.*, 1985). Despite wide interest in the use of pertussis toxin as a tool to unravel the mechanisms of signal transduction, few studies have been performed *in vivo*.

Administered intravenously to guinea-pigs, pertussis toxin suppresses the pulmonary effects of fMLP, including thromboxane-mediated bronchoconstriction following its intratracheal instillation to isolated lungs (Kadiri *et al.*, 1992). Inhibition of alveolar macrophages (and also of circulating granulocytes) persisted for at least ten days and correlated with ADP-ribosylation of G proteins. By contrast, pertussis toxin did not inhibit bronchoconstriction and related effects of PAF, indicating that, under *in vivo* conditions, the G proteins which transduce the effects of fMLP are not necessarily the same for PAF-induced activation (Kadiri *et al.*, 1992). Pertussis toxin can thus be used *in vivo* as a tool to study the pathways involved with the specific effects of different secretagogues.

In the present study, the effects of fMLP and of PAF on lung sequestration of inflammatory cells, on PIP and on vasopermeability and pulmonary blood volume were compared. We demonstrate that different pathways are involved with the transduction of the signals generated by these agonists. The intravenous injection of fMLP or of PAF to guinea-pigs induced bronchoconstriction, a prolonged pulmonary sequestration of leucocytes and a transient sequestration of platelets, long lasting leucopenia and thrombocytopenia, increased capillary exchanges and a decrease of the lung blood volume, indicating pulmonary vasoconstriction (Bureau *et al.*, 1989; Boukili *et al.*, 1989). Imaizumi *et al.* (1992) demonstrated that bronchoconstriction by intravenous fMLP in guinea-pigs is prevented by pertussis toxin, as is the case here for intra-tracheal fMLP (Kadiri *et al.*, 1992). In contrast, bronchoconstriction by intravenous PAF was unaffected. Similarly, bronchoconstriction induced by acetylcholine is not modified, providing further evidence that, *in vivo*, the transductional mechanisms of direct smooth muscle effects are not prevented by pertussis toxin, thus supporting its selectivity.

Bronchoconstriction by intravenous fMLP is cyclo-oxygen-

ase-dependent (Boukili et al., 1989), involving essentially the formation of thromboxane A_2 (TxA_2) (Bureau et al., 1992). Kadiri et al. (1992) showed that the release of TxB_2 induced by the intra-arterial or intra-tracheal administration of fMLP to guinea-pig isolated lungs is suppressed by the pretreatment of animals by pertussis toxin. Furthermore, alveolar macrophages collected from pertussis toxin-treated guinea-pigs fail to release TxB_2 and superoxide anions when stimulated by fMLP and indeed contained ADP-ribosylated G_i proteins (Kadiri et al., 1992). Thus, pertussis toxin does induce a long-lasting suppression of the transduction mechanisms for fMLP *in vivo* and *ex vivo*, as was described *in vitro* for human neutrophils (Lad et al., 1985). In contrast, this protective effect does not extend to PAF, as indicated by the inability of pertussis toxin to inhibit PAF-induced bronchoconstriction. Since pertussis toxin inhibited the PAF-induced release of TxB_2 by alveolar macrophages (Kadiri et al., 1992), chemotaxis, superoxide generation, aggregation and release of lysozyme by human neutrophils (Lad et al., 1985), as well as leucopenia (this paper), neither alveolar macrophages nor leucocytes mediated the *in vivo* bronchoconstrictor effect of PAF. However, PAF induces early recruitment of platelets to lungs (McManus et al., 1981; Lellouch-Tubiana et al., 1985) and to other tissues (Bourgain et al., 1985) and their depletion suppresses further PAF-induced bronchoconstriction (Vargaftig et al., 1980). The fact that PAF operates through a guanine nucleotide regulatory protein distinct from the G_i protein (Houslay et al., 1986; Hwang & Lam, 1986) may explain why pertussis toxin treatment did not affect PAF-induced lung platelet sequestration in our experiments. The preserved platelet activation *in vivo*, under these conditions, is consistent with the fact that bronchoconstriction requires intact circulating platelets (Vargaftig et al., 1980).

A similar difference between fMLP and PAF was noted for the increased trans-endothelial albumin exchanges and for the decreased lung blood volume, since in both instances, pertussis toxin inhibited the effects of fMLP, but not those of PAF. The inhibition by pertussis toxin of fMLP-induced leucocyte sequestration and increased vasopermeability is consistent with the hypothesis that both events are closely related (Wedmore & Williams, 1981). Nevertheless, we did not confirm such a correlation with PAF-induced vasopermeation since it was not affected by pertussis toxin, under conditions where leucopenia was partially reduced. This result is consistent with the fact that PAF-induced leucocyte activation is inhibited by pertussis toxin (Lad et al., 1985). PAF-induced lung platelet sequestration was refractory to pertussis toxin and, accordingly, platelets might mediate the PAF-induced increase of vasopermeability. However, platelet activation and PAF-induced increase of vasopermeability are probably independent events (Morley et al., 1983), since neither platelet depletion nor reserpine-induced 5-hydroxytryptamine depletion interfered with PAF-induced plasma leakage (Braquet et al., 1984). In summary, PAF-induced vasopermeation is independent from leucocyte and from platelet activation and is mediated by G proteins resistant to pertussis toxin treatment. A likely target is the endothelium itself, as proposed by Morley et al. (1983) for the PAF-induced cutaneous oedema in the guinea-pig and by Björk & Smedegard (1983) for the hamster cheek pouch preparation, even though a direct effect of PAF on lung endothelial permeability has not been shown (Bolin et al., 1987).

The PAF-induced pertussis toxin-resistent lung leucocyte sequestration may not result from direct leucocyte activation, but involve their passive entrapment in platelet aggregates or their activation by other cells. Nevertheless, since PAF-induced intrathoracic sequestration of neutrophils is preserved under anti-platelet serum or iloprost pretreatment (Hultkvist-Bengtsson et al., 1991), a role for their indirect recruitment via platelet activation is unlikely.

In conclusion, our results indicate that all studied effects of fMLP are pertussis toxin-sensitive and G-protein-dependent, probably G_i-protein, whereas those of PAF are only partially so.

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Endothelin-1 does not mediate hypoxic vasoconstriction in canine isolated blood vessels: effect of BQ-123

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1 The role of endothelin-1 in mediating the phenomenon of hypoxic vasoconstriction was examined in canine, isolated pulmonary, circumflex coronary and femoral arterial rings.

2 In tissues with an intact endothelium, the exogenous application of endothelin-1 (0.1–300 nM) caused concentration-dependent increases in canine, isolated pulmonary artery tone. Endothelin-3 (1–300 nM) was approximately 30 fold less potent than endothelin-1 as a vasoconstrictor in this tissue. In contrast, the selective ET_B-receptor agonist, sarafotoxin S6c (0.01–1 μM), failed to elicit vasoconstriction in this tissue. Thus, endothelin isopeptide-induced vasoconstriction of the canine isolated pulmonary artery is mediated exclusively by the ET_A-receptor subtype.

3 The concentration-dependent increases in isometric tension induced by endothelin-1 (0.1–300 nM) were antagonized by the ET_A-selective antagonist, BQ-123 (10 μM); this concentration of antagonist caused a shift to the right in the concentration-response curve for endothelin-1 of approximately two orders of magnitude. This concentration of BQ-123 did not unmask any ET_B-receptor-mediated vasoconstriction since sarafotoxin S6c (0.01–1 μM) still failed to elicit contraction in the presence of this concentration of BQ-123.

4 The hypoxia-induced vasoconstriction of canine, isolated pulmonary, circumflex coronary and femoral arterial rings was unaffected by pretreatment with the endothelin receptor antagonist, BQ-123 (10 μM), a concentration shown previously to antagonize the contractile actions of exogenously applied endothelin-1 in the isolated pulmonary artery.

5 These results are the first to provide direct evidence showing that the endothelium-dependent vasoconstriction observed during acute periods of hypoxia *in vitro* is not mediated by an endothelin-related isopeptide.

Keywords: Endothelin-1; BQ-123; EDCF; hypoxic vasoconstriction; endothelium; canine pulmonary artery; canine coronary artery; canine femoral artery

Introduction

The characterization of endothelium-derived relaxing factors such as prostacyclin and nitric oxide has illustrated the critical role of the intimal layer of blood vessels in the regulation of vascular smooth muscle tonus. Until quite recently, however, less attention has been focussed on the possible release of endothelium-derived vasoconstrictor substances. Amongst the first indications that the endothelium produced such factors was the observation that hypoxia-induced contraction of porcine, isolated pulmonary arteries was attenuated by endothelial denudation (Holden & McCall, 1984). Subsequently, Rubanyi & Vanhoutte (1985) showed that canine coronary endothelium released a diffusible, non-eicosanoid, contractile substance, termed endothelium-derived contracting factor (EDCF), when subjected to anoxia. Following the isolation of endothelin-1 from cultured porcine aortic endothelial cells (Yanagisawa *et al.*, 1988) it was suggested that this peptide might be responsible for mediating such a response, thus, raising the possibility that it played an important role in controlling organ blood flow during acute periods of ischaemia. Further support for this this hypothesis comes from the findings that hypoxia enhances endothelin-1 release (Hieda & Gomez-Sanchez, 1990) and augments vascular smooth muscle reactivity to this peptide (Douglas *et al.*, 1991). Nevertheless, in view of such differences as the susceptibility to wash out, the role of this peptide in mediating hypoxic vasoconstriction remains controversial (Vanhoutte *et al.*, 1989). However, with the recent development of the specific endothelin antagonist, BQ-123 (Ihara *et al.*, 1991; Douglas *et al.*, 1992a; Nakamichi *et al.*, 1992; Ohlstein *et al.*, 1992), it is now possible to examine directly the role of endothelin-1 in mediating this phenomenon.

A preliminary account of some of the data presented in this paper was given to the American Heart Association (Douglas *et al.*, 1992b).

Methods

Pulmonary, circumflex coronary and femoral arterial rings (5 mm) were isolated from male mongrel dogs (10–15 kg) following barbiturate overdose (65 mg kg^{-1}, i.v. sodium pentobarbitone; Steris Laboratories Inc., Phoenix, AZ, U.S.A). Vessels were suspended in 10 ml organ baths under 4 g resting tension and bathed in Krebs solution of the following composition (mM): NaCl 112.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, dextrose 11.0 and EDTA 0.03. Normoxic Krebs (37 ± 1°C) was gassed with 95% O_2:5% CO_2; tissues were made hypoxic by bubbling this solution with 95% N_2:5% CO_2. In order to avoid contamination of the hypoxic Krebs solution with atmospheric O_2, the surface of the Krebs solution was exposed to 95% N_2:5% CO_2. Variations in isometric tension were recorded with a Grass FT03 force-displacement transducer (Grass Instrument Co., Quincy, MA, U.S.A.) linked to a Beckman Model R-611 Dynograph (Beckman Electronic Instruments Division, Schiller Park, IL, U.S.A.). The functional integrity of the endothelium was assessed by examining the ability of 1 μM acetylcholine (chloride; Sigma Chemical Co., St Louis, MO, U.S.A.) to reverse tone induced in the vessels by 1 μM noradrenaline (bitartrate; Sigma). Since noradrenaline fails to elicit reproducible vasoconstriction in the canine coronary

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artery, possibly due to concomitant endothelium-dependent (α_2-adrenoceptor-mediated EDRF release) and endothelium-independent (β_2-adrenoceptor-mediated smooth muscle relaxation) vasodilator activity, the selective α_1-adrenoceptor agonist, phenylephrine, was used to induce tone in this vessel (1 μM, hydrochloride; Sigma). Tissues which responded with a relaxant response to acetylcholine of $<50\%$ were discarded from the study. After 30 min equilibration, tissues were constricted with either 1 μM noradrenaline or phenylephrine. Once the ensuing contraction had reached a plateau, the normoxic Krebs was gassed with 95% N_2:5% CO_2. The hypoxic contraction was allowed to reach a maximum, after which tissues were washed and returned to normoxic Krebs solution. After establishing this control response, tissues were then exposed to either 10 μM BQ-123 (cyclo-[D-Asp-L-Pro-D-Val-L-Leu-D-Trp]; synthesized by SmithKline Beecham, King of Prussia, U.S.A.) or an equivalent volume of vehicle (5% w/v Na_2CO_3) and 30 min later a second hypoxic vasoconstriction was induced. The P_{O_2} and P_{CO_2} were monitored with a Radiometer Copenhagen ABL 330 blood gas analyser.

In order to characterize the receptor subtype(s) responsible for mediating endothelin-1-induced vasoconstriction in the canine pulmonary artery, an initial series of experiments were performed where concentration-response curves were constructed to endothelin-1 (0.1–300 nM), endothelin-3 (0.1–300 nM) and sarafotoxin S6c (0.01–1 μM) in isolated, endothelium-intact pulmonary arterial rings (peptides were from American Peptide Co., Inc., Santa Clara, CA, U.S.A.). The effect of 30 min pretreatment with 10 μM BQ-123 on the contractile responses to endothelin-1 (0.1–300 nM) and sarafotoxin S6c (0.01–1 μM) were also assessed to demonstrate that this concentration of antagonist was sufficient to attenuate endothelin isopeptide-mediated vasoconstriction.

Values are expressed as the mean ± s.e.mean and n represents the number of animals studied. Statistical comparisons were made by paired Student's t test and differences were considered significant where $P<0.05$.

Results

Endothelin-1 (0.1–300 nM; $n=10$) produced concentration-dependent vasoconstriction of canine, isolated pulmonary arterial rings (Figure 1a). Endothelin-3 (0.1–300 nM; $n=3$), however, was approximately 30 times less potent as a vasoconstrictor in this tissue, relative to endothelin-1 (Figure

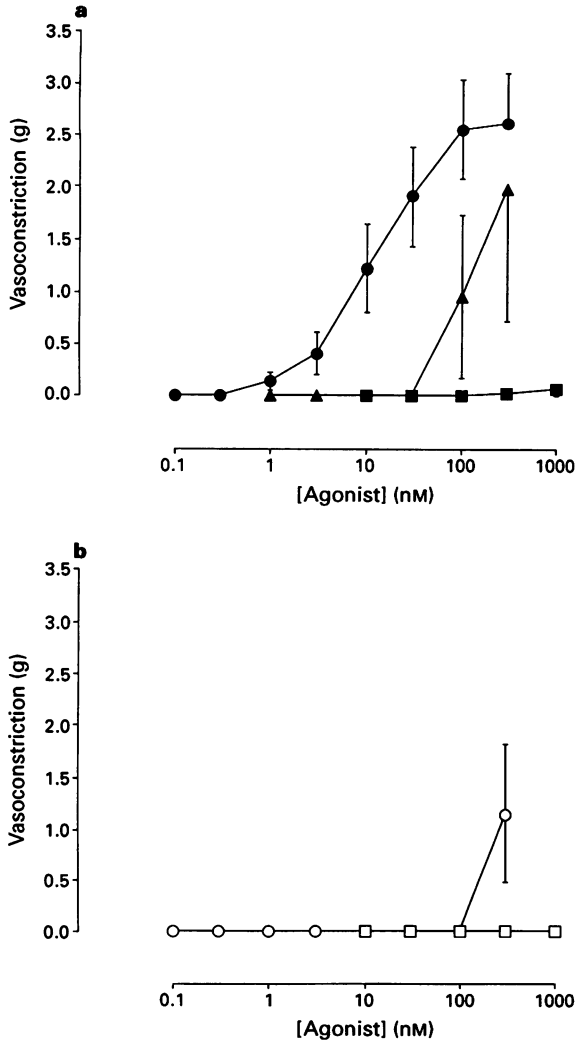

Figure 1 (a) Concentration-dependent vasoconstrictor responses to endothelin-1 (●, 0.1–300 nM; $n=10$), endothelin-3 (▲, 0.1–300 nM; $n=3$) but not sarafotoxin S6c (■, 0.01–1 μM; $n=10$) in endothelium-intact canine, isolated pulmonary arterial rings in the absence of BQ-123. (b) Concentration-dependent responses to endothelin-1 (○, 0.1–300 nM; $n=4$) but not sarafotoxin S6c (□, 0.01–1 μM; $n=2$) in endothelium-intact canine, isolated pulmonary arterial rings in the presence of 10 μM BQ-123.

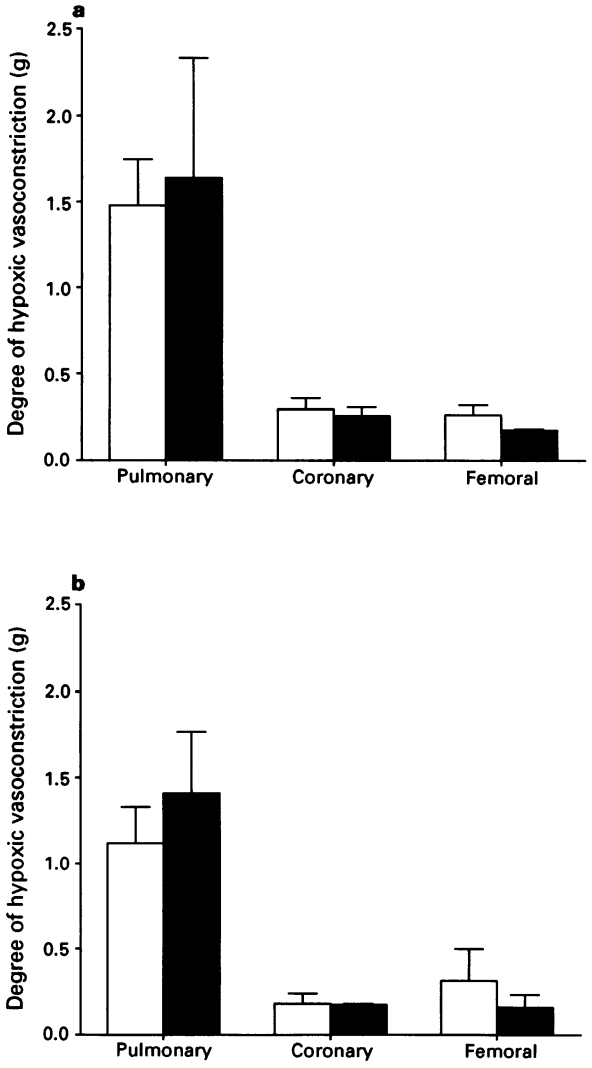

Figure 2 Degree of hypoxic vasoconstriction observed in pulmonary ($n=3$), coronary ($n=4$) and femoral ($n=3$) arterial rings under control conditions or following 30 min pretreatment with (a) antagonist vehicle (5% Na_2CO_3, w/v) or (b) 10 μM BQ-123. Open columns show the control responses and the closed columns those obtained in the same tissues following exposure to vehicle or antagonist.

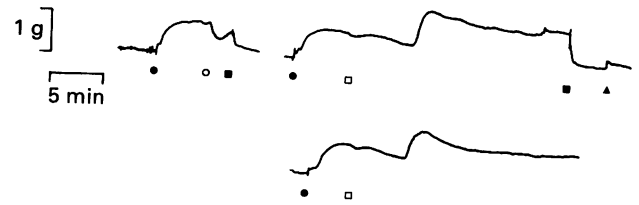

Figure 3 Representative trace demonstrating that the degree of hypoxic vasoconstriction observed in the canine, isolated pulmonary artery is unaffected by pretreatment with the endothelin antagonist BQ-123 (10 μM): (●) indicates the point at which tone was induced with 1 μM noradrenaline. The presence of a functional endothelium is confirmed by the ability of 1 μM acetycholine (○) to cause significant vasorelaxation of this induced tone. Following this procedure, the tissue was washed (■) and allowed to equilibrate a further 30 min in normoxic Krebs solution (gassed with 95% O_2:5% CO_2) before it was recontracted with noradrenaline; once this response had reached a plateau, the tissue was exposed to hypoxic Krebs (gassed with 95% N_2:5% CO_2; □). Once the ensuing control hypoxic vasoconstriction was established, the tissue was washed and exposed to 10 μM BQ-123 (▲); 30 min later is was re-exposed to the hypoxic stimulus.

1a). In contrast, the ET_B-selective agonist, sarafotoxin S6c, did not elicit contraction at the concentrations examined (0.01–1 μM; $n = 10$). Figure 1b shows that a concentration of 10 μM BQ-123 was sufficient to cause significant antagonism of the contractile actions of endothelin-1 (0.1–300 nM; $n = 4$), shifting the concentration-response curve to this peptide to the right by approximately two orders of magnitude. This concentration of antagonist failed to uncover any ET_B-mediated vasoconstriction; sarafotoxin S6c (0.01–1 μM; $n = 2$) still failed to elicit vasoconstriction in this tissue after 30 min pretreatment with 10 μM BQ-123 (Figure 1b).

The initial exposure of the isolated arterial rings to either 1 μM noradrenaline or phenylephrine caused a similar degree of vasoconstriction in all of the vessels examined; i.e. 0.92 ± 0.04 ($n = 3$), 0.70 ± 0.13 ($n = 4$) and 1.00 ± 0.17 ($n = 3$) g tension in the pulmonary, coronary and femoral arteries, respectively. Acetylcholine (1 μM) caused 68.8 ± 7.6, 89.8 ± 4.3 and 95.7 ± 4.3% reversal of this tone, demonstrating that all the tissues used possessed a functional endothelium. The partial pressures of O_2 and CO_2, [HCO_3^-] and the pH of the normoxic Krebs were 653 ± 16 mmHg, 36 ± 1 mmHg, 19 ± 1 mM and 7.35 ± 0.02, respectively ($n = 10$). Bubbling the Krebs solution with 95% N_2:5% CO_2 did not change pH (7.30 ± 0.01) or [HCO_3^-] (19 ± 1 mM) but did significantly reduce P_{O_2} (25 ± 1 mmHg; $P < 0.001$). This effect was associated with a moderate rise in P_{CO_2} (40 ± 1 mmHg; $P < 0.05$).

Pretreatment of pulmonary, coronary or femoral arterial rings with antagonist vehicle (5% w/v Na_2CO_3) did not affect the degree of vasoconstriction observed when the tissues were exposed to hypoxic Krebs solution (Figure 2a). Similarly, this contractile response was unaffected by BQ-123 (10 μM; Figure 2b and Figure 3). The phenomenon of hypoxic vasoconstriction was not observed in tissues which did not possess an intact endothelium.

Discussion

Vascular endothelin receptors are currently divided into two main functional subtypes (Masaki, 1991); the 'endothelin-1-selective', ET_A-receptor subtype (rank order or isopeptide potency: endothelin-1 = endothelin-2 = sarafotoxin S6b > endothelin-3 >> sarafotoxin S6c) and the 'non-isopeptide selective', ET_B-receptor subtype (rank order of isopeptide potency: endothelin-1 = endothelin-2 = sarafotoxin S6b = endothelin-3 = sarafotoxin S6c). Until recently, the ET_A-receptor was regarded as being the exclusive receptor subtype linked to direct vascular smooth muscle constriction. In contrast, the ET_B-receptor was believed to be responsible for mediating indirect vasodilatation through the release of endothelium-derived nitric oxide (Douglas & Hiley, 1990). However, evidence is now accumulating to suggest that 'non-ET_A-receptors', possibly ET_B-receptors, are also directly linked to vasoconstriction (Douglas *et al.*, 1992a; Fukuroda *et al.*, 1992). This is exemplified by the endothelin receptor subtype(s) which mediate isolated pulmonary artery vasoconstriction; in rat, isolated pulmonary arterial rings endothelin-1 is approximately 50 fold more potent than endothelin-3 as a vasoconstrictor (Watanabe *et al.*, 1991) suggesting that, as is also seen in porcine pulmonary vessels (Nakamichi *et al.*, 1992), this response is mediated by the ET_A-receptor subtype. In contrast, however, endothelin-1 is only an order of magnitude more potent than endothelin-3 as a vasoconstrictor in rabbit pulmonary arterial rings (Panek *et al.*, 1992). More importantly, the ET_B-selective agonist, sarafotoxin S6c, is equipotent with endothelin-1 as a vasoconstrictor in the rabbit, isolated pulmonary arterial rings indicating that in this species ET_B-receptors are capable of mediating pulmonary vasoconstriction (Panek *et al.*, 1992).

This study demonstrates that in the canine, isolated pulmonary artery the rank order of potency of exogenously applied endothelin isopeptides is endothelin-1 > endothelin-3 >> sarafotoxin S6c, indicating that the receptor responsible for mediating canine pulmonary artery vasoconstriction, like that in the pig and rat pulmonary artery, is the ET_A-receptor subtype. Since sarafotoxin S6c does not cause pulmonary artery contraction, it appears that there are no functional ET_B-receptors present in this tissue linked to vascular smooth muscle contraction. Furthermore, a concentration of 10 μM BQ-123, a selective antagonist at the ET_A-receptor subtype (Ihara *et al.*, 1991), causes a shift to the right in the concentration-contraction curve to endothelin-1 of approximately two orders of magnitude. However, the hypoxic vasoconstriction observed in the pulmonary, coronary and femoral arteries is insensitive to this concentration of BQ-123.

It has been suggested that hypoxic vasoconstriction results from a loss in agonist-stimulated/basal nitric oxide release (Archer *et al.*, 1989; Vanhoutte *et al.*, 1989; Rodman *et al.*, 1990; Adnot *et al.*, 1991). However, tissue and species variations exist; for example, nitric oxide production is not abolished during acute episodes of hypoxia in the rat, isolated mesentery since inhibition of nitric oxide synthase augments the contractile actions of endothelin-1 in both normoxic and hypoxic tissues (Douglas *et al.*, 1991). Furthermore, endothelin isopeptides (and acetylcholine) cause endothelium-dependent vasorelaxation in this preparation during similar periods of hypoxia (S.A. Douglas & C.R. Hiley; unpublished observations). Nevertheless, the results of this study are the first to provide direct evidence that the vasoconstrictor substance, or EDCF, released by hypoxia from the canine vascular endothelium is not an endothelin-related isopeptide. The precise identity of the factor(s) responsible for mediating the phenomenon of hypoxic vasoconstriction remains to be elucidated.

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Actions of agonists of metabotropic glutamate receptors on synaptic transmission and transmitter release in the olfactory cortex

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1 The effects of agonists of metabotropic glutamate receptors on the evoked N-wave complex in slices of mouse olfactory cortex have been studied: most experiments were carried out using slices perfused with Mg^{2+}-free solution to which 10 μM of either 6,7-dinitroquinoxaline-2,3-dione or 6-cyano-7-nitroquinoxaline-2,3-dione was applied.

2 Following agonist washout, a slowly developing, long lasting potentiation of the complex occurred which was confined to the N-methyl-D-aspartate (NMDA) receptor-mediated component of the potential. The relative agonist potencies were 1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD, 5–250 μM) = quisqualate (5–50 μM) > 1RS,3RS-*cis*-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD, 25–1000 μM) > L-glutamate (0.25–2.5 mM); NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and L-aspartate were inactive.

3 Potentiation of the NMDA receptor-mediated component by 1S,3R-ACPD (0.1 mM) was non-competitively antagonised by S-(+)- but not R-(−)-2-amino-3-phosphonopropionate (AP3, 0.125 mM), equally by D-(−) and L-(+)-2-amino-4-phosphonobutyrate (0.25 mM) and also by the protein kinase C inhibitors sphingosine, (25 μM), sangivamycin (25 μM) and 5-(isoquinolinylsulphonyl)-3-methylpiperazine (50 μM).

4 In a series of input-output experiments, 1S,3R-ACPD (0.1 mM) reversibly reduced the latency to peak of the NMDA receptor-mediated component at submaximal stimulus intensities, an effect blocked by S-(+)-AP3 (0.125 mM). On agonist washout, there was an increase in the area of the NMDA receptor-mediated component over all stimulus intensities, an effect blocked by the inhibitors of protein kinase C and by S-(+)-AP3 (0.125 mM). 4-β-Phorbol-12,13-diacetate (2.5 μM) also potentiated the component, an action inhibited by protein kinase C inhibitors but not by S-(+)-AP3.

5 1S,3R-ACPD (0.1 mM) had no significant effect on postsynaptic responses evoked by NMDA, AMPA and kainate, but significantly reversed a partial antagonism of NMDA responses produced by 7-chlorokynurenate (2.5 μM).

6 The K^+-evoked release of glycine was selectively and significantly increased in the presence of 0.1 mM 1S,3R-ACPD (antagonized by 0.125 mM S-(+)-AP3) whereas following agonist washout, release of glycine fell to control levels but there was a significant increase in release of aspartate (antagonized by 25 μM sangivamycin and 0.125 mM S-(+)-AP3).

7 It is concluded that metabotropic glutamate receptors mediate (i) a reduction in the latency of the NMDA receptor-mediated component of potentials by a mechanism that is independent of protein kinase C but which may depend on increased glycine release and (ii) a long lasting increase in the total area of the potential by increasing transmitter (possibly aspartate) release by a mechanism that is protein kinase C-dependent.

Keywords: Metabotropic glutamate receptors; amino acid transmitters, NMDA receptors; olfactory cortex

Introduction

The excitatory neurotransmitter, glutamate, mediates its central effects by activation of two major classes of amino acid receptor, the co-called ionotropic and metabotropic receptors (Collingridge & Lester, 1989; Monaghan *et al.*, 1989). The ionotropic receptors, which are gated ion channels, are named after their selective agonists and include the N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtypes. In contrast, metabotropic glutamate receptors, for which 1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) is a selective agonist (Irving *et al.*, 1990; Schoepp *et al.*, 1991), are linked by guanine nucleotide binding proteins to their effectors. At least in some cases, activation of the receptors triggers the formation of the second messengers *myo*-inositol-1,4,5-trisphosphate (Challis *et al.*, 1988; Baird *et al.*, 1991) and, presumbly, diacylglycerol which, in turn, mobilises intracellular Ca^{2+} (Murphy & Miller, 1989; 1990) and activates protein kinase C (Manzoni *et al.*, 1990), respectively.

Although the functions of metabotropic glutamate receptors are poorly understood, there is growing evidence suggesting an important role in modulating neurotransmission. Presynaptically localized receptors in the hippocampus (Baskys & Malenka, 1991) and striatum (Lovinger, 1991) inhibit transmitter release when activated by 1RS,3RS-*cis*-1-aminocyclopentane-1,3,-dicarboxylic acid (ACPD). Metabotropic glutamate receptors are also found postsynaptically, for ACPD directly potentiates excitatory responses of hippocampal pyramidal cells (Desai & Conn, 1991), probably by inhibition of a Ca^{2+}-activated K^+ conductance (Stratton *et al.*, 1989; 1990). In the cerebellum, ACPD causes a transient depolarization of Purkinje cells superimposed on a long last-

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ing depression of transmission (Crepel *et al.*, 1991) and a role for metabotropic glutamate receptors in hippocampal long term potentiation has also been proposed (McGuiness *et al.*, 1991a; Otani & Ben-Ari, 1991; Zheng & Gallagher, 1992).

In spite of this growing list there are few studies which define whether the effects of metabotropic glutamate receptor activation are mediated primarily by release of intracellular Ca^{2+} or by activation of protein kinase C. Charpak *et al.* (1990) showed that inhibition of hippocampal K^+ conductances by agonists of metabotropic glutamate receptors was independent of any changes in intracellular Ca^{2+}, suggesting a primary role for protein kinase C. In contrast, the oscillatory currents evoked by activation of metabotropic receptors in *Xenopus* oocytes injected with rat brain mRNA (Sugiyama *et al.*, 1987) are typical of responses caused by inositol phosphate-evoked Ca^{2+} release; a similar mechanism may underlie the enhancement of hippocampal long term potentiation by ACPD, a phenomenon which is unaffected by co-application of the protein kinase C inhibitor, sphingosine (McGuiness *et al.*, 1991b). The study presented here was undertaken to investigate the roles and mechanism by which metabotropic glutamate receptors modulate excitatory amino acid-mediated transmission in the mouse olfactory cortex. A preliminary account of this work has been published elsewhere (Collins, 1992).

Methods

Field potential experiments

Surface slices of olfactory cortex, nominal thickness of 300 μm, were prepared from freshly killed, adult male white mice and preincubated and perfused at room temperature in a solution which was continuously gassed with 95% O_2 and 5% CO_2 and which contained (mM): NaCl 118, $NaHCO_3$ 25, D-glucose 11, $CaCl_2$ 2.5, KCl 2.1 and KH_2PO_4 0.9 (Mg^{2+}-free solution). In some experiments, $MgSO_4$ (1 mM) was present. Surface, extracellular field potentials were evoked by stimulation of the lateral olfactory tracts of slices (0.2 Hz, 100 μs, various voltages) and recorded with techniques described elsewhere (Pickles & Simmonds, 1976; Collins, 1991). At the end of each experiment, a mixture of 25 μM D-(−)-2-amino-5-phosphonopentanoate (AP5) and either 10 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was applied to block NMDA and AMPA/kainate receptor-mediated potentials, respectively. The residual waveform was subtracted from the potential under investigation using a Gould 260 digital waveform processor and the resultant potential usually quantified by measuring its total area. Agonist drug solutions were applied to the pial surface of slices at a rate of 1 drop every min for a sufficient time period for a plateau effect to be produced whereas antagonist drugs were applied throughout an experiment. Most agonist drug effects have been expressed as percentage changes in the area of potentials measured 15 min after agonist washout.

In some experiments, an analysis of the differential effects of 1S,3R-ACPD on the NMDA and AMPA/kainate receptor mediated components of field potentials was made (Collins, 1991). Recordings were made until the potentials were of constant area and peak amplitude. Drugs were then applied in the sequence: AP5 (25 μM for 6 min), drug-free solution (30–40 min for recovery), 1S,3R-ACPD (0.1 mM for 15 min), drug-free solution for 15 min, AP5 (25 μM for 6 min) followed immediately by the simultaneous application of CNQX or DNQX (10 μM for 15 min) and the NMDA and AMPA/kainate components isolated by the digital subtraction procedures described by Collins (1991). The experimental design assumed that the residual potential recorded in the presence of AP5 plus CNQX/DNQX was unchanged over the 100 min of a typical experiment; in control experiments, the area of this potential at the end of 100 min perfusion was 97.8 ± 2.8% (mean ± s.e.mean; $n = 4$) of that recorded at the beginning.

Input-output studies

Drug effects on the relationship between the stimulus input and evoked output of the NMDA receptor-mediated components of potentials were investigated in slices perfused with Mg^{2+}-free solution containing 25 μM picrotoxin and to which 10 μM CNQX was applied. Briefly, slices were stimulated with a range of voltages (see Collins & Richards, 1990 for details) and graphs plotted of (i) stimulus voltage *versus* amplitude of tract action potential, (ii) action potential amplitude *versus* area of potential and (iii) area of the potential *versus* latency to peak. The procedure was repeated 30 min later and if the graphs derived from the 2 runs could not be superimposed, the slice was discarded. The procedure was then repeated after application of 1S,3R-ACPD (15 min) or 4-β-phorbol-12,13-diacetate (PDAc, 30 min) and after drug washout for 15 min. When PDAc or sphingosine were tested, slices were perfused throughout with the respective solvents, dimethylsulphoxide (3.45 mM) and ethanol (18 mM). The effects of sangivamycin on adenosine receptors were abolished by including 0.3 mM theophylline in all solutions (Collins & Richards, 1990). The relationship between action potential amplitude and area of the NMDA receptor-mediated component of potentials was quantified by measuring the area under the curve of the graph, the upper limit of the action potential amplitude being defined by a vertical line crossed by all the curves.

Excitatory amino acid-evoked depolarizations

A series of experiments was carried out to ascertain the effect of 1S,3R-ACPD on responses evoked by single, submaximal concentrations of NMDA (50 μM), AMPA (5 μM) and kainate (50 μM). Slices were preincubated in Mg^{2+}-free solution and the d.c. potential across each slice monitored with extracellular electrodes (method of Brown & Galvan, 1979, modified by Collins & Surtees, 1986). Agonists were applied for 1 min every 30 min and depolarizations quantified by measuring peak deflections on a chart recorder. Responses were measured once they had stabilized, again at the end of a 10 min application of 1S,3R-ACPD (0.1 mM) and finally after perfusion of drug-free solution for 30 min.

Release experiments

The K^+-evoked release of endogenous aspartate, glutamate, glutamine, glycine and γ-aminobutyric acid (GABA) from cubes of olfactory cortex was monitored and assayed as described previously (Clark & Collins, 1976). Briefly, 40–50 mg wet wt. of 0.5 × 0.5 mm cubes of olfactory cortical tissues were perfused with Mg^{2+}-free solution at 35°C. Following perfusion for 15 min, 5 min samples were collected, the first 3 to monitor resting levels of amino acid release and a further 3 during which the tissue was continuously challenged with 50 mM KCl. The following protocols were used in 3 series of experiments: (1) 1S,3R-ACPD (0.1 mM) with or without S-(+)-2-amino-3-phosphonopropionate (S-(+)-AP3, 0.125 mM) present during the 15 min prior to sample collection; (2) 1S,3R-ACPD (0.1 mM) with or without S-(+)-AP3 (0.125 mM) present throughout the K^+ challenge; (3) S-(+)-AP3 (0.125 mM) alone present throughout the K^+; challenge. The amino acid contents of 5 μl aloquots of the samples were estimated by a double isotope microdansylation assay, full details of which are given by Clark & Collins (1976). The mean amino acid content of the 3 pre-K^+ samples was subtracted from each of the contents of the K^+ challenged samples and the differences summed to give the total increase in release.

Drugs and chemicals

Sphingosine, 5-(isoquinolinylsulphonyl)-3-methylpiperazine, AMPA, NMDA, kainate and PDAc were purchased from Sigma whilst ACPD, 1S,3R-ACPD, DNQX, CNQX, D-(−)- and L-(+)-2-amino-4-phosphonobutyrate and S-(+)- and R-(−)-AP3 were from Tocris. Sangivamycin was a gift from the Natural Products Branch, National Cancer Institute, U.S.A. Sphingosine was dissolved in absolute ethanol, DNQX and CNQX in dimethylsulphoxide and PDAc in 25% v/v dimethylsulphoxide and diluted with the perfusion medium to give the working solutions; appropriate solvent controls were carried out as necessary. All other drugs were directly dissolved in the perfusion solution. Radiochemicals used in the assay for endogenous amino acid release were purchased from Amersham International plc and included L-[2,3-^{3}H]-aspartic acid (707 TBq mmol^{-1}), L-[G-^{3}H]-glutamic acid (1.85 TBq mmol^{-1}), L-[G-^{3}H]-glutamine (1.59 TBq mmol^{-1}), L-[G-^{3}H]-glycine (610 GBq mmol^{-1}), 4-amino-*n*-[2,3-^{3}H]-butyric acid (2.26 TBq mmol^{-1}) and [N-methyl-^{14}C]-dansyl chloride (4.12 GBq mmol^{-1}).

Data analysis

All data are presented as means ± s.e.mean for n experiments. In the agonist concentration-effect studies, EC_{25} values (agonist concentration causing a 25% increase in the area of the NMDA receptor-mediated component of the N-wave complex) were determined by non-linear regression analysis and comparison of the values was carried out by analysis of variance followed by Dunnett's t test. Comparison of other mean values was carried out using either a paired or unpaired Student t test as appropriate. The significance level was set at $P<0.05$.

Results

Supramaximal stimulation of the lateral olfactory tract of slices perfused with Mg^{2+}-containing solution evoked a characteristic surface potential, the N-wave complex. When components insensitive to AP5 and DNQX/CNQX were subtracted, the complex consisted of a short latency peak, which reflected monosynaptic excitation of a population of pyramidal cells (Gilbey & Wooster, 1979; Haberly, 1985) and which was largely mediated by AMPA/kainate receptors (Figure 1a and Collins, 1991) followed by a longer duration, low amplitude potential which reflected a disynaptic excitation of other pyramidal cells (Haberly, 1985) and to which NMDA receptors made a major contribution (Figure 1a). In many slices, a positive-going population spike was superimposed on the complex (Figure 1a and Pickles & Simmonds, 1978). Polysynaptic events were abolished by the stimulus parameters employed (Collins, 1991).

During application of neither ACPD (0.5 mM, 5 slices) nor 1S,3R-ACPD (0.1 mM, 5 slices) was there any consistent change in the form of the complex. Following washout, there was a progressive increase in the total area of the complex which required 15 min fully to develop and was confined to the NMDA receptor-mediated component (Figure 1a). In slices perfused with Mg^{2+}-free solution, the NMDA receptor-mediated component was augmented, presumably due to relief of the Mg^{2+} block of the ion channel (Nowak *et al.*, 1984; Mayer & Westbrook, 1987). Both ACPD (0.5 mM, 4 slices) and 1S,3R-ACPD (0.1 mM, 5 slices) selectively enhanced the NMDA receptor-mediated component although, as before, this only occurred following drug washout (Figure 1b). The maximum increase was achieved only with a drug contact time of 15 min followed by application of drug-free solution for a further 15 min although the effect persisted for at least 1 h (not shown). Because of these findings, agonist contact and washout times of 15 min were used in most subsequent experiments.

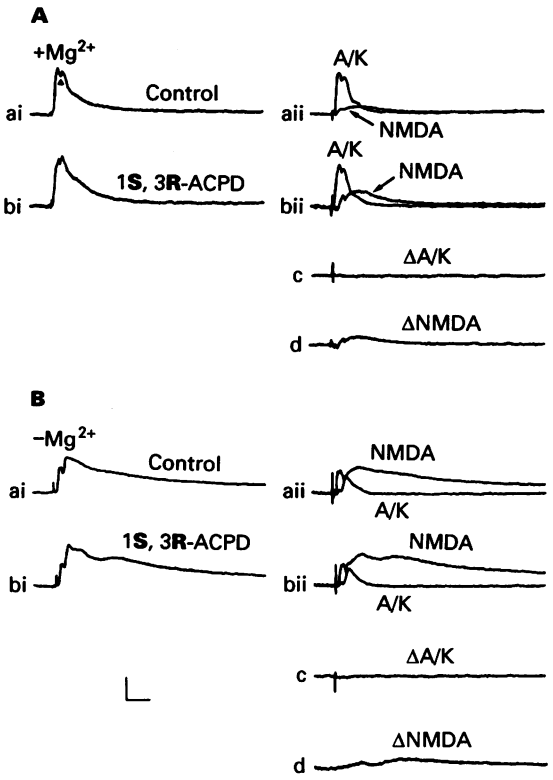

Figure 1 1S,3R-1-aminocyclopentane-1,3-dicarboxylate (1S,3R-ACPD; 0.1 mM) selectively enhances the N-methyl-D-aspartate (NMDA) receptor-mediated components of potentials evoked on supramaximal stimulation of the lateral olfactory tract of olfactory cortical slices. The results are from one slice perfused with solution containing 1 mM Mg^{2+} (A) and a second with Mg^{2+}-free solution (B). Each tracing is an average of 4 sweeps. Tracings labelled (ai) (Control) illustrate the pre-drug controls (solid arrow head indicates population spike) whilst (bi) (1S,3R-ACPD) shows the potentials evoked at the end of a 15 min washout period following application of 1S,3R-ACPD for 15 min. The NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate/kainate (A/K) components of the control and post 1S,3R-ACPD potentials are shown in (aii) and (bii). Subtraction of the individual components in (aii) from those in (bii) gives the 1S,3R-ACPD induced changes in the A/K (c; ΔA/K) and NMDA (d; ΔNMDA) components of the potential. Calibration bars 0.4 mV, 20 ms.

The ability of various excitatory amino acids to potentiate the NMDA receptor-mediated components of the N-wave complex was studied in slices perfused with Mg^{2+}-free solution and to which 10 μM DNQX or CNQX was applied (Figure 2). The relative potencies (μM concentrations producing a 25% increase in the area of the potential in parentheses) were 1S,3R-ACPD (8.0 ± 1.6, $n=4$) = quisqualate (11.2 ± 2.7, $n=7$) > ACPD (82.3 ± 7.7, $n=6$) > L-glutamate (1120 ± 207, $n=4$) and the corresponding maximum percentage increases in the area of the component were 65.1 ± 4.2, 38.2 ± 3.8, 44.2 ± 3.7 and 26.9 ± 1.9, respectively (means ± s.e.mean). Note that kainate, NMDA and L-aspartate were inactive. The presence or otherwise of Mg^{2+}, DNQX or picrotoxin had no significant effect on the action of ACPD (Table 1; 1S,3R-ACPD not tested). The increase caused by 1S,3R-ACPD occurred equally at short and long latencies and was unaffected by co-application of glycine (50 μM; Table 1). 1S,3R-ACPD applied to 4 slices perfused with AP5 (25 μM), followed by washout of both drugs, caused a 56.7 ± 6.9% increase in the area of the potential, the μM concentration causing a 25% increase being 9.7 ± 1.1 (means ± s.e.means). Finally, the effects of repeated applications of a single concentration of 10 μM 1S,3R-ACPD were not additive; the increase in area following washout of the first dose (28.6 ± 3.6%) was not significantly different following

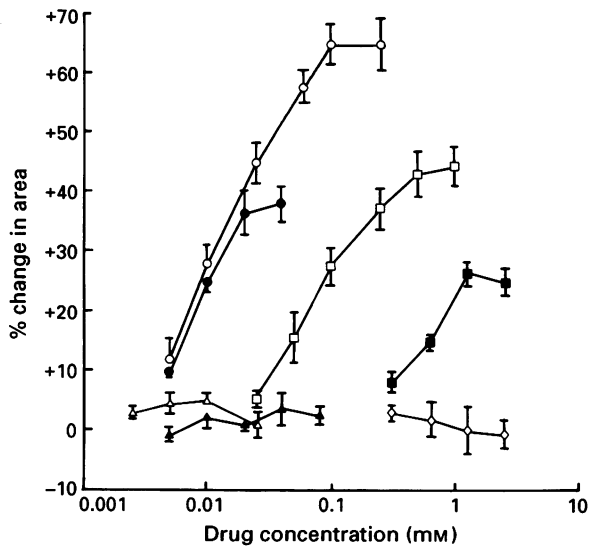

Figure 2 Concentration-effect curves for 1S,3R-1-aminocyclopentane-1,3-dicarboxylate (1S,3R-ACPD, ○), quisqualate (Q, ●), 1RS,3RS-1-aminocyclopentane-1,3-dicarboxylate (ACPD, □), L-glutamate (Glu, ■), N-methyl-D-aspartate (NMDA, △), α-amino-3-hydroxy-5-methyl-4-ioxazolepropionate (AMPA, ▲) and L-aspartate (Asp, ◇) showing the percentage increase in the area of the NMDA receptor mediated component of potentials evoked in slices perfused with Mg^{2+}-free solution and to which 10 μM 6,7-dinitroquinoxaline-2,3-dione was applied throughout. The vertical lines represent the s.e.mean and the points are the means of 5–8 values.

the second (27.4 ± 2.7%; means ± s.e.means, $n = 4$).

Of the potential antagonists tested, RS-(+)-AP3 antagonized the effects of ACPD and quisqualate (Figure 3a, b) on the NMDA receptor-mediated component of potentials and 0.125 mM S-(+)-AP3 antagonized the effect of 1S,3R-ACPD, the R-(−)-isomer being essentially inactive (Figure 3c). S-(+)-AP3 alone had no effect on the potential and was ineffective if applied after the effect of 1S,3R-ACPD had developed (4 slices, not shown). In contrast, AP4 caused a reversible reduction in the amplitude and area of the potential (see Anson & Collins, 1987) and its enantiomers were equipotent antagonists of the effects of 1S,3R-ACPD (Figure 3d). The protein kinase C inhibitors, 5-(isoquinolinylsulphonyl)-3-methylpiperazine, sangivamycin and sphingosine (Hidaka *et al.*, 1984; Loomis & Bell, 1988), all blocked the effect of 1S,3R-ACPD on the NMDA receptor-mediated component of the N-wave complex (Table 1).

Input-output experiments

Application of 0.1 mM 1S,3R-ACPD had no effect on the stimulus voltage-tract action potential relationship (5 slices, not shown) but increased the area under the graph of action potential *versus* area of potential (Figure 4a); this effect was only observed following washout of the 1S,3R-ACPD and was significantly antagonized by S-(+)-AP3 and the 3 protein kinase C inhibitors (Table 2). In the presence of 1S,3R-ACPD, the latency to peak of the NMDA receptor-mediated component was reduced at submaximal stimulus intensities, an effect readily reversed on washout (Figure 4c), mimicked by 0.5 mM D-serine (4 slices, not shown) but not antagonized by sangivamycin or 5-(isoquinolinylsulphonyl)-3-methylpiperazine (each tested on 4 slices at 25 μM, not shown). PDAc (2.5 μM) increased the area of the potential for a given amplitude of tract action potential, an effect blocked by protein kinase C inhibitors (Table 2) but not S-(+)-AP3. PDAc had no effect on the latency to peak of the potential (Figure 4d).

Excitatory amino acid-evoked depolarizations

Application of 1S,3R-ACPD alone (0.1 mM) did not evoke a cellular depolarization (6 slices) neither did it significantly affect the responses evoked by NMDA, AMPA and kainate (4 slices each, not shown). 7-Chlorokynurenate (2.5 μM) reduced the response to NMDA to 16.4 ± 2.9% of control whereas during co-perfusion of 1S,3R-ACPD (0.1 mM) responses to NMDA were reduced to only 40.7 ± 3.7% (means ± s.e.mean, $n = 8$; significant difference, $P < 0.05$).

K^+-evoked release of amino acids

Exposure of olfactory cortical slices to K^+ (50 mM) significantly increased release of aspartate and glutamate ($P < 0.05$) which was largely Ca^{2+}-dependent (Table 3). Co-perfusion with S-(+)-AP3 (0.125 mM) significantly reduced release of glycine only. 1S,3R-ACPD in the presence of K^+ caused a highly significant ($P < 0.001$) potentiation of glycine release and a smaller increase in aspartate release. Addition of S-(+)-AP3 significantly reduced the effect of 1S,3R-ACPD on glycine release. In experiments in which the K^+ challenge was given following washout of 1S,3R-ACPD, aspartate release was significantly increased ($P < 0.05$) whereas glycine release had returned to control levels; the presence of S-(+)-AP3 throughout completely prevented the increase in aspartate release caused by 1S,3R-ACPD. Sangivamycin (25 μM) had no effect on the potentiation of glycine release by 1S,3R-ACPD but significantly reduced the increase in release of aspartate (Table 3).

Discussion

1S,3R-ACPD had two distinct effects on the NMDA receptor-mediated component of the N-wave complex. First, it caused a long lasting increase in the area of the potential. That the effect was blocked by S-(+)-AP3, the active emantiomer in antagonizing metabotropic receptors (Irving *et al.*, 1990; Schoepp *et al.*, 1990a) and that the potencies of the agonists tested mirrored their potencies in stimulating phosphoinositide turnover (Sladeczek *et al.*, 1985; 1988; Schoepp *et al.*, 1990b) strongly suggests that the effect was mediated by metabotropic glutamate receptors. The potentiation was also antagonized by AP4, another potential antagonist of metabotropic glutamate receptors (Schoepp *et al.*, 1990b). However, unlike AP3, AP4 reversibly reduces excitatory transmission in the olfactory cortex (Anson & Collins, 1987) so that antagonism of the effects of 1S,3R-ACPD by AP4 cannot be used as a diagnostic test of a role for metabotropic receptors. The second effect was to reduce the latency to peak of the potential. This action occurred only at submaximal stimulus intensities, was readily reversible on drug washout and was also antagonized by S-(+)-AP3, again suggesting a role for metabotropic glutamate receptors. In addition to these electrophysiological actions, the K^+-evoked release of endogenous glycine was increased in the presence of 1S,3R-ACPD whereas following washout, there was a selective increase in aspartate release. Both these effects were also sensitive to S-(+)-AP3.

The results provide some evidence of the mechanisms by which 1S,3R-ACPD might increase the area of the potential. Activation of the NMDA receptor complex *per se* was unnecessary, for NMDA itself did not affect the potential and 1S,3R-ACPD potentiated the area of the potential even when applied to slices perfused with AP5. The inability of picrotoxin to block the effects of ACPD suggests that changes in GABA-mediated inhibition were not involved (Desai & Conn, 1991). One possibility is that the glycine released by the 1S,3R-ACPD could displace any DNQX/CNQX which might antagonize the strychnine-insensitive glycine site of the NMDA receptor complex (Johnson & Ascher, 1987; Birch *et al.*, 1988). This would seem an

Table 1 Some characteristics of the effect of 1**RS**, 3**RS**-*cis*-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) and 1S, 3R-ACPD on the NMDA receptor-mediated component of evoked potentials

Agonist	*Experimental conditions*	*n*	*Drug*	*Concentration which increased area by 25% (μM)*[a]	*P*	*Maximum percentage increase in area*[a]	*P*
ACPD	$-Mg^{2+}$, + DNQX	6	–	82.3 ± 7.5	–	44.2 ± 3.7	–
(25 to 1000 μM)	$-Mg^{2+}$, + DNQX	4	–	96.5 ± 10.5	NS[b]	40.5 ± 7.2	NS[b]
	$-Mg^{2+}$, – DNQX	4	–	81.7 ± 6.7	NS[b]	38.6 ± 3.6	NS[b]
	$-Mg^{2+}$, + DNQX	4	Picrotoxin	80.4 ± 6.4	NS[b]	32.7 ± 6.4	NS[b]
1S,3R-ACPD	$-Mg^{2+}$, + CNQX	4	–				
(5–250 μM)	total			8.0 ± 1.6	<0.001[b]	65.1 ± 4.2	<0.05[b]
	0–40 ms			7.2 ± 1.4	NS[c]	67.0 ± 2.9	NS[c]
	40–450 ms			10.1 ± 2.7	NS[c]	66.1 ± 6.3	NS[c]
	$-Mg^{2+}$, + CNQX	4	Glycine	10.2 ± 1.8	NS[c]	58.2 ± 6.6	NS[c]
	$-Mg^{2+}$, + CNQX	4	Sangivamycin	>250	–	19.3 ± 1.2	<0.001
	$-Mg^{2+}$, + CNQX	5	5-Isoquinolinyl-sulphonyl)-3-methyl-piperazine	>250	–	13.6 ± 2.6	<0.001
	$-Mg^{2+}$, + CNQX + ethanol	4	–	12.3 ± 2.8	NS[c]	44.2 ± 6.8	NS[c]
	$-Mg^{2+}$, + CNQX + ethanol	4	Sphingosine	>250	–	12.0 ± 0.24	<0.005[d]

When present, Mg^{2+} was at a concentration of 1 mM, CNQX/DNQX 10 μM, picrotoxin 25 μM, glycine 50 μM, sangivamycin 25 μM, 5-isoquinolinylsulphonyl-3-methylpiperazine 50 μM, and sphingosine 25 μM.

[a]Measured 15 min after agonist washout (see Methods).

[b]Compared to value for ACPD ($-Mg^{2+}$, + DNQX).

[c]Compared to value for the effect of 1S,3R-ACPD ($-Mg^{2+}$, + CNQX) on the total potential.

[d]Compared to ethanol control.

All values are means ± s.e.means. Statistical analysis was performed using Dunnett's *t* test. NS, not significant.

unlikely basis for the potentiation in that; (i) potentiation by 1S,3R-ACPD occurred on co-application of exogenous glycine and in the absence of DNQX, (ii) release of endogenous glycine only occurred *during* application of 1S,3R-ACPD whereas potentiation of the potential was maintained at times *after* glycine release had returned to control levels and (iii) the effects of 1S,3R-ACPD on the potential, but not on glycine release, were sensitive to inhibitors of protein kinase C. In contrast, the reduction in the latency to peak of the NMDA receptor-mediated component of the potential seems to have a fundamentally different mechanism in that the effect was recorded only in the presence of the agonist and was not antagonized by inhibitors of protein kinase C or mimicked by PDAc, an activator of the enzyme (Castagna *et al.*, 1982); indeed, its time course and pharmacology closely paralleled those of the

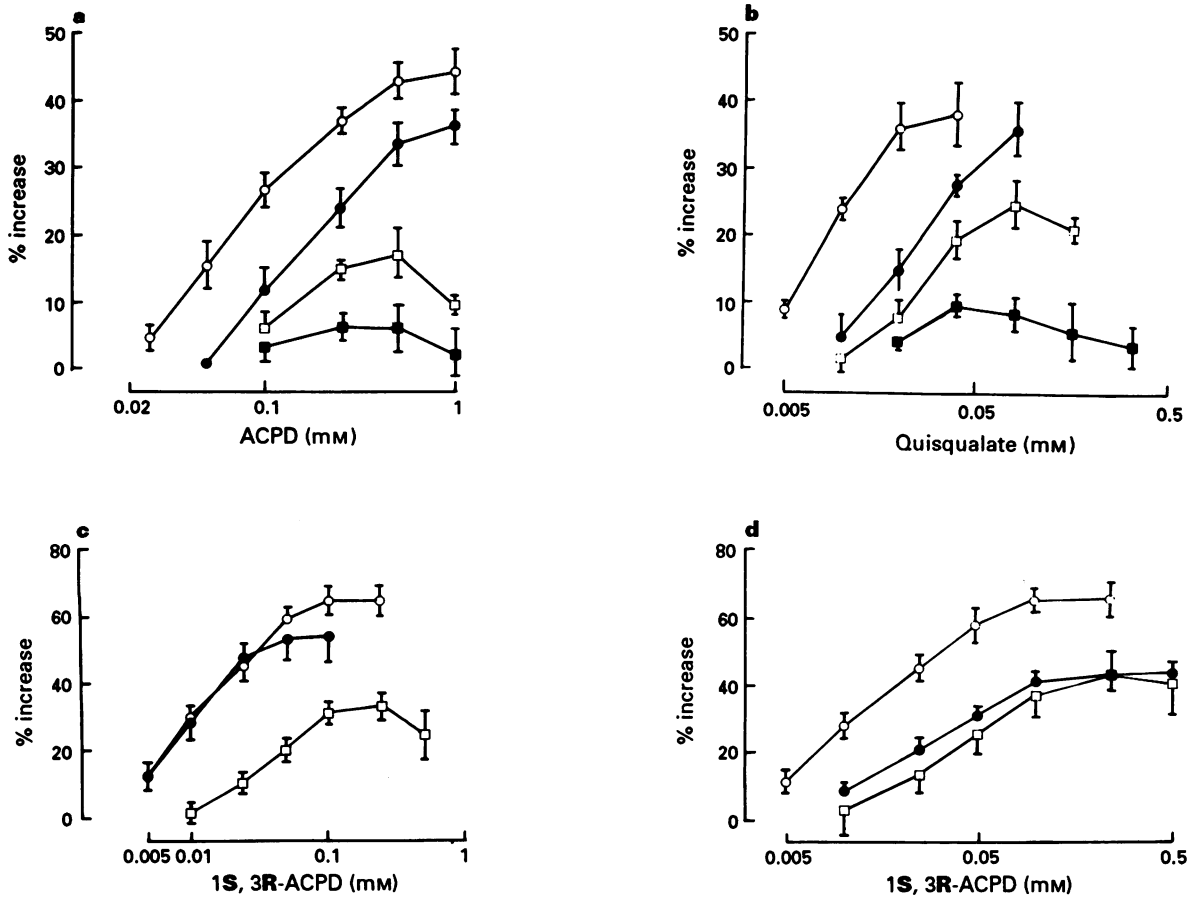

Figure 3 Effects of 2-amino-3-phosphonopropionate (AP3) and 2-amino-4-phosphonobutyrate (AP4) on the increase in the area of the N-methyl-D-aspartate receptor-mediated component of slices perfused in Mg^{2+}-free solution and to which 10 μM of either 6,7-dinitroquinoxaline-2,3-dione (a,b) or 6-cyano-7-nitroquinoxaline-2,3,-dione (c,d) was applied. Increasing concentrations of RS-(+)-AP3 (0.5 mM, ●; 1 mM, □; 2 mM, ■) antagonize the effects of (a) 1RS,3RS-aminocyclopentane-1,3-dicarboxylate (ACPD) and (b) quisqualate. (c) Effects of 0.125 mM R-(−)- (●) and S-(+)- (□) AP3 on the concentration effect curve to 1S,3R-1-aminocyclopentane-1,3-dicarboxylate (1S,3R-ACPD; ○). (d) Effects of 0.25 mM D-(−) (●) and L-(+)- (□) AP4 on the concentration-effect curve to 1S,3R-ACPD (○). The vertical lines represent the s.e.mean and the points the mean of 4–7 values.

Table 2 Drug effects on the increase in the area of NMDA receptor-mediated potential by 1S,3R-1-amino-cyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) and 4-β-phorbol-12,13-diacetate (PDAc) in a series of input-output experiments

	% increase in area under the curve[a]					
Drug	*1S,3R-ACPD* (0.1 mM)	n	P	*PDAc* (2.5 μM)	n	P
None (control)	58.4 ± 6.2	5	–	50.7 ± 5.2	5	–
S-(+)-AP3 (0.125 mM)	26.4 ± 1.9	5	<0.05	54.1 ± 6.1	5	NS
Sangivamycin (25 μM)	18.2 ± 3.6	5	<0.05	26.4 ± 3.8	5	<0.05
1-(5-Isoquinolinylsulphonyl)-3-methylpiperazine (50 μM)	29.7 ± 5.2	5	<0.05	38.0 ± 3.4	5	<0.05
Sphingosine solvent control	47.2 ± 4.9	4	–	46.4 ± 6.8	4	–
Sphingosine (25 μM)	16.0 ± 2.7	4	<0.01	24.2 ± 3.9	4	<0.05

All experiments were carried out using slices perfused with Mg^{2+}-free solution and to which CNQX (10 μM) and picrotoxin (25 μM) was applied.

[a]Measured 15 min after washout of 1S,3R-ACPD or PDAc, as appropriate. Values are means ± s.e.means of the areas under the curve of action potential amplitude *versus* area of the NMDA receptor-mediated component. Statistical analysis was performed using an unpaired Student's *t* test, comparing each mean to the appropriate control. NS, not significant.

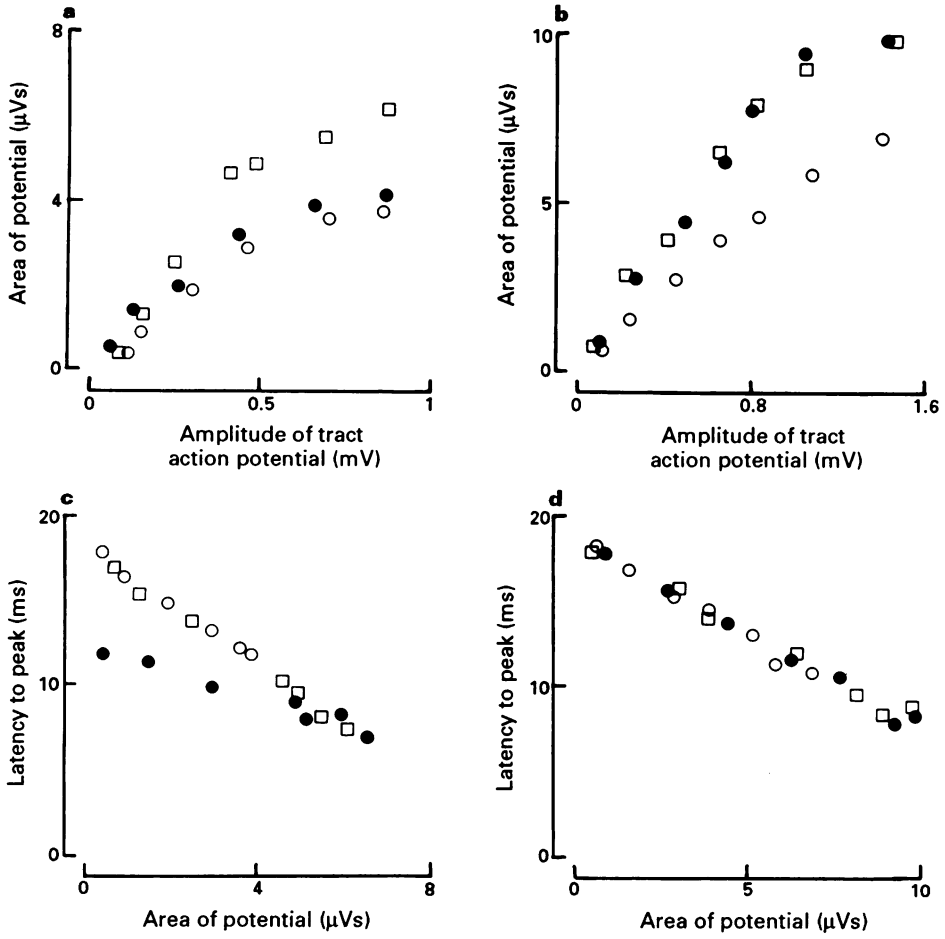

Figure 4 Effects of 0.1 mM 1S,3R-1-aminocyclopentane-1,3-dicarboxylate (1S,3R-ACPD; a and c) and 2.5 μM 4-β phorbol-12,13-diacetate (PDAc; b and d) in two stimulus input-evoked output experiments carried out in slices perfused in Mg^{2+}-free solution containing picrotoxin (25 μM) and to which 6-cyano-7-nitro-quinoxaline-2,3-dione (10 μM) was applied. Each slice was stimulated using a range of voltages before (○), during application of 1S,3R-ACPD for 15 min or PDAc for 30 min (●) and after perfusion with drug-free solution for 15 min (□). Mean results are given in Table 2.

Table 3 Effect of 1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) on the K^+-evoked release of endogenous amino acids from cubes of mouse olfactory cortex

	K^+-evoked release (pmol min^{-1} mg^{-1} wet weight)								
Experimental conditions	*Aspartate*	n	P	*Glutamate*	n	P	*Glycine*	n	P
K^+ (50 mM) control	27.2 ± 6.1	9	–	131 ± 19.7	12	–	11.4 ± 5.6	12	–
K^+ minus Ca^{2+}	6.3 ± 2.7	4	<0.05	26 ± 7.2	4	>0.05	4.8 ± 2.2	4	NS
K^+ plus S-(+)-AP3 (0.125 mM)	20.3 ± 4.6	4	NS	122 ± 13.8	6	NS	−24.6 ± 3.7	6	<0.05
K^+ in presence of 1S,3R-ACPD (0.1 mM)									
1S,3R-ACPD alone	55.4 ± 8.5	6	<0.05	126 ± 19.5	6	NS	87.6 ± 7.6	6	<0.001
plus S-(+)-AP3 (0.125 mM)	40.3 ± 7.7	5	<0.05	89.9 ± 9.4	5	NS	56.8 ± 6.2[a]	3	<0.05
plus sangivamycin (25 μM)	42.8 ± 6.2	3	NS	94.6 ± 12.1	3	NS	72.7 ± 9.4	3	NS
K^+after washout of 1S,3R-ACPD (0.1 mM)									
1S,3R-ACPD alone	53.0 ± 8.8	7	<0.05	144 ± 10.6	10	NS	1.19 ± 8	10	NS
plus S-(+)-AP3 (0.125 mM)	27.6 ± 6.6	6	NS	88.3 ± 10.2[a]	6	NS	−16.4 ± 2.4[a]	6	<0.05
plus sangivamycin (25 μM)	30.2 ± 6.1	6	NS	131 ± 12.9	6	NS	10.1 ± 3.4	6	NS

All experiments were carried out using preparations perfused with Mg^{2+}-free solution.
Values are means ± s.e.means. Statistical analyses were performed by comparing values to the control using an unpaired Student's *t* test. NS, not significant.
[a]Significantly different from 1S,3R-ACPD alone ($P<0.05$).

increase in glycine release.

It is proposed that the potentiation in the NMDA receptor-mediated component of potentials was caused by an increase in transmitter release from the terminals of the lateral olfactory tract which was triggered, and possibly maintained, by activation of protein kinase C. Evidence for a presynaptic locus is provided by the findings that; (i) 1S,3R-ACPD had no effect on the postsynaptic responses evoked by NMDA, (ii) the effects of 1S,3R-ACPD on the relationship between the tract action potential and evoked potential are best explained by an increase in transmitter release (Collins & Richards, 1990) and (iii) protein kinase C is located in the tract terminals and, when activated, increases transmitter release (Collins & Richards, 1990). It is tempting to speculate that the increased release of aspartate, a transmitter candidate of the lateral olfactory tract (Collins, 1986), plays a role in the phenomenon. The mechanisms by which 1S,3R-ACPD reduced the latency to peak of the potential is problematical for although an increase in postsynaptic excitability is consistent with the finding (see Constanti & Libri, 1992), 1S,3R-ACPD failed to potentiate postsynaptic responses to the excitants tested. It is possible that the increased levels of glycine acting on the strychnine-insensitive site on the NMDA receptor complex were involved for not only did D-serine, an agonist of the site (McBain *et al.*, 1989) mimic the effect of 1S,3R-ACPD on the latency of the potential, but 1S,3R-ACPD significantly antagonized the reduction in NMDA responses caused by 7-chlorokynurenate, an antagonist of the strychnine insensitive glycine site (Kemp *et al.*, 1988).

Metabotropic glutamate receptor-mediated increases in NMDA receptor-mediated responses have been reported by others (Aniksztejn *et al.*, 1991; Harvey *et al.*, 1991; Kinney & Slater, 1992) although increases in transmitter release were not thought to be involved. Indeed, with the exception of the present study, presynaptically located metabotropic glutamate receptors have been reported to inhibit transmitter release (Baskys & Malenka, 1991; Crepel *et al.*, 1991; Lovinger, 1991). However, both ACPD and phorbol esters broaden the action potential of hippocampal neurones (Hu & Storm, 1992), effects which are compatible with a metabotropic glutamate receptor-induced increase in transmitter release mediated by protein kinase C.

In conclusion, it is proposed that metabotropic glutamate receptors in the olfactory cortex mediate a short term increase in pyramidal cell excitability, a long term and selective increase in NMDA receptor-mediated transmission, together with temporally similar increases in the release of glycine and aspartate, respectively. The long duration effects on transmission and aspartate release are dependent on activation of protein kinase C. There is no direct evidence that the electrophysiological and neurochemical events are related, neither is it clear that the early increase in excitability is related to the longer term changes. The results to do not preclude a role for metabotropic receptor-mediated changes in phosphoinositide turnover, cyclic AMP synthesis or release of arachidonic acid (Aramori & Nakanishi, 1992). Finally, it is likely that the reported potentiation of NMDA receptor-mediated events would affect long-term potentiation (Kanter & Haberly, 1990) and hence modulate the associative memory processes of the olfactory cortex (Haberly & Bower, 1989).

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Effects of capsaicin and 5-HT_3 antagonists on 5-hydroxytryptamine-evoked release of calcitonin gene-related peptide in the guinea-pig heart

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1 The effect of 5-hydroxytryptamine (5-HT) on the release of calcitonin gene-related peptide (CGRP) was studied directly in the isolated perfused heart and indirectly in the isolated left atria of guinea-pig.

2 5-HT injection into the guinea-pig isolated and perfused heart evoked a dose-dependent (1–100 μM) release of CGRP-like immunoreactivity (LI) that was abolished by *in vitro* pretreatment with capsaicin and was not affected by indomethacin.

3 Chlorophenyldiguanide (CPD, 100 μM), but not 8-hydroxy-dipropylaminotetralin (8-OH-DPAT, 100 μM), sumatriptan (100 μM) or 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, 100 μM) evoked a release of CGRP-LI. Ondansetron (10 μM) or ICS205-930 (20 μM) completely abolished the 5-HT (100 μM)-evoked CGRP-LI release.

4 In the isolated electrically driven left atria of the guinea-pig 5-HT (1–10 μM) and CPD (3–100 μM) produced a positive inotropic response, which was abolished by capsaicin pretreatment. 8-OH-DPAT (10 μM) and DOI (10 μM) were inactive. Ondansetron inhibited the response to 5-HT with a pA_2 of 6.50 (CL 6.08–6.91).

5 It is concluded that 5-HT causes a release of CGRP in the whole heart and a positive inotropic response in the isolated atria of guinea-pig. Both these effects are sensitive to capsaicin pretreatment and to 5-HT_3 antagonists.

Keywords: 5-HT receptors; capsaicin; sensory nerves; calcitonin gene-related peptide (CGRP); guinea-pig heart

Introduction

Stimulation of sensory receptors in the heart and lungs by 5-hydroxytryptamine (5-HT) elicits reflex responses which include bradycardia, hypotension, tachypnoea and nausea. One of such reflexes, the von Bezold-Jarisch reflex, evoked mainly from cardiac receptors on C-fibre vagal afferents, results in bradycardia, hypotension and apnoea (Thoren, 1979) and, when produced by 5-HT and related drugs, involves the activation of 5-HT_3 receptors (Collins & Fortune, 1983; Richardson *et al.*, 1985). The increase in permeability to Na^+ and K^+ evoked by 5-HT in dorsal root ganglion neurones in culture is antagonized by 5-HT_3 receptor antagonists (Robertson & Bevan, 1991). Peripherally administered 5-HT_3 receptor antagonists reduce inflammatory pain (Giordano & Rogers, 1989) and 5-HT_3 receptors appear also to mediate the algesic response evoked by 5-HT applied to the blister base of human skin (Richardson *et al.*, 1985). Together, these findings support the view that 5-HT, by stimulating 5-HT_3 receptors localized on primary afferents, generates a sensory impulse, leading to reflex cardiovascular responses, nociceptive responses and pain perception.

A sub-population of peptide-containing primary afferent neurones has been distinguished for its sensitivity to capsaicin (Jancso' *et al.*, 1977; see for review Holzer, 1991; Maggi, 1991). These neurones, in addition to conveying sensory impulses to the central nervous system, release peptide transmitters, namely tachykinins and calcitonin gene-related peptide (CGRP), from their peripheral endings, thus eliciting a variety of responses in effector cells (see for review Holzer, 1991; Maggi, 1991).

A variety of autacoids have been shown to release sensory neuropeptides from the peripheral terminals of capsaicin-sensitive nerve fibres (Geppetti *et al.*, 1988b; 1991; Saria *et al.*, 1988). CGRP increases the force and rate of contraction in the guinea-pig isolated atrium and is considered the mediator of the inotropic response produced in this preparation by the activation of sensory nerves (Franco-Cereceda & Lundberg, 1985; Saito *et al.*, 1986; Sigrist *et al.*, 1986; Giuliani *et al.*, 1989). Indirect evidence suggests that in the guinea-pig isolated atria, 5-HT can release CGRP from capsaicin-sensitive sensory nerves, since the inotropic response evoked by 5-HT is reduced by *in vitro* capsaicin desensitization (Bernoussi & Rioux, 1989). Recently, release of substance P by 5-HT has been shown from dorsal root ganglion neurones in culture (Vedder & Otten, 1991). However, direct evidence that 5-HT releases sensory neuropeptides from peripheral tissues is lacking. Here, we present evidence showing that 5-HT induces a release of CGRP from the peripheral nerve endings of capsaicin-sensitive neurones, and suggest that this effect may be due to the stimulation of 5-HT_3 receptors.

Methods

Isolated and perfused heart

Male albino guinea-pigs (Dunkin-Hartley strain, 240–300 g) were used. Animals were killed by cervical dislocation and the heart rapidly removed and squeezed a few times to remove any blood in the coronary vasculature. A cannula was immediately inserted into the aorta for perfusion via the coronary arteries according to the Langendorff procedure. Hearts were perfused under constant pressure at 37°C with oxygenated (96% O_2:4% CO_2) physiological salt solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$

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2.5, $MgCl_2$ 0.54, NaH_2PO_4 1.06, $NaHCO_3$ 24.5 and glucose 10. Coronary flow was measured electronically (Marb Gx-84), by counting drops of the perfusate leaking from the perfusion apparatus. The electrocardiogram, contractile strength (monitored by connecting the apex of the heart to a force displacement transducer) and coronary flow were continuously recorded on a three channel polygraph (Basile).

Preparations were allowed to equilibrate for at least 30 min. Drug administration was by injection through a side arm of the perfusion apparatus immediately above the cannula. A volume of 1 ml of the required concentration of the drug was slowly injected over 2 min. Drug concentrations reported throughout the paper refer to those contained in the syringe. Because basal coronary flow was about 5 ml min^{-1}, drugs were diluted approximately 10 times when injected into the coronary circulation. Preliminary experiments showed that the injection of 1 ml of the physiological salt solution did not affect either the cardiac coronary parameters, or produce any detectable release of CGRP-LI. *In vitro* desensitization of capsaicin-sensitive nerves was obtained by injecting 10 μM capsaicin (0.5 ml min^{-1} over 2 min) three times at 10 min intervals. By using this protocol (P. Geppetti, unpublished observation), both functional responses and CGRP-LI release observed at the first capsaicin administration were abolished at the third exposure to the drug. Indomethacin was added to the perfusion medium 40 min before 5-HT administration. 5-HT receptor antagonists were added to the perfusion medium 30 min before the administration of 5-HT.

One min fractions were collected in plastic tubes. Acetic acid was added to 2 ml aliquots to a final concentration of 2 N, and samples were freeze-dried. Two fractions were collected immediately before, two during and eight after the administration of the stimulus. CGRP-LI values measured by radioimmunoassay were corrected to account for the volume collected in each fraction.

CGRP-LI radioimmunoassay

After reconstitution with the assay buffer (0.1 M, pH 7.4 phosphate buffer, containing 0.9% NaCl, 0.1% bovine serum albumin and 0.01% NaN_3), CGRP-LI was measured as reported previously (Geppetti *et al.*, 1988a). Briefly, rat α-CGRP standard or samples were incubated with rabbit anti-CGRP antiserum (BAS 6012) for 48 h at 4°C. The [^{125}I]-iodohistidyl CGRP was added and further incubated for 48 h at 4°C. After the addition of 1 ml of buffer containing 7.5% polyethylene glycol, 1/2000 goat anti-rabbit antiserum and 1/200 normal rabbit serum, free from bound antigen, were separated by centrifugation at 2000 *g* for 30 min at 4°C. The coefficient of variation was less than 10% for values between 20 and 300 pmol l^{-1}. The lowest detection limit was 2 fmol per tube. The antiserum fully cross-reacted with rat β-CGRP and with human CGRP-I and CGRP-II. 5-HT and any of the drug used in the study up to 100 μM did not produce significant displacement of the labelled antigen from the antiserum.

Isolated left atrium

Inotropic responses of the electrically driven guinea-pig left atrium following exposure to various agents were obtained by using preparations similar to those described previously (Saito *et al.*, 1986; Guiliani *et al.*, 1989; Geppetti *et al.*, 1990). Male albino guinea-pigs (250–300 g) received reserpine (5 mg kg^{-1}, i.p.) 48–96 h before they were stunned and bled. The whole heart was rapidly removed and placed in Tyrode solution of the following composition (mM): NaCl 137, KCl 2.68, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, glucose 5.5. The left atrium was cleaned of adhering tissues and placed in a 5 ml organ bath containing oxygenated (96% O_2:4% CO_2) Tyrode solution at 37°C in the presence of 1 μM atropine. The atrium was connected under a resting tension of 5 mN to an isometric force transducer connected to a Basile 7050 Unirecord.

The atrium was stimulated by means of two platinum wire electrodes placed at the top and the bottom of the organ bath and connected to a GRASS S88 stimulator. The atria were driven at a frequency of 3 Hz (0.5 ms pulse width, maximal voltage). The experiments started after at least 2 h had been allowed for equilibration. Each substance was administered in a cumulative manner. To obtain desensitization to capsaicin in the atrium this preparation was exposed to the drug (10 μM) for 10 min (Giuliani *et al.*, 1989).

Statistical analysis

All data in the text, tables and figures are means ± s.e.mean. Statistical analysis was performed by means of analysis of variance and the Bonferroni's procedure for multiple *t* test, Student's *t* test for paired and unpaired data and analysis of variance and Dunnett's test when applicable. Total evoked release was calculated as the sum of values observed during and after the administration of the stimulus subtracted by the mean basal value. The method of Arunlakshana & Schild (1959) was used to investigate the competitive nature of the interaction of the antagonists. pA_2 values were calculated by the constrained plot method.

Drugs and reagents

The following were used: 5-hydroxytryptamine and indomethacin (Sigma); 8-hydroxy-dipropylaminotetralin (8-OH-DPAT) and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminpropane (DOI) (RBI); *m*-chlorophenyldiguanide, sumatriptan and ondansetron (Glaxo); ICS 205-930 [(3α-tropanyl)-1H-indole-3-carboxylic acid ester] (Sandoz); peptides and the CGRP antiserum (Peninsula); [^{125}I]-iodohistidyl CGRP (Amersham).

Results

Release of calcitonin gene-related peptide-like immunoreactivity from guinea-pig isolated perfused heart

Basal release of CGRP-LI from the guinea-pig isolated perfused heart was 98 ± 14 pg ml^{-1} min^{-1} ($n = 16$). Administration of 5-HT increased the outflow of CGRP-LI in a dose-related manner (1 μM–100 μM, 1 ml, 0.5 ml min^{-1}). The increase in CGRP-LI outflow evoked by 5-HT (100 μM) was completely abolished in guinea-pig hearts pretreated *in vitro* with capsaicin. (Figure 1 and Table 1). CGRP-LI release induced by 5-HT (100 μM) from hearts which were exposed for 40 min to indomethacin (10 μM) was not different from that observed in control preparations (Table 1).

8-OH-DPAT or sumatriptan (up to 100 μM) failed to evoke release of CGRP-LI. Likewise, DOI (100 μM), a selective agonist of the 5-HT_{1C}/5-HT_2 receptors did not produce any detectable increase in CGRP-LI outflow (Table 2). CPD (100 μM) a selective, 5-HT_3 receptor agonist however, increased significantly ($P<0.01$) the CGRP-LI outflow. As observed for 5-HT, CGRP-LI release evoked by CPD (100 μM) was completely abolished in hearts desensitized with capsaicin (Table 2).

ICS 205-930 (20 μM), which blocks 5-HT_3 and less potently 5-HT_4 receptors totally prevented the increase in CGRP-LI outflow evoked by 5-HT. Likewise, the selective 5-HT_3 receptor antagonist, ondansetron (10 μM), blocked the 5-HT (100 μM)-induced release of CGRP-LI (Table 1).

Functional responses in the guinea-pig isolated perfused heart

After 30 min equilibration the resting parameters for the guinea-pig isolated perfused hearts were as follows: frequency 222 ± 6 beats min^{-1}; force of contraction 1.96 ± 0.02 g; mean

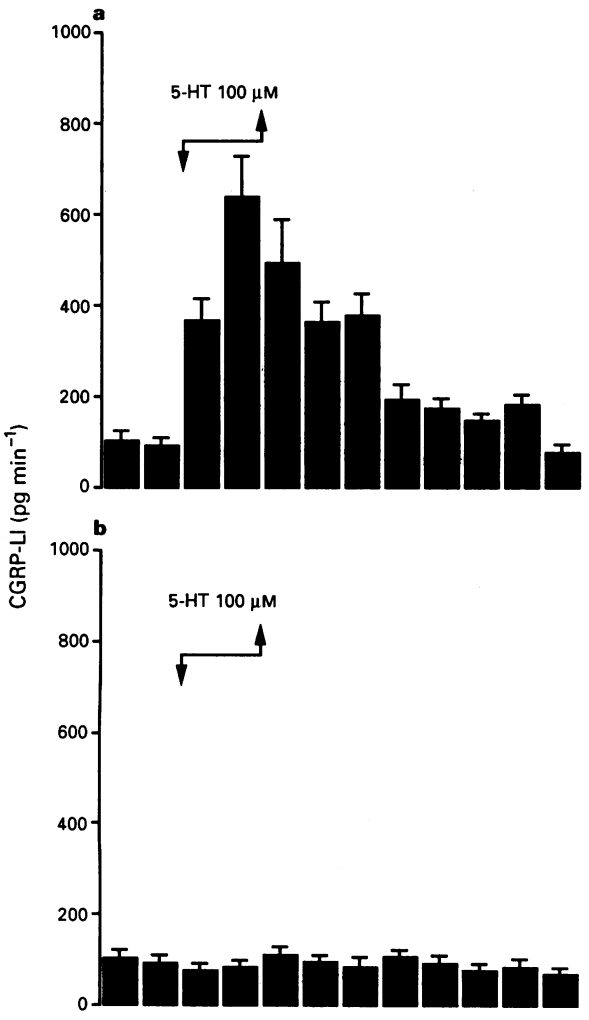

Figure 1 Outflow of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) from guinea-pig isolated perfused hearts after injection of 5-HT (100 μM, 1 ml, 0.5 ml min^{-1}) in control preparations (a) or in preparations desensitized with capsaicin (10 μM, 1 ml, 0.5 ml min^{-1}, given three times at 10 min intervals before 5-HT) (b). Each column is the mean ± s.e.mean (vertical bars) of 5 experiments.

Table 1 Effect of 5-hydroxytryptamine (5-HT) on the release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) from guinea-pig isolated perfused heart

	n	*CGRP-LI* (pg 10 min^{-1})
5-HT 1 μM	4	ND
5-HT 10 μM	5	1215 ± 198
5-HT 100 μM	5	2121 ± 176
5-HT 100 μM + capsaicin desens.[a]	5	129 ± 55*
5-HT 100 μM + indomethacin 10 μM[b]	5	1987 ± 215
5-HT 100 μM + ICS 205,930 (20 μM)[c]	5	228 ± 74*
5-HT 100 μM + ondansetron (10 μM)[c]	4	156 ± 35*

[a]Capsaicin desensitization was obtained by the administration of the drug (10 μM, 1 ml, 0.5 ml min^{-1}) three times (at 10 min intervals) before 5-HT. Indomethacin [b] or 5-HT antagonists [c] were added to the perfusion medium 40 and 30 min before 5-HT, respectively.
*$P < 0.01$ vs. 5-HT (100 μM).

Table 2 Effect of selective agonists of 5-HT_1, 5-HT_2 and 5-HT_3 receptors on the release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) from guinea-pig isolated perfused heart

	n	*CGRP-LI* (pg 10 min^{-1})
CPD 10 μM	5	653 ± 132
CPD 100 μM	5	1110 ± 172
CPD 100 μM + capsaicin desens.[a]	5	158 ± 41*
8-OH-DPAT (100 μM)	4	196 ± 25
Sumatriptan (100 μM)	4	287 ± 32
DOI (100 μM)	4	134 ± 51

CPD = chlorphenyldiguanide;
8-OH-DPAT = 8-hydroxy-dipropylaminotetralin;
DOI = 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane.
[a]Capsaicin desensitization was obtained by the administration of the drug (10 μM, 1 ml, 0.5 ml min^{-1}) three times (at 10 min intervals) before 5-HT.
*$P < 0.01$ vs. CPD 100 μM.

Table 3 Effect of 5-hydroxytryptamine (5-HT) and chlorophenyldiguanide (CPD) on the cardiac performance of guinea-pig isolated perfused heart

	Heart rate (% change)	*Contractile strength* (% change)	*Coronary blood flow* (% change)
5-HT (100 μM)	+ 28 ± 6%	− 9 ± 2%	+ 20 ± 3%
5-HT (100 μM) + caps. desens.[a]	+ 18 ± 3%	− 12 ± 2%	+ 15 ± 3%
CPD (100 μM)	+ 18 ± 3%	− 7 ± 2%	+ 14 ± 4%
CPD (100 μM) + caps. desens.[a]	+ 11 ± 3%	− 10 ± 2%	+ 10 ± 2%

[a]Capsaicin desensitization was obtained by the administration of the drug (10 μM, 1 ml, 0.5 ml min^{-1}) three times (at 10 min intervals) before 5-HT. Each value is the mean ± s.e.mean of 5 experiments.

coronary flow 4.8 ± 0.2 ml min^{-1} ($n = 12$). Injection of 5-HT evoked a positive chronotropic response, increased the coronary blood flow, and slightly decreased the contractile strength. These responses were not significantly affected by capsaicin pretreatment (Table 3). CPD evoked similar, although less pronounced effects to those induced by 5-HT. Capsaicin pretreatment did not significantly affect either the increase in blood flow and heart rate or the decrease in contractility evoked by CPD (Table 3). Since capsaicin desensitization did not significantly affect the responses to 5-HT or CPD, the effect of the other agonists and antagonists of 5-HT receptors were not further considered in this preparation.

Inotropic response in the isolated left atrium of guinea-pigs

In the electrically driven (3 Hz) left atria from reserpine-pretreated guinea-pigs and in the presence of atropine (1 μM), administration of 5-HT (1–10 μM) produced a concentration-dependent positive inotropic response, which started after a latency of 5–10 s and peaked after 1–2 min for each concentration. The 5-HT_3 receptor agonist, CPD, also produced a concentration-dependent inotropic response. CPD was approximately ten times less potent than 5-HT. 8-OH-DPAT as well as DOI up to 100 μM failed to increase the contractile force developed by the guinea-pig atrium. In contrast, at this concentration, 8-OH-DPAT produced a small negative inotropic response (Figure 2). The inotropic response produced by 5-HT was reduced to 25% of that

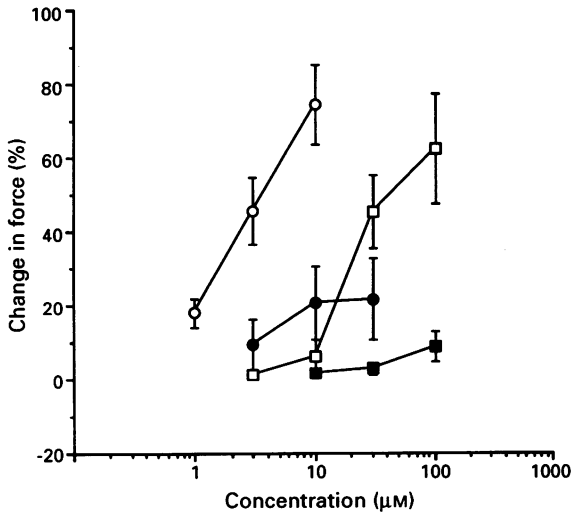

Figure 2 Inotropic response of the guinea-pig isolated left atria to 5-hydroxytryptamine (○, 5-HT) and different 5-HT receptor agonists. 8-Hyroxy-dipropylaminotetralin (●), 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (△), chlorophenyldiguanide (□). Responses are expressed as percentage variation of the basal value. Each point is the mean ± s.e.mean (vertical bars) of at least four experiments.

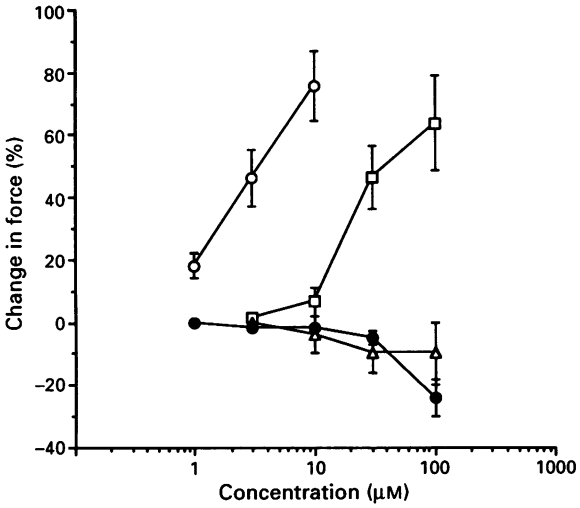

Figure 3 Effect of desensitization with capsaicin (10 μM, for 10 min) on the positive inotropic response produced by 5-hydroxytryptamine (5-HT) and chlorophenyldiguanide (CPD) in the guinea-pig isolated left atria: (○) 5-HT; (●) 5-HT plus capsaicin desensitization; (□) CPD; (■) CPD plus capsaicin desensitization. Responses are expressed as percentage variation of the basal value. Each point is the mean ± s.e.mean (vertical bars) of at least four experiments.

observed in controls by pretreatment with capsaicin (1 μM for 10 min) (Figure 3). The response to CPD was virtually abolished by capsaicin pretreatment (Figure 3).

The 5-HT_3 receptor antagonist ondansetron (0.3–1 μM), effectively inhibited the positive inotropic response produced by 5-HT. Schild analysis gave a slope of − 0.86 (CL − 1.18 and − 0.51). The pA_2 calculated with the constrained method was 6.50 (CL 6.08 and 6.91).

Discussion

Previous work has indicated that 5-HT activates the 'efferent' function of capsaicin-sensitive sensory neurones. Evidence for this has been obtained indirectly by showing that at least part of the inotropic response produced by 5-HT in the guinea-pig isolated atrium is abolished by capsaicin pretreatment *in vitro* (Bernoussi & Rioux, 1989). Our present data confirm these previous findings. We have also shown by direct measurement of CGRP outflow from the guinea-pig heart, that 5-HT evokes release of this peptide from capsaicin-sensitive nerve terminals.

The actions of 5-HT on effector cells are mediated by four types of receptors, the 5-HT_1, 5-HT_2, 5-HT_3 and the most recently identified 5-HT_4 (Kaumann *et al.*, 1990). In addition, the 5-HT_1 type can be further divided into at least five other types (Bradley *et al.*, 1986; Hoyer, 1990; Connor *et al.*, 1991). 5-HT_3 receptors mediate the depolarizing action of 5-HT on sensory nerves (Thoren, 1979; Collins & Fortune, 1983; Richardson *et al.*, 1985; Giordano & Rogers, 1989) and hence it was reasonable to hypothesize that 5-HT_3 receptors could mediate the release of peptide transmitters from capsaicin-sensitive afferents. The results of the present experiments using selective agonists and antagonists for 5-HT receptors, support this view.

Since 8-OH-DPAT, and sumatriptan are agonists at 5-HT_1 receptors the lack of effect of these compounds rules out the possibility that these receptors have a role in the 5-HT-evoked release of CGRP-LI. In contrast, the involvement of 5-HT_3 receptors is indicated by the following two observations. CPD, a moderately selective 5-HT_3 receptor agonist, evoked a capsaicin-sensitive release of CGRP-LI. CGRP-LI release evoked by 5-HT was inhibited by the two 5-HT_3 receptor antagonists, ICS 205-930 and ondansetron. ICS 205-930 has also been shown to act as a weak antagonist of the 5-HT_4 receptor. However, it is unlikely that ICS 205-930 is inhibiting the release of CGRP-LI by this mechanism since the selective 5-HT_3 receptor antagonist ondansetron (Butler *et al.*, 1988), which does not act on 5-HT_4 receptors (Baxter *et al.*, 1991), also blocked the 5-HT-mediated release.

Fully consistent with the release experiments, studies in the isolated atria showed that the inotropic response produced by 5-HT is mediated by activation of 5-HT_3 receptors. In this preparation CPD, although less potent than 5-HT, was found to be active, while 8-OH-DPAT and sumatriptan failed to exert any response. Ondansetron, antagonized the 5-HT-evoked inotropic response. The pA_2 observed for ondansetron in our experiments (6.5) is in good agreement with the observation that various 5-HT_3 antagonists show lower affinities in guinea-pig tissues than in rat tissues (Butler *et al.*, 1990). However, while we found that CPD was able to release CGRP from the guinea-pig isolated heart, Butler *et al.* (1990) failed to observe depolarization of the guinea-pig vagus nerve by a rather high concentration of CPD. The discrepancy may be due to several reasons, including different ways of administering the drug (intracoronary perfusion vs. superfusion), or differences between the vagus nerve trunk and the terminal region of the nerve. Finally, the possibility that the CGRP released by 5-HT originates mainly from sympathetic and not from vagal afferents could be considered.

The responses to 5-HT in the whole heart include increases in heart rate and coronary flow and small decreases in force. These effects were mimicked by CPD. Interestingly, capsaicin infusion into the coronary circulation of the guinea-pig evokes a similar response (Manzini *et al.*, 1989). Capsaicin desensitization did not affect the response to 5-HT or CPD. In contrast, in the isolated atria the 5-HT and CPD-evoked increase in force of contraction was insensitive to capsaicin. One of the possible reasons that can explain the apparent discrepancy regarding sensitivity to capsaicin between the whole heart and the isolated atrium is that the response in this latter preparation was obtained in tissues taken from animals pretreated with reserpine and in the presence of atropine (Franco-Cereceda & Lundberg, 1985; Saito *et al.*, 1986; Giuliani *et al.*, 1989), while experiments in the whole heart were performed with organs taken from untreated animals. Therefore, it is possible that in the whole heart responses to 5-HT were due to sensory nerve activation

which was masked by more pronounced effects mediated by 5-HT receptors present on other neuronal and non-neuronal structures. For instance, in the rabbit heart, the positive chronotropic and inotropic response to 5-HT, and 5-HT_3 agonists, is due to sympathetic activation (Fozard & Mobarok Ali, 1978).

Release of CGRP-LI from the guinea-pig heart by bradykinin is inhibited by indomethacin and the possibility that prostanoids can activate the efferent function of capsaicin-sensitive nerve fibres stems from the direct (Geppetti *et al.*, 1991) and indirect (Mapp *et al.*, 1991) observation that prostaglandins release CGRP or tachykinins from these nerves. However, in the present study the ability of 5-HT to promote CGRP release from sensory nerves via release of other mediators, such as prostaglandins, is excluded by the observation that the effect of 5-HT was completely resistant to indomethacin.

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Characterization of 5-HT_3 receptors of N1E-115 neuroblastoma cells by use of the influx of the organic cation [^{14}C]-guanidinium

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1 The 5-HT_3 receptor-mediated cation influx into N1E-115 mouse neuroblastoma cells has been studied by the use of the organic cation [^{14}C]-guanidinium.

2 5-Hydroxytryptamine (5-HT, 30 μM) caused a time-dependent influx of [^{14}C]-guanidinium which, in contrast to the influx elicited by veratridine (100 μM), was not inhibited by tetrodotoxin (TTX, 10 μM). The 5-HT-induced influx was potentiated by substance P and inhibited by ondansetron.

3 5-HT and the selective 5-HT_3 receptor agonists, *m*-chloro-phenylbiguanide, phenylbiguanide and 2-methyl-5-HT caused bell-shaped concentration-response curves; the rank order of potency was *m*-chlorophenylbiguanide > 5-HT > phenylbiguanide = 2-methyl-5-HT. Among these agonists, 5-HT elicited the highest influx of [^{14}C]-guanidinium. 5-Methoxytryptamine, an agonist at 5-HT_4 receptors, showed no effect.

4 The [^{14}C]-guanidinium influx induced by 100 μM 5-HT was not affected by methysergide (10 μM) and ketanserin (10 μM) but was inhibited by 5-HT_3 receptor antagonists with the following rank order of potency: ICS 205-930 > ondansetron > MDL 72222 >> metoclopramide.

5 The 5-HT-induced [^{14}C]-guanidinium influx was increased in the absence of Ca^{2+} and/or Na^+ and by a reduction of the temperature from 36° to 20°C.

6 Preincubation with 5-HT (100 μM) caused a time-dependent and rapidly reversible decrease of the 5-HT-induced [^{14}C]-guanidinium influx.

7 It is concluded that [^{14}C]-guanidinium influx measurement in N1E-115 cells is a convenient method to study properties of the cation channel of the 5-HT_3 receptor. This influx is independent of the fast sodium channel.

Keywords: 5-HT_3 receptor; [^{14}C]-guanidinium influx; N1E-115 neuroblastoma cells; cation channel; veratridine; substance P; fast sodium channel; desensitization

Introduction

5-Hydroxytryptamine (5-HT) exerts its action via four classes of 5-HT receptors denoted as 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4 (for recent reviews see Frazer *et al.*, 1990; Peroutka, 1990; Göthert, 1992). In contrast to 5-HT_1, 5-HT_2 and 5-HT_4 receptors which are linked to G-proteins and second messenger signalling pathways, the 5-HT_3 receptor is a ligand gated cation channel (Yakel & Jackson, 1988; Derkach *et al.*, 1989).

5-HT_3 receptors are located exclusively on neurones and are widely distributed throughout the peripheral and central nervous system (Wallis, 1989; Waeber *et al.*, 1989; Kilpatrick *et al.*, 1987). Electrophysiological studies have shown that in sympathetic neurones (Wallis & North, 1978) and in several neuronal clonal cells such as the murine neuroblastoma cells N1E-115 and the neuroblastoma × glioma hybrid cells NG 108-15 (Peters & Usherwood, 1983; Neijt *et al.*, 1988; 1989; Lambert *et al.*, 1989; Yakel *et al.*, 1990), 5-HT_3 receptor activation elicits a rapidly desensitizing membrane depolarization due to an increase in membrane permeability to sodium and potassium ions. 5-HT_3 receptors on N1E-115 and NG 108-15 cells have also been identified by means of ligand binding studies (Hoyer & Neijt, 1987; Lummis *et al.*, 1990).

5-HT_3 receptor-mediated effects are inhibited by the selective 5-HT_3 receptor antagonists, ICS 205-930 [(3α-tropanyl)-1H-indole-3-carboxylic acid ester], ondansetron and MDL 72222 (1αH, 3α, 5αH-tropan-3yl-3,5-dichlorobenzoate) (Richardson *et al.*, 1985; Butler *et al.*, 1988; Fozard, 1984) and mimicked by the selective agonists 2-methyl-5-HT, phenylbiguanide (Wallis & Nash, 1981) and *m*-chlorophenylbiguanide (Kilpatrick *et al.*, 1990).

In the present study, the 5-HT_3 receptor in N1E-115 mouse neuroblastoma cells has been characterized by means of the 5-HT_3 receptor-mediated influx (uptake) of the organic cation [^{14}C]-guanidinium. This cation has been shown to be a suitable probe for studying the cation permeabilities of sodium channels (Catterall & Nirenberg, 1973; Reith, 1990) and of the 5-HT_3 receptor of NG 108-15 cells (Reiser & Hamprecht, 1989).

Preliminary accounts of some of this work have appeared in abstract form (Barann *et al.*, 1991).

Methods

Cell culture

Mouse neuroblastoma cells of the clone N1E-115 (Amano *et al.*, 1972) and of passage numbers 40–50 were grown in Dulbecco's modified Eagle's medium (DMEM) with HEPES (7.6 mM) and sodium bicarbonate (30 mM). The growth medium was supplemented (as described by Hoyer & Neijt, 1988) with the antibiotics penicillin (100 i.u. ml^{-1}) and streptomycin (100 μg ml^{-1}), 10% foetal calf serum (Gibco) and the following amino acids (in mM concentrations): cystein hydrochloride (0.3), L-alanine (0.4), asparagine (0.45), L-aspartic acid (0.4), L-proline (0.4) and L-glutamic acid (0.4). Cells were cultured in a humified atmosphere containing 5% CO_2 at 37°C in vent flasks (Nunc, 750 ml) and fed every third day. Three days before starting experiments, cells were subcultured in 24-well cell culture clusters (Falcon).

[^{14}C]-guanidinium influx measurements

After removal of the growth medium, cells were washed and then preincubated (for 20 min, 1.5 ml per well) with warmed

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(36°C) incubation buffer containing (in mM): 25 HEPES-Tris (pH 7.4), 135 choline chloride, 5.4 KCl, 0.98 $MgSO_4$, 5.5 D-glucose and bovine serum albumin (1 mg ml^{-1}). When compounds were tested for inhibition of [^{14}C]-guanidinium influx (e.g. TTX or 5-HT receptor antagonists), they were already present during this preincubation period. After preincubation the cells were incubated for 2 min (unless stated otherwise) with the same incubation buffer which, in addition, contained 5 μM [^{14}C]-guanidinium chloride (specific activity 59 mCi mmol^{-1}) and the drug to be tested. The incubation was terminated by sucking off the incubation medium and rapidly washing the cells three times with ice-cold albumin-free incubation buffer in which choline chloride was replaced by 135 mM sodium chloride. Thereafter the cells were dissolved in 0.5 ml 0.1% Triton X-100 and the [^{14}C]-guanidinium content of this solution was determined by liquid scintillation counting. An aliquot of the cell lysate was used for determination of the protein content according to the method of Lowry *et al.* (1951); the mean protein content per well was 213 ± 6 μg (n = 57). All experiments were carried out in duplicate or triplicate.

Data analysis

Calculations of EC_{50}, E_{max}, IC_{50} values and slope factors (apparent Hill-coefficients, nH) were performed with a commercially available computer programme (GRAPHPAD Inplot). Statistical analysis were performed with an unpaired Student's t test, with significance at $P < 0.05$.

Materials

Constituents of cell culture media were obtained from Gibco BRL or from Sigma. The following compounds were used in experiments: 5-HT (5-hydroxytryptamine creatinine sulphate, serotonin; Sigma), [^{14}C]-guanidinium chloride (specific activity = 59 mCi mmol^{-1}; CEA), 5-methoxytryptamine hydrochloride (Sigma), 2-methyl-5-HT (2-methyl-5-hydroxytryptamine maleate; Sigma), phenylbiguanide (1-phenylbiguanide; Aldrich), *m*-chlorophenylbiguanide (1-(*m*-chlorophenyl)-biguanide dihydrochloride; Glaxo), ondansetron (Glaxo), ICS 205 930 ((3α-tropanyl)-1H-indole-3-carboxylic acid ester; Sandoz), MDL 72222 (1αH, 3α, 5αH-tropan-3yl-3,5-dichlorobenzoate; Merrell Dow), metoclopramide hydrochloride (Sigma), TTX (tetrodotoxin; Sigma), veratridine (Sigma), methysergide bimaleinate (Sandoz), ketanserin tartrate (Sigma), substance P (Sigma), (+)-tubocurarine chloride (Sigma).

Results

Time course of 5-HT-induced [^{14}C]-guanidinium influx

Exposure of N1E-115 cells to 5-HT (30 μmol l^{-1}) caused a time-dependent increase in the intracellular content of [^{14}C]-guanidinium within N1E-115 cells. While the basal influx (in the absence of 5-HT) tended to increase linearly with time, the 5-HT-mediated influx of [^{14}C]-guanidinium approached a steady-state after about 2 min (Figure 1a). In all subsequent experiments [^{14}C]-guanidinium influx was determined after 2 min.

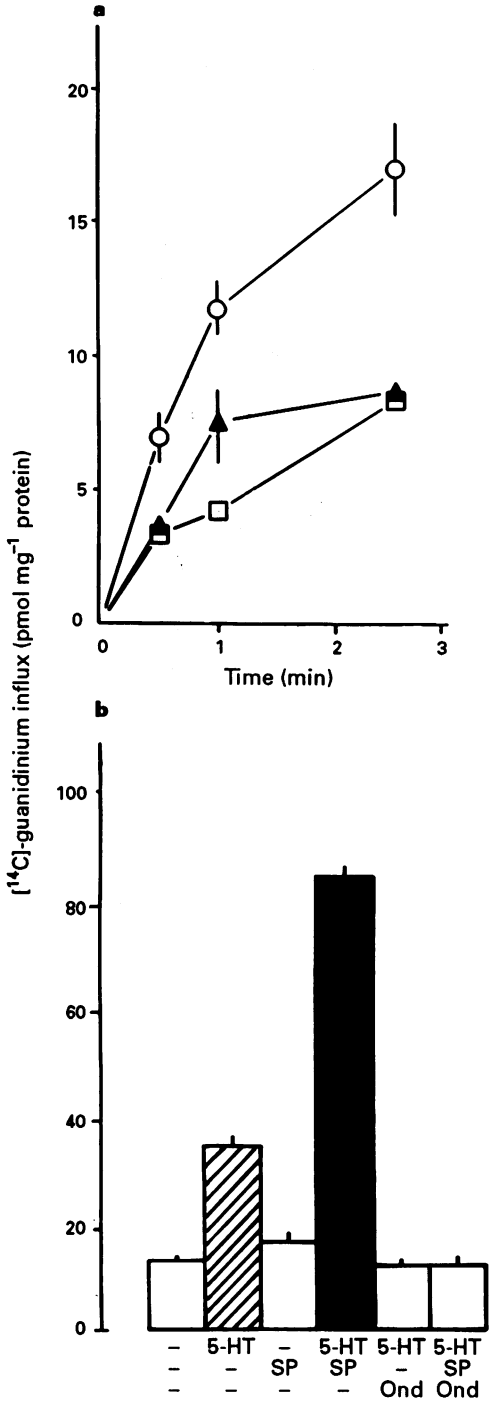

Figure 1 (a) Time course of basal (□), total (○) and specific (▲), i.e. 5-hydroxytryptamine (5-HT, 30 μM)-induced, [^{14}C]-guanidinium influx into N1E-115 cells. (b) Effect of substance P (SP, 10 μM) on basal and on 5-HT (30 μM)-induced 2-min influx of [^{14}C]-guanidinium, and inhibition by ondansetron (Ond, 0.3 μM) of the influx mediated by 5-HT alone or 5-HT plus substance P. Shown are means ± s.e.mean (vertical lines) from 3–5 experiments.

Effects of substance P, veratridine and tetrodotoxin (TTX)

Substance P (10 μM) which had been shown to induce pronounced 5-HT$_3$ receptor-mediated influx of [^{14}C]-guanidinium in NG108-15 cells (Reiser & Hamprecht, 1989) caused only marginal influx of [^{14}C]-guanidinium in N1E-115 cells. However, this concentration of substance P potentiated the influx evoked by 30 μM 5-HT (Figure 1b). The influx mediated by either 5-HT or 5-HT plus substance P was inhibited by the selective 5-HT$_3$ receptor antagonist, ondansetron (0.3 μM; Figure 1b). Influx of [^{14}C]-guanidinium evoked by 100 μM 5-HT was not affected by TTX (10 μM) whereas [^{14}C]-guanidinium influx elicited by 1 mM veratridine was abolished by 10 μM TTX (Figure 2). 5-HT-induced influx of [^{14}C]-guanidinium remained also unaffected by 1 mM ouabain (data not shown).

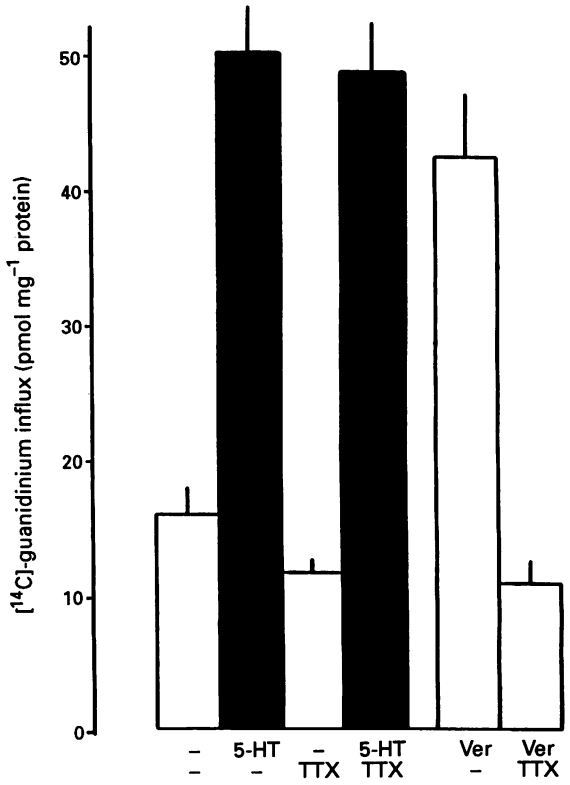

Figure 2 Effect of tetrodotoxin (TTX, 10 μM) on basal influx of [^{14}C]-guanidinium and on the influx evoked by 2 min exposure to 100 μM 5-hydroxytryptamine (5-HT) or 1 mM veratridine (Ver); TTX was present for 20 min before and during stimulation. Shown are means ± s.e.mean (vertical lines) of 3–4 experiments.

Concentration-response curves for 5-HT$_3$-receptor agonists

When the influx of [^{14}C]-guanidinium was determined at increasing concentrations of 5-HT or the selective 5-HT$_3$ receptor agonists, 2-methyl-5-HT, phenylbiguanide or *m*-chlorophenylbiguanide, all compounds caused bell-shaped concentration-response curves with slope factors of the ascending part of the concentration-response curve of about 2 (Figure 3). With 5-HT a maximal effect was obtained at 100 to 300 μM; the maximal responses of the other agonists were significantly smaller than that of 5-HT (Figure 3 and Table 1). The rank order of potency was *m*-chlorophenylbiguanide > 5-HT > phenylbiguanide = 2-methyl-5-HT (Table 1). 5-Methoxytryptamine (10–1000 μM) which activates 5-HT$_4$ receptors (and at less potency 5-HT$_1$ receptors), but not 5-HT$_3$ receptors, caused no influx of [^{14}C]-guanidinium (data not shown).

Antagonists

The selective 5-HT$_3$-receptor antagonist, ondansetron (100 and 300 nM), produced a concentration-dependent rightward shift of the 5-HT concentration-response curve (Figure 4). The pA$_2$-value estimated from this shift was 7.3. A still higher concentration of ondansetron (1 μM) caused a further rightward shift (Figure 4).

As shown in Figure 5 and Table 1, the [^{14}C]-guanidinium influx elicited by 100 μM 5-HT was concentration-dependently inhibited by the 5-HT$_3$ receptor antagonists, ICS 205-930, ondansetron, MDL 72222 and by metoclopramide; the rank order of potency was ICS 205 930 > ondansetron > MDL 72222 >> metoclopramide. The response was also inhibited by (+)-tubocurarine (pIC$_{50}$ = 6.12 ± 0.12, n = 3).

The mixed 5-HT$_1$/5-HT$_2$ receptor antagonist methysergide (10 μM) and the 5-HT$_2$ receptor antagonist ketanserin (10 μM) had no significant effects on the 5-HT-evoked [^{14}C]-guanidinium influx (105.0 ± 4.0 and 91.6 ± 1.1% of control, respectively; n = 4 each).

Influence of temperature, Ca^{2+} and Na^+

5-HT-induced [^{14}C]-guanidinium influx was influenced by the incubation temperature; influx increased by a decrease in the temperature from 36°C to 20°C, whereas it decreased again when the temperature was further reduced to 11°C or to 4°C (Figure 6a).

5-HT-evoked influx of [^{14}C]-guanidinium (determined at

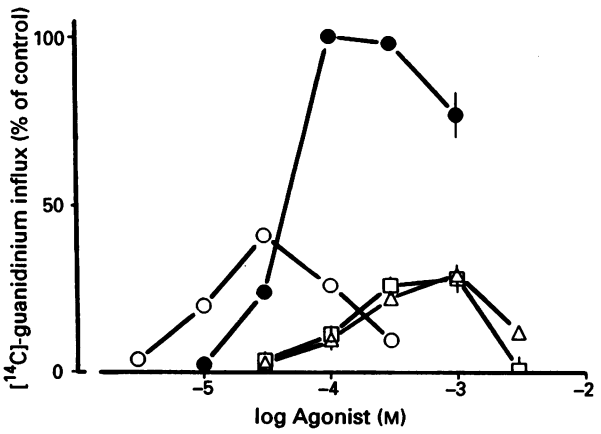

Figure 3 Concentration-response curves for the [^{14}C]-guanidinium influx evoked by 2-min exposure to the 5-HT$_3$ receptor agonists *m*-chlorophenylbiguanide (○); 5-hydroxytryptamine (5-HT) (●); phenylbiguanide (□) and 2-methyl-5-HT (△). The responses are expressed as percentages of the effect elicited by 100 μM 5-HT (= control). Shown are means ± s.e.mean (vertical lines) of 6 experiments.

Table 1 pEC$_{50}$ values and maximal stimulation (E$_{max}$) of [^{14}C]-guanidinium influx into N1E-115 cells for 5-HT$_3$ receptor agonists, and pIC$_{50}$ values of 5-HT$_3$ receptor antagonists for inhibition of 5-hydroxytryptamine (5-HT, 100 μM)-induced influx of [^{14}C]-guanidinium (determined from the concentration-response curves in Figures 3 and 5)

Agonists	n	*pEC$_{50}$*	± *s.e.mean*	*E$_{max}$**	± *s.e.mean*
m-Chlorophenylbiguanide	6	4.89	0.06	50.3	3.0
5-Hydroxytryptamine (5-HT)	6	4.35	0.03	106.7	3.9
Phenylbiguanide	6	3.69	0.08	33.1	3.2
2-Methyl-5-HT	6	3.63	0.05	37.2	2.9
Antagonists	n	*pIC$_{50}$*	± *s.e.mean*	*pK$_i$***	
ICS 205-930	6	7.85	0.05	8.33	
Ondansetron	8	6.91	0.07	7.38	
MDL 72222	6	6.39	0.02	6.90	
Metoclopramide	6	5.00	0.01	5.49	

*Given as percentage of the [^{14}C]-guanidinium influx obtained at 100 μM 5-HT (control).
**Calculated from mean pIC$_{50}$ values according to Cheng & Prusoff (1973) on the assumption of a competitive interaction.

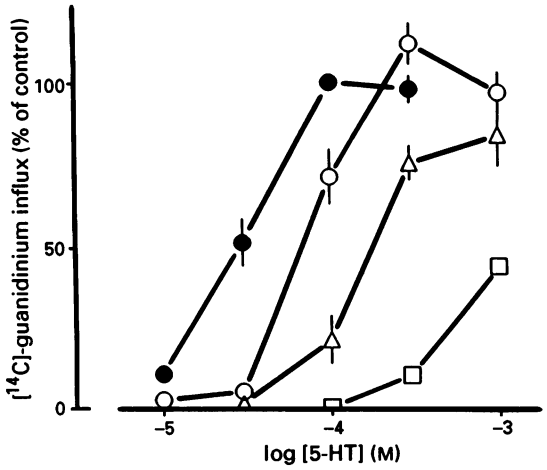

Figure 4 Rightward shift of the concentration-response curve for the 5-hydroxytryptamine (5-HT)-induced influx of [^{14}C]-guanidinium by 100 nM (○), 300 nM (△) and 1000 nM (□) ondansetron. Ondansetron was present for 20 min before and during 2-min exposure to 5-HT. The responses are expressed as percentages of the effect elicited by 100 μM 5-HT in the absence of ondansetron (= control). Shown are means ± s.e.mean (vertical lines) of 4 experiments.

20°C) was reduced by about 50% when the incubation buffer was supplemented with a physiological concentration of Ca^{2+} (1.8 mM) and it was further reduced when, in addition, 135 mM choline chloride was replaced by 135 mM sodium chloride (Figure 6b).

Effect of preincubation with 5-HT

Preincubation of the cells with 100 μM 5-HT for 0.5 to 16 min caused a decrease of the 5-HT-induced [^{14}C]-guanidinium influx when the experiments were performed at 36°C or 20°C. This decline increased with the duration of preincubation and was faster the higher the temperature at which the experiments were carried out (Figure 7a). The half-time of decline of response was 0.4 min and 2.5 min at 36°C and 20°C, respectively (Figure 7a, inset). Interestingly, at 4°C and at 11°C, preincubation with 5-HT for up to 5 min caused an increase in [^{14}C]-guanidinium influx (Figure 7a).

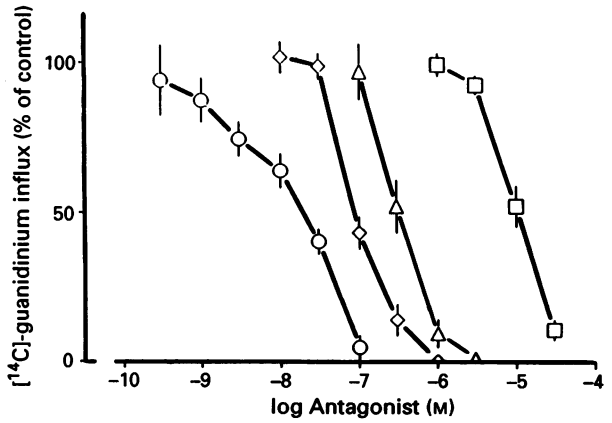

Figure 5 Inhibition of 5-hydroxytryptamine (5-HT, 100 μM)-induced influx of [^{14}C]-guanidinium by the 5-HT$_3$ receptor antagonists ICS 205 930 (○); ondansetron (◇); MDL 72222 (△) and by metoclopramide (□). The respective antagonist was present for 20 min before and during the 2-min exposure period to 5-HT. The responses are expressed as percentages of the effect elicited by 100 μM 5-HT in parallel reference experiments carried out in the absence of antagonists (= control). Shown are means ± s.e.mean (vertical lines) of 6–8 experiments.

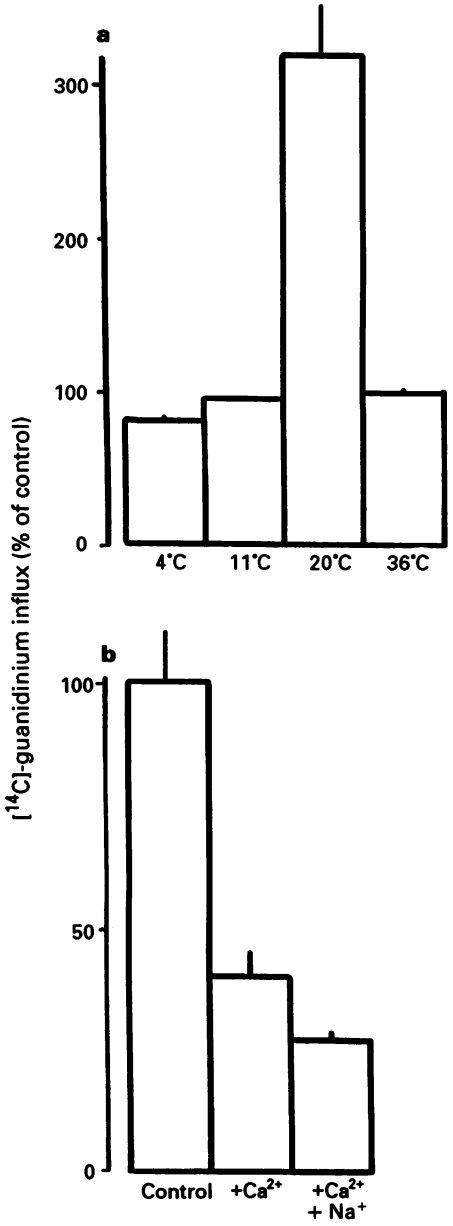

Figure 6 (a) Effect of temperature on [^{14}C]-guanidinium influx evoked by 2-min exposure to 100 μM 5-hydroxytryptamine (5-HT). Control = 5-HT-induced influx at 36°C. (b) Inhibition by Ca^{2+} (1.8 mM) and Na^+ of [^{14}C]-guanidinium influx elicited by 2-min exposure to 100 μM 5-HT at 20°C. Ca^{2+} (1.8 mM) was added to the Ca^{2+}- and Na^+-free incubation buffer, and Na^+ was introduced by substituting choline chloride (135 mM) by equimolar concentrations of NaCl 20 min before and during exposure to 5-HT. Control = 5-HT-induced influx in the absence of Ca^{2+} and Na^+ at 20°C. Shown are means ± s.e.mean (vertical lines) of 6 experiments.

The response to 5-HT (at 20°C) was rapidly restored by washing out 5-HT before starting the incubation with [^{14}C]-guanidinium plus 5-HT. The preincubation-induced desensitization of the response disappeared after only a 1-min wash of the cells (Figure 7b).

Discussion

The present results have shown that the organic cation [^{14}C]-guanidinium is a useful tool to study cation flux through the cation channel of 5-HT$_3$ receptors. This influx is independent of the cation flux through the veratridine-activated fast

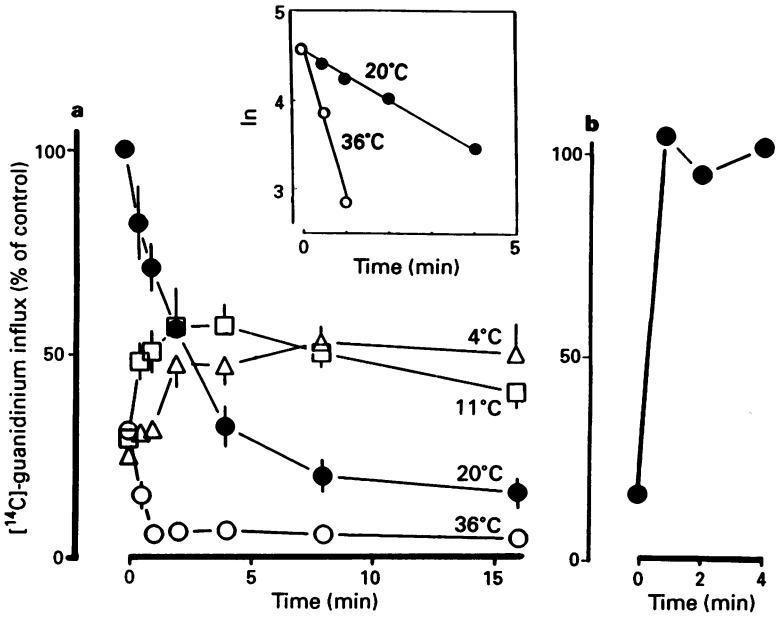

Figure 7 (a) Effect of the duration (min) of preincubation with 100 μM 5-hydroxytryptamine (5-HT) and of the temperature (4°C–36°C) on the 2-min influx of [^{14}C]-guanidinium elicited by 100 μM 5-HT. The 5-HT-induced influx at 20°C without preincubation with 5-HT (producing the highest response) was taken as 100% (= control). In the inset, the respective maximal responses at 36°C and 20°C (without preincubation) were taken as 100% and the preincubation-induced decline of response (%) was transformed to logarithmic values and plotted against the duration of preincubation; the slopes of the two lines were − 0.2769 (at 20°C) and − 1.7148 (at 36°C). (b) Effect of omission of 5-HT for various intervals (min; abscissa scale) after 16 min of preincubation with 100 μM 5-HT on the [^{14}C]-guanidinium influx evoked by 2-min exposure to 100 μM 5-HT at 20°C. Shown are means ± s.e.mean (vertical lines) of 3–9 experiments.

sodium channel. In contrast to NG 108-15 cells in which a reliable response to 5-HT was obtained only in the presence of substance P (Reiser & Hamprecht, 1989) and in which maximal effects of substance P (10 μM) were much higher than those of 5-HT, N1E-115 cells exhibited a pronounced and reproduceable influx of [^{14}C]-guanidinium in response to activation of 5-HT$_3$ receptors in the absence of substance P. In addition, the maximum response to 5-HT was much higher than that of 10 μM substance P which caused only marginal influx. The fact that the 5-HT-induced influx of [^{14}C]-guanidinium was unaffected by TTX indicates that this influx did not occur through voltage-dependent fast sodium channels (which could be assumed to be opened as a consequence of activation of 5-HT$_3$ receptors). The experimental conditions, namely the absence of Na^+ and Ca^{2+} and the use of a low concentration of guanidinium (5 μM) might have prevented a depolarization of N1E-115 cells and, thus a contribution of voltage or Ca^{2+}-dependent channels in influx of [^{14}C]-guanidinium. Furthermore, the use of choline as a substitute for sodium might also have prevented 5-HT-mediated depolarization of N1E-115 cells, since the permeability of choline through the cation channel of 5-HT$_3$ receptors has been shown to be relatively small (Yakel *et al.*, 1990). Finally, the lack of effect of ouabain indicates that Na^+/K^+-ATPase was obviously not involved in influx of [^{14}C]-guanidinium.

5-HT-evoked influx of [^{14}C]-guanidinium was not affected by either methysergide, a mixed 5-HT$_1$/5-HT$_2$ receptor antagonist, or ketanserin, a selective 5-HT$_2$ receptor antagonist, but it was inhibited by a series of selective 5-HT$_3$ receptor antagonists. Therefore, it may be concluded that 5-HT-evoked influx was due to activation of 5-HT$_3$ receptors on N1E-115 cells. Expression of 5-HT$_3$ receptors in N1E-115 cells has previously been demonstrated using electrophysiological as well as receptor binding techniques (see Introduction).

The rank order of potency for inhibition of 5-HT-induced influx of [^{14}C]-guanidinium by selective 5-HT$_3$ receptor antagonists found in the present study (ICS 205-930 > ondansetron > MDL 72222) conforms to the pharmacological properties of 5-HT$_3$ receptors found in previous experiments, e.g. in afferent neurones of the rabbit vagus (Fozard, 1990) or in N1E-115 cell membranes for competition with [^{3}H]-GR65630 binding (Lummis *et al.*, 1990). In addition, Lummis *et al.* (1990) also found (+)-tubocurarine to be about equipotent with MDL 72222 and more potent than metoclopramide as an antagonist at 5-HT$_3$ receptors of N1E-115 cells. The relatively low pIC_{50} values for the above mentioned antagonists are at least partially a consequence of the high concentration of 5-HT (100 μM) used to determine IC_{50} values. Assuming competitive antagonism, a pK_i-value for, e.g., ondansetron of about 7.4 can be calculated by means of the equation of Cheng & Prusoff (1973); this value is in close agreement with the pA_2 value (7.3) calculated from the parallel rightward shift of the 5-HT concentration-response curve by ondansetron. However, the antagonist affinities are by a factor of about 8 lower than previously published values for 5-HT$_3$ receptors of N1E-115 cells (Hoyer & Neijt, 1988; Neijt *et al.*, 1988; Lummis *et al.*, 1990). The reason for this discrepancy remains unknown. It may be due to the cells used in our laboratory and/or the assay conditions (sodium replaced by choline; see also below). Furthermore, the rather low antagonist potencies may be related to the possibility that with the present method a different receptor state, probably a partially desensitized 5-HT$_3$ receptor, was studied.

Not only 5-HT but also the selective 5-HT$_3$ receptor agonists 2-methyl-5-HT, phenylbiguanide and *m*-chlorophenylbiguanide caused concentration-dependent influx of [^{14}C]-guanidinium into N1E-115 cells, whereas 5-methoxytryptamine, an agonist at 5-HT$_4$ receptors, was inactive. The rank order of potencies (*m*-chlorophenylbiguanide > 5-HT > phenylbiguanide = 2-methyl-5-HT) and the fact that all of the 5-HT$_3$ receptor agonists tested (except 5-HT) behaved as partial agonists are in agreement with results obtained for 5-HT$_3$ receptors of the rat vagus nerve (Kilpatrick *et al.*, 1990). Again in agreement with the literature is the steep slope factor (of about 2) of the concentration-response curve for the 5-HT$_3$ receptor agonists, indicating cooperativity in

agonist binding (Peters & Lambert, 1989). However, the absolute potency values for the agonists are by almost one order of magnitude lower than expected from the literature (see e.g. Boess *et al.*, 1992). However, a low potency of 5-HT (EC_{50} of about 30–40 μM) was also found in a recent electrophysiological study in NG108-15 cells. A change in agonist potency due to 5-HT$_3$ receptor desensitization (after 2 min exposure to agonists) might be one reason for such low agonist potencies (see below).

Results different from those of the present investigation were also obtained in a previous study on NG108-15 cells in which Reiser & Hamprecht (1989) used the [^{14}C]-guanidinium influx method to determine concentration-response curves for 5-HT. In the absence of substance P, those curves were characterized by a higher potency (EC_{50} of about 5 μM) than in the present study (and that of Newberry *et al.*, 1992) but a very low maximal effect (the addition of substance P was necessary to obtain a reliable efficacy of 5-HT). Since these authors used not only another cell line but also different assay conditions (e.g., no replacement of sodium chloride by choline chloride), it remains to be clarified whether the low agonist potencies might be a characteristic of the cells used in our experiments and/or a consequence of the use of choline chloride as a substitute for sodium chloride.

A further characteristic of 5-HT$_3$ receptors, namely rapid desensitization (Peters & Lambert, 1989; Neijt *et al.*, 1989), was also observed in the present study. Bell-shaped concentration-response curves, seen for all 5-HT$_3$ receptor agonists, are a typical feature (although rarely shown in a figure) of rapidly desensitizing receptors such as the nicotinic acetylcholine receptor and have also been demonstrated in the literature for 5-HT$_3$ receptors (see, e.g., Fozard, 1990). Moreover, preincubation of N1E-115 cells with 5-HT caused a decrease of [^{14}C]-guanidinium influx which was dependent on the duration of preincubation and which was rapidly reversed by washing. A similar phenomenon has been observed by Neijt *et al.* (1989) using the same cell line but electrophysiological methods. The rate at which the 5-HT$_3$ receptor-induced [^{14}C]-guanidinium influx decreased (i.e. the 5-HT$_3$ receptors desensitized) was dependent on the temperature and could be described by a single exponential function. Lowering of the temperature from 36°C to 20°C caused a more than 6 fold increase in the half-time of decay. Interestingly, at very low temperatures (11°C and 4°C) preincubation with 5-HT caused a transient increase in the influx of [^{14}C]-guanidinium. The reason for this phenomenon is unknown and remains to be clarified.

Finally, sodium and calcium ions were found to reduce the rate of [^{14}C]-guanidinium influx in N1E-115 cells. The effect of sodium ions may be due to a competition of this ion with the guanidinium ion for flux through the 5-HT$_3$ receptor channel. The effect of calcium ions, on the other hand, seems to be due to another mechanism. In a recent study, Peters *et al.* (1988) have shown that a decrease in the concentration of Ca^{2+} (or Mg^{2+}) from their standard values augmented both the amplitude and duration of the 5-HT-induced current in N1E-115 cells, whereas an increase in the concentration of either divalent cation produced the opposite effect. These authors could exclude a voltage-dependent blockade of the 5-HT-gated ion channel by Ca^{2+} (or Mg^{2+}) and they assumed that Ca^{2+} and Mg^{2+} influence the desensitization kinetics of the 5-HT$_3$ receptor. In agreement with the results of Peters *et al.* (1988) we also recently demonstrated a decrease of 5-HT-induced [^{14}C]-guanidinium influx in N1E-115 cells by Mg^{2+} (unpublished observations). Whether the 5-HT-potentiating effect of substance P is also due to a change in the desensitization kinetics of 5-HT$_3$ receptors of N1E-115 cells is currently under investigation.

In conclusion, [^{14}C]-guanidinium influx measurements in N1E-115 cells are a convenient and reliable method to study the pharmacological and conductive properties of the cation channel of the 5-HT$_3$ receptor independently of the fast Na^+ channel.

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Effect of opioid receptor subtype-selective agonists on purinergic and adrenergic components of neurogenic contractions of mouse vas deferens

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1 Effects of opioid agonists on the purinergic and adrenergic components of neurogenic contractions and in some experiments on transmitter overflow were studied in the mouse isolated vas deferens.

2 When the vas deferens was stimulated every 2 min by pairs of pulses 2 s apart in the presence of prazosin 0.3 μM (to isolate the purinergic component) or α,β-methylene-ATP 3 μM (to isolate the adrenergic component), each pulse elicited a separate twitch. The opioid agonists [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO, μ-receptor-selective), [D-Pen^2,D-Pen^5]enkephalin (DPDPE, δ-selective) and *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide (U-50488, κ-selective) concentration-dependently reduced both purinergic and adrenergic contractions. For each agonist, maximal effects and concentrations causing half-maximal effects were very similar for inhibition of the purinergic component on the one hand and for inhibition of the adrenergic component on the other hand, although the adrenergic component was inhibited with a slight preference. Moreover, effects on contractions elicited by the first and the second pulse of the pairs were very similar.

3 When vasa deferentia preincubated with [^{3}H]-noradrenaline were stimulated with trains of 100 pulses delivered at 20 Hz, morphine 10 μM reduced significantly both evoked tritium overflow and evoked contractions. Its effect was antagonized by naloxone.

4 It is concluded that, in contrast to drugs acting at some other presynaptic receptors, opioid μ-, δ- and κ-agonists inhibit purinergic and adrenergic neurogenic contractions of the mouse vas deferens in a similar manner. In contrast to a previous report, no enhancement by morphine of the release of noradrenaline elicited by high frequency pulse trains was observed.

Keywords: Mouse vas deferens; adrenergic transmission; purinergic transmission; co-transmission; opioid receptors; morphine

Introduction

Since 1972 (Henderson *et al.*, 1972), the mouse isolated vas deferens has often been used as a standard model in studies on opioids. Opioid agonists reduce contractions elicited by sympathetic nerve stimulation. They also reduce the evoked overflow of noradrenaline (Henderson *et al.*, 1972). Hence, presynaptic inhibition of noradrenaline release has been thought to be the basis for the inhibition of contractions (see Starke, 1977 and Henderson *et al.*, 1979 for review). All three major subtypes of opioid receptor – μ, δ, κ, – occur at the sympathetic terminal axons of the mouse vas deferens (see Illes, 1989 for review).

Sympathetic transmission in the mouse vas deferens is now known to be due to at least two co-transmitter substances, noradrenaline and adenosine 5'-triphosphate (ATP). Both contribute to the tetanic response to trains of high frequency pulses (e.g., 5 Hz). In the individual biphasic twitches elicited by single or low frequency pulses (e.g., 0.5 Hz), ATP mediates the intitial rapid phase, whereas noradrenaline mediates the secondary slow phase (Stjärne & Åstrand, 1985; Allcorn *et al.*, 1986; von Kügelgen *et al.*, 1989; see Burnstock, 1990 and von Kügelgen & Starke, 1991a for review).

The question arises as to whether opioid agonists modulate purinergic and adrenergic components of this transmission process similarly or differentially. The question is all the more pertinent because some drugs acting at presynaptic receptors seem to modulate the release of noradrenaline and that of ATP in a differential or even opposite manner (e.g., angiotensin peptides: Trachte, 1988; Ellis & Burnstock, 1989a; Ziogas & Cunanne, 1991; calcitonin gene-related peptide: Ellis & Burnstock, 1989b; prostaglandin E_2: Ellis & Burnstock, 1990; nicotine: Bültmann *et al.*, 1991a; von Kügelgen & Starke, 1991b). In fact it has been suggested that, in mouse vas deferens, morphine reduces the non-adrenergic-non-cholinergic (i.e., purinergic) but enhances the adrenergic component of the tetanic response to electrical stimulation at 20 Hz, presumably due to inhibition of the release of the non-adrenergic-non-cholinergic transmitter (i.e., ATP) but enhancement of the release of noradrenaline (Forsyth & Pollock, 1988). In the present study the possibility of a differential modulation was examined by means of selective agonists at all three subtypes of opioid receptor (see Illes, 1989), namely the μ-receptor-selective [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol] enkephalin (DAMGO; Handa *et al.*, 1981), the δ-receptor-selective [D-Pen^2,D-Pen^5] enkephalin (DPDPE; Mosberg *et al.*, 1983) and the κ-receptor-selective *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide (U-50488; Lahti *et al.*, 1982). Purinergic and adrenergic phases of low frequency stimulation-evoked contractions were isolated by the α_1-adrenoceptor antagonist, prazosin and the P_2-purinoceptor agonist and desensitizing agent, α,β-methylene-ATP (Meldrum & Burnstock, 1983; von Kügelgen *et al.*, 1989), respectively. The stimulation-evoked overflow of noradrenaline (tritiated compounds after treatment with [^{3}H]-noradrenaline) was also examined.

Methods

Adult male NMRI mice (Savo, Kissleg, Germany; 30–51 g) were killed by cervical dislocation and exsanguination. Both vasa deferentia were dissected out and desheathed. Smooth muscle tension was recorded by means of an isometric force transducer (Q11, Hottinger Baldwin, Darmstadt, Germany) on a Rikadenki pen recorder (Rikadenki, Freiburg, Germany). The initial tension applied was 9.8 mN. Incubation

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and superfusion media contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 0.9, $NaHCO_3$ 25, glucose 11, ascorbic acid 0.3, and disodium EDTA 0.03. They were saturated with 95% O_2/5% CO_2 and kept at 37°C.

Contractions elicited by pairs of pulses

Single vasa deferentia were suspended vertically in an organ bath containing 5.7 ml medium which was replaced every 15 min (at shorter intervals when drugs or solvent were washed out). The lower end was fixed and the upper end attached to the transducer. Platinum electrodes were located at the top and the bottom of the organ bath. During an initial 60-min equilibration period, the tissues were allowed to relax to a resting tension of approximately 3 mN. Thereafter, they were stimulated electrically every 2 min by pairs of pulses 2 s apart (Stimulator II, Hugo Sachs Elektronik, Hugstetten, Germany; constant current mode; 0.3 ms pulse width, current strength 200 mA; supramaximal with respect to evoked contractions). In order to isolate purinergic and adrenergic contraction components, prazosin 0.3 μM and α,β-methylene-ATP 3 μM, respectively, was present in the medium from the 30th min of the equilibrium period onwards. Beginning 15 min after the onset of electrical stimulation, non-cumulative concentration-response curves of DAMGO, DPDPE or U-50488 were determined as follows. Increasing concentrations of the agonist (0.5 log unit increments) were added to the organ bath at 30-min intervals and washed out after the effect had reached its maximum (7 min for DAMGO and DPDPE, 11 min for U-50488). Inhibition of contractions by the opioids was calculated in each experiment as a percentage of the contraction obtained immediately before addition of the respective agonist concentration, and was corrected for the mean change occurring in solvent controls. For further evaluation of concentration-response relationships, sigmoid curves were fitted to the weighted mean percentage inhibition values using equation No. 25 of Waud (1976) and non-linear regression analysis (Motulsky & Ransnas, 1987). The calculation yielded an E_{max} value, i.e. the maximal agonist effect obtained in the same curve, an EC_{50} value, i.e. the concentration of agonist producing 50% of E_{max}, and a slope value. Concentration-response curves for inhibition of the purinergic component and the corresponding (same agonist, same position of pulse in pair) concentration-response curves for inhibition of the adrenergic component were always analysed together, under the assumption of either different or common E_{max} or EC_{50} values (slopes never differed and, hence, were considered identical). Differences between EC_{50} (E_{max}) values for inhibition of the purinergic component and EC_{50} (E_{max}) values for inhibition of the adrenergic component were considered significant if the assumption of identity significantly ($P < 0.05$) decreased the goodness of fit (pp. 371–372 of Motulsky & Ransnas, 1987).

Tritium overflow and contractions elicited by pulse trains

Two vasa deferentia were tied to each other at their epididymal ends and preincubated at 37°C for 60 min in 2 ml medium containing 0.2 μM (−)-[^{3}H]-noradrenaline, specific activity 43.7 Ci mmol^{-1}. They were then washed, suspended between two parallel electrodes with the lower end fixed and the upper end attached to the force transducer, and superfused for 167 min with [^{3}H]-noradrenaline-free medium at a rate of 4 ml min^{-1} by means of a roller pump.

There were six periods of electrical stimulation (Stimulator II, constant current mode). An initial stimulation period was applied at $t = 60$ min ($t = 0$ min being the start of superfusion; 120 pulses, 0.5 Hz, 1 ms pulse width, 40 mA) in order to test the viability of the preparation; the ensuing increase in tension averaged 3.30 ± 0.31 mN ($n = 34$). The subsequent five stimulation periods (S_1 to S_5) were applied at regular intervals, beginning at $t = 85$, 105, 125, 145 and 165 min. Each consisted of a train of 100 pulses at 20 Hz (0.5 ms pulse width; 40 mA; supramaximal with respect to evoked contractions). Since contraction responses to S_3–S_5 but not to S_1 and S_2 were well reproducible, only S_3–S_5 were evaluated. The superfusate was collected in 0.5- (1 or 2 sampling periods before and 4 sampling periods after onset of S_3 to S_5), 8.5- or 9-min periods from $t = 124$ min (i.e., 1 min before S_3) onwards. At the end of each experiment, tissues were solubilized in 1 ml Soluene-350 (Canberra Packard, Frankfurt am Main, Germany). Tritium was measured in superfusate samples and solubilized tissues by liquid scintillation spectrometry.

Electrically evoked contractions consisted of an initial twitch and a secondary slower and smaller phase; only the predominating twitch phase was measured. The outflow of tritium was expressed as fraction of the amount of tritium in the tissue at the onset of the respective collection period (fractional rate of outflow, min^{-1}). The stimulation-evoked overflow of tritium was calculated as the difference between the total outflow in the 1.5 min after onset of a stimulation period and the estimated basal outflow during this time. The basal outflow was assumed to decline linearly from the 0.5-min interval before, to the interval 1.5–2 min after, onset of stimulation. The electrically evoked overflow (total minus basal; nCi) was then expressed as a percentage of the tritium content (nCi) of the tissue at the start of the respective stimulation period. For evaluation of the effect of drugs that were added before S_5, ratios were calculated of basal tritium efflux immediately before S_5 (or overflow and contraction responses to S_5) over basal outflow immediately before S_4 (responses to S_4).

Materials

The following drugs were used: *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methane sulphonate (U-50488) (Biotrend, Köln, Germany), naloxone HCl (Gödecke, Freiburg, Germany), morphine HCl (Merck, Darmstadt, Germany), (−)-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 43.7 Ci mmol^{-1} (NEN, Dreieich, Germany), prazosin HCl (Pfizer, Karlsruhe, Germany), α,β-methylene-adenosine 5′-triphosphate lithium salt (α,β-methylene-ATP), [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol] enkephalin acetate (DAMGO), [D-Pen^2,D-Pen^5] enkephalin (DPDPE), tetrodotoxin (Sigma, Deisenhofen, Germany). Solutions of drugs were prepared with distilled water (tetrodotoxin: sodium acetate buffer 0.1 M, pH 4.85).

Statistics

Unless stated otherwise, arithmetic means ± s.e.mean are given. Differences between means were tested for statistical significance by the Mann-Whitney test. $P < 0.05$ or lower was taken to be statistically significant. For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni.

Results

Contractions elicited by pairs of pulses

When the vas deferens was stimulated every 2 min by pairs of pulses 2 s apart in the presence of prazosin 0.3 μM (to isolate the purinergic phase) or α,β-methylene-ATP 3 μM (to isolate the adrenergic phase), each pulse elicited a separate twitch. Twitches were abolished by tetrodotoxin 0.5 μM, thus indicating their neurogenic character ($n = 3$; not shown). The purinergic responses to pulse 1 and pulse 2 of the last pair of pulses, just before the first addition of agonist (or solvent), were similar, amounting to 2.0 ± 0.1 mN and 2.2 ± 0.1 mN, respectively ($n = 54$). The adrenergic responses, in contrast,

declined from pulse 1 to pulse 2, amounting to 1.8 ± 0.1 mN and 0.9 ± 0.1 mN, respectively (n = 52).

DAMGO, DPDPE and U-50488 all reduced both purinergic and adrenergic contractions elicited by both the first (Figure 1) and second pulse. Values derived from concentration-response curves for inhibition of both twitch 1 and twitch 2 are summarized in Table 1. DPDPE was the most potent inhibitor; DAMGO and U-50488 were about equipotent. Whereas the maximal effects of DAMGO and DPDPE were complete inhibition, the effect of U-50488 levelled off at 61 to 86% inhibition. Overall, the three opioids influenced purinergic contractions on the one hand, and adrenergic contractions on the other hand, in a very similar manner. However, EC_{50} values for inhibition of the adrenergic component were consistently somewhat lower, and for the inhibition by DAMGO and DPDPE of the first twitch this tendency reached statistical significance. Moreover, U-50488 produced greater maximal inhibition of the adrenergic than the purinergic component, and for the first twitch of the pair this difference also was significant (Table 1).

Tritium overflow and contractions elicited by pulse trains

In the absence of drugs, the fractional rate of tritium efflux immediately before S_3, the first stimulation period evaluated, amounted to 0.00302 ± 0.00012 min^{-1}, the overflow of tritium elicited by S_3 (100 pulses, 20 Hz) was 0.398 ± 0.014% of the tritium content of the tissue, contractions at S_3 averaged 13.1 ± 0.7 mN (n = 24), and basal tritium efflux as well as responses to stimulation remained approximately constant for the remainder of the experiment (n = 6). Tetrodotoxin 1 μM, added 10 min before S_5, almost abolished tritium overflow and contractions at S_5 (n = 6; not shown). Effects of morphine and naloxone are shown in Figure 2. Morphine 1 μM tended to reduce, and morphine 10 μM reduced significantly, both evoked tritium overflow and evoked contractions. Naloxone 1 μM, when added 20 min before S_3, did not by itself affect tritium overflow and contractions (S_3 and S_4; not shown) but abolished the effect of morphine (Figure 2). Morphine and naloxone did not change basal tritium efflux.

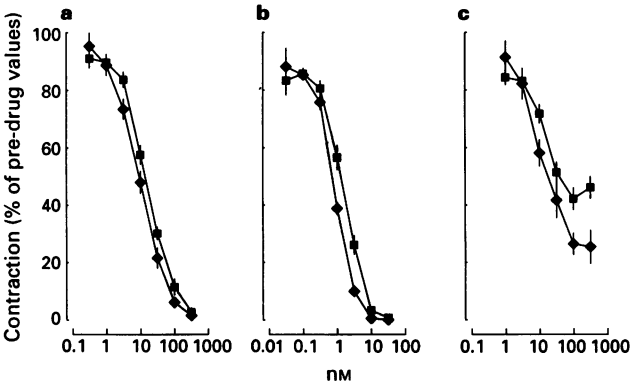

Figure 1 Effects of [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (a), [D-Pen^2,D-Pen^5]enkephalin (b) and *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide (c), on purinergic and adrenergic neurogenic contractions elicited by pulse 1 of pairs of pulses. The pulse pairs, pulse intervals 2 s, were applied every 2 min. The medium contained either prazosin 0.3 μM or α,β-methylene-ATP 3 μM throughout in order to isolate the purinergic and adrenergic phase, respectively. Non-cumulative concentration-response curves were determined by addition of increasing agonist concentrations at 30-min intervals and washout after the response had reached its maximum. Ordinate scale: purinergic (■) and adrenergic (◆) contractions elicited by pulse 1, expressed as a percentage of the contraction obtained immediately before addition of the respective agonist concentration. Means ± s.e.mean (vertical lines) from 9 or 10 experiments.

Discussion

Contraction responses of the mouse vas deferens to single or low frequency electric pulses are biphasic (McGrath, 1978), consisting of a rapid phase, now known to be purinergic, and a slow adrenergic phase. Purinergic and adrenergic components were isolated in the present study by prazosin and α,β-methylene-ATP, respectively (von Kügelgen *et al.*, 1989). The purinergic contractions changed little, whereas the adrenergic contractions fell markedly from pulse 1 to pulse 2; this pattern is probably due to the fact that transmitter release is free from prejunctional α_2-adrenergic autoinhibition at pulse 1, and that the autoinhibition at pulse 2 (which is due to noradrenaline released by pulse 1) depresses the adrenergic component far more than the purinergic component (von Kügelgen *et al.*, 1989; Bültmann *et al.*, 1991b).

Our results agree with the view that μ-, δ- and κ-receptors all are present at the sympathetic terminal axons of the mouse vas deferens: the selective agonists DAMGO, DPDPE and U-50488 reduced the neurogenic contractions at low concentrations, and the EC_{50} values obtained resemble those previously determined in the mouse vas deferens with non-cumulative agonist addition (McKnight *et al.*, 1983; Mosberg *et al.*, 1983; Corbett *et al.*, 1984; Takemori *et al.*, 1986; Berzetei *et al.*, 1987; Kramer *et al.*, 1991). In those previous studies, only effects on mixed purinergic-adrenergic contractions were examined. The present experiments show that all three opioid agonists also depressed the isolated purinergic and adrenergic contraction components. Moreover, each agonist reduced the purinergic component on the one hand,

Table 1 EC_{50} and E_{max} values for inhibition by opioid agonists of contractions elicited by pairs of pulses

Drug	Contraction component	pulse 1 EC_{50} (nM)	pulse 1 E_{max} (%)	pulse 2 EC_{50} (nM)	pulse 2 E_{max} (%)	n
DAMGO	purinergic	14.47 ± 1.30	102.0 ± 2.0	16.35 ± 1.55	102.9 ± 2.3	10
	adrenergic	9.02 ± 0.82[b]	101.8 ± 1.1	15.29 ± 2.14	102.1 ± 2.6	9
DPDPE	purinergic	1.02 ± 0.28	101.4 ± 2.8	1.08 ± 0.21	101.1 ± 2.2	10
	adrenergic	0.56 ± 0.08[a]	100.9 ± 0.6	0.99 ± 0.13	101.4 ± 1.0	10
U-50488	purinergic	12.10 ± 5.93	61.1 ± 6.6	19.25 ± 9.40	60.6 ± 6.6	10
	adrenergic	8.14 ± 3.55	85.5 ± 10.2[b]	8.41 ± 3.34	78.0 ± 10.9	10

For abbreviations, see text.

Pairs of pulses, pulse interval 2 s, were applied every 2 min. The medium contained either prazosin 0.3 μM or α,β-methylene-ATP 3 μM throughout in order to isolate the purinergic and adrenergic phase, respectively. EC_{50} and E_{max} values were calculated by simultaneous sigmoid curve fitting to data for inhibition of the purinergic component and corresponding (same agonist, same position of pulse in pair) data for inhibition of the adrenergic component; slopes but not EC_{50} and E_{max} values for the two concentration-response curves were assumed to be identical.

[a]Significant difference from corresponding value for purinergic component ($P < 0.05$).

[b]Significant difference from corresponding value for purinergic component ($P < 0.01$).

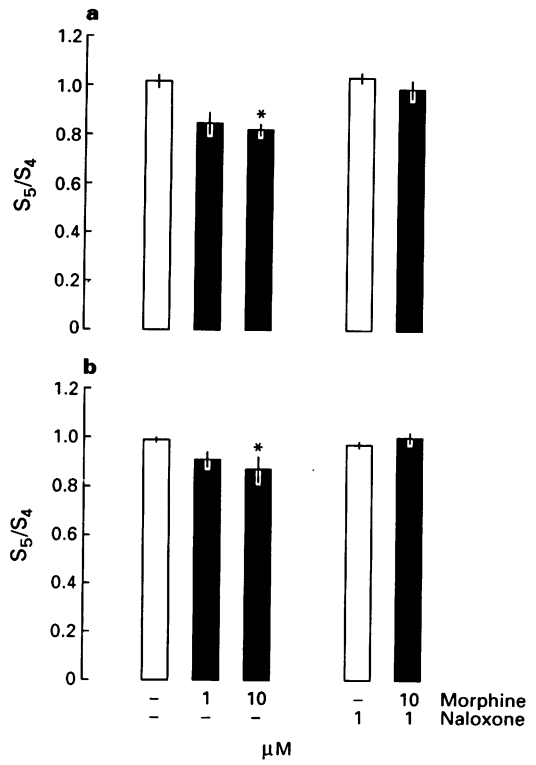

Figure 2 Effect of morphine on electrically evoked tritium overflow (a) and contraction (b) in vasa deferentia preincubated with [^{3}H]-noradrenaline, and interaction with naloxone. Tissues were stimulated 5 times with trains of 100 pulses, 20 Hz; only responses to S_3 to S_5 were evaluated. Solvent (open columns) or morphine at concentrations indicated (solid columns) was added to the medium 10 min before S_5. Naloxone 1 μM, when used, was added 20 min before S_3. Figure shows ratios of tritium overflow and of contraction elicited by S_5 over responses elicited by S_4 (S_5/S_4). Means ± s.e.mean (vertical lines) from 5 to 7 experiments.
Significant differences from corresponding control: $^*P < 0.01$.

and the adrenergic component on the other hand, in a closely similar manner, with only a few small statistically significant differences in favour of an inhibition of the adrenergic component. There was also very little difference in the inhibition of (autoinhibition-free) responses to pulse 1 and (α_2-autoinhibited) responses to pulse 2, indicating that there was little of the prejunctional opioid receptor-α_2-adrenoceptor interaction observed in other tissues (Hölting & Starke, 1986; Jackisch *et al.*, 1986; Limberger *et al.*, 1986). Although postjunctional responses are only very indirect estimates of transmitter release (see Starke, 1977), a conservative conclusion from these findings is that, so far, there is no evidence for a differential modulation by opioids, in mouse vas deferens, of the release of ATP and the release of noradrenaline. In this respect, opioid agonists may differ from several other compounds that have been suggested to modulate release of ATP and release of noradrenaline in a differential and even opposite manner (see Introduction).

It has been suggested that morphine reduces the high frequency-induced release of the non-adrenergic-non-cholinergic transmitter (i.e., ATP) in mouse vas deferens but increases the release of noradrenaline. The view was based on a differential modulation by morphine of the two phases of the tetanic contraction elicited by 100 pulses delivered at 20 Hz; moreover, morphine increased the evoked overflow of total tritiated compounds (after preincubation with [^{3}H]-noradrenaline; Forsyth & Pollock, 1988). However, the purinergic and adrenergic components of the (mixed purinergic and adrenergic) tetanus were not isolated in that study; the evoked overflow of tritium was unusual in that it declined markedly under control conditions (to about 1/30 in the course of three successive stimulation periods in one group) to be subsequently increased no less markedly by morphine (at least 30 fold in the same group; Figure 5 of Forsyth & Pollock, 1988). Our finding of a modest reduction of both contractions (non-differentiated with respect to components) and tritium overflow elicited by 100 pulses/20 Hz is more in line with the traditional view that morphine causes pure inhibition of the release of noradrenaline, the degree of inhibition declining with increasing frequency (Montel *et al.*, 1974; Henderson & Hughes, 1976). Strain differences, known for opioid effects on the mouse vas deferens (Henderson & Hughes, 1976), may explain the discrepancy.

It was planned to study the outflow of ATP simultaneously with the outflow of tritiated compounds. A measurable basal outflow and electrically evoked overflow of ATP was in fact obtained (luciferin-luciferase technique). However, the evoked overflow of ATP was very variable as also noticed by others in mouse vas deferens (Drake & Petersen, 1992). Moreover, attempts to exclude extra-neural sources for ATP (see von Kügelgen & Starke, 1991b) by suppression of evoked contractions failed, since two compounds used to block the purinergic contraction component interfered with the luciferase reaction: the P_2-purinoceptor antagonist, suramin, reduced the luminescence signal given by the luciferase reaction, and α,β-methylene-ATP gave a luminescence signal itself which in mouse vas deferens, in contrast to guinea-pig vas deferens (von Kügelgen & Starke, 1991b) greatly exceeded the signal from endogenous ATP (results not shown).

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A novel ET_A-receptor antagonist, FR 139317, inhibits endothelin-induced contractions of guinea-pig pulmonary arteries, but not trachea

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1 The effects of a proposed endothelin-receptor antagonist, FR 139317, on the contraction induced by endothelin-1, endothelin-2 and endothelin-3, were analysed on isolated circular segments of pulmonary arteries and rings of trachea from the guinea-pig.

2 The pharmacological profiles of endothelin-1 and endothelin-2 were almost identical in the guinea-pig pulmonary artery, whereas endothelin-3 demonstrated a weaker and less potent contractile effect. The contractions induced by endothelin-1 and endothelin-2 were competitively antagonized by FR 139317. Schild plot analysis revealed a straight line with a slope that did not differ from unity. The pA_2 value was 6.65. In contrast, the endothelin-3 induced contractile response was unaffected by FR 139317.

3 In tracheal segments endothelin-1, endothelin-2 and endothelin-3 evoked contractions of similar magnitude and sensitivity. FR 139317 had no effect on the endothelin-induced contractions in tracheal segments.

4 In ring segments of pulmonary artery and trachea, potassium, noradrenaline and histamine caused concentration-dependent contractile effects. These contractions were not modified by FR 139317 in the concentration range 10^{-7} to 3×10^{-6}M.

5 FR 139317 seems to be a selective ET_A-receptor antagonist which competitively antagonizes the endothelin-1- and endothelin-2-induced contractions of guinea-pig isolated pulmonary arteries. Thus, the guinea-pig pulmonary artery appears to be endowed with one receptor type (ET_A) which is antagonized by FR 139317 and with another endothelin-receptor subtype which responds to endothelin-3, but is not antagonized by FR 139317. In the trachea, all three peptides act on a homogeneous population of receptors which is unaffected by FR 139317. This suggests an ET_A-receptor in the guinea-pig pulmonary artery and another receptor, probably of ET_B-type, in the guinea-pig trachea.

Keywords: ET_A-receptors; endothelin; FR 139317; pulmonary artery; trachea

Introduction

Three endothelin genes with vasoactive products have been described in the human genome (Inoue *et al.*, 1989). The products expressed by these genes are distinct from each other but display a considerable homology. The 'original' endothelin, endothelin-1, is the product originally isolated from porcine aortic endothelial cells (Yanagisawa *et al.*, 1988a). Endothelin-2 bears a close resemblance to endothelin-1, whereas endothelin-3 differs from endothelin-1 in 6 out of 21 residues (Yanagisawa *et al.*, 1988b; Inoue *et al.*, 1989). The existence of at least two distinct endothelin-receptor subtypes has been postulated, they are termed ET_A (endothelin-1-selective; Arai *et al.*, 1990) and ET_B (equally sensitive to isopeptides of the endothelin family; Sakurai *et al.*, 1990). The endothelin family of peptides and their receptors are widely distributed both in peripheral tissues and in the central nervous system, where it has been suggested that they are involved in numerous biological responses (Nayler, 1990; Whittle & Moncada, 1990; Hemsén & Lundberg, 1991; Takayanagi *et al.*, 1991; Webb, 1991; Rubanyi, 1992).

We have previously presented pharmacological evidence for different endothelin receptor populations in the guinea-pig trachea and pulmonary artery by the use of desensitization experiments (Cardell *et al.*, 1991; 1992). The recent development of endothelin antagonists (Ihara *et al.*, 1991; Saeki *et al.*, 1991) has provided suitable tools for a more strict receptor classification. In the present study, we have examined the endothelin-induced responses of pulmonary artery and tracheal segments in relation to the new endothelin ET_A-receptor antagonist, FR 139317 (Sogabe *et al.*, 1992) in order to characterize further these receptors.

Methods

Young male guinea-pigs (200–300 g) were killed by a blow on the neck. The lungs, including the heart and trachea, were quickly removed and immersed in a cold (+4°C) buffer solution (for composition, see below). The main pulmonary artery and a distal portion of the trachea were dissected free of surrounding tissues. The vessels and the trachea were used in the experiments either immediately or, occasionally, following overnight storage in a cold buffer solution. Circular segments were mounted on two L-shaped metal prongs. One prong was connected to a force displacement transducer attached to a computer for continuous registration of isometric tension and the other to a displacement device. The mounted segments were immersed in small (2.5 ml) temperature-controlled (37°C) tissue baths containing the buffer solution. The solution was equilibrated with 5% CO_2 in O_2, giving a pH of 7.4.

Initially, a tension of 1–2 mN was applied to the arterial segments and 2–3 mN was applied to the tracheal segments. The segments were subsequently allowed to stabilize at this level of tension for 90 min. The contractile ability of each segment was then examined by exposure to a potassium-rich (60 mM) buffer solution (for composition, see below). Only when two reproducible contractions could be elicited was the individual segment used in further studies. The integrity of the vascular endothelium was assessed at the end of the

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experiments by obtaining a dilator response to 10^{-6} M acetylcholine (Furchgott, 1984). The presence of tracheal epithelium was confirmed by staining with a 5% silver nitrate solution followed by light microscopy (Abrol *et al.*, 1984). Preparations which showed signs of being without endothelium or epithelium were rejected. There were no differences in the responses to the endothelins when concentration-response curves obtained by cumulative application were compared to those obtained by a single dose procedure (Cardell *et al.*, 1990).

The responses to potassium and to the maximally effective concentration of noradrenaline and histamine were completely reversible. The responses in the absence and presence of the endothelin antagonist, FR 139317 could consequently be carried out by successive cycles of agonist exposures in each segment. This could not be done in experiments with endothelin due to the virtually sustained responses to this vasoconstrictor. Therefore, these experiments were carried out in matched pairs of segments, one segment in each series being incubated with the vehicle (0.9% saline, control) and the others incubated with the antagonist, FR 139317.

The log concentration-response relationship was approximated by linear regression analysis of the data within the 20–80% interval and the pD_2 value (i.e. the negative logarithm of the concentration eliciting half the maximum response, EC_{50}) was calculated for each experiment. Since the linear regressions in many experiments only were reliant on 3 to 4 points, the data were also fitted to a logistic hypobolic equation with tension as a function of concentration (Acheves *et al.*, 1985; Randall *et al.*, 1989):

$$t(k) = \frac{t_{max} \times k}{A + k}$$

where t represents the tension, k the concentration and A the concentration at half-maximal tension.

Since only small differences in the pD_2 were seen between the two methods (e.g. ET-1-induced contraction of the pulmonary artery; linear regression, $pD_2 = 8.11 \pm 0.28$ and fitted to a logistic equation, $pD_2 = 8.03 \pm 0.33$) only the values from linear regression are presented in the tables. E_{max}% (the maximal contraction elicited by an agonist expressed as a percentage of the contraction induced by 60 mM K^+) was calculated for each experiment. The concentration ratio (CR) was defined as the ratio of the EC_{50} value in the presence and absence of a given concentration of agonist (B). The pA_2 was calculated as described by Arunlakshana & Schild (1959) and modified by Tallarida *et al.* (1979); log (CR-1/B).

Solutions and drugs

The following solutions were used: (a) standard buffer solution (mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2, glucose 11. (b) 60 mM K^+ buffer solution: as above, but substituting equimolar amounts of NaCl with KCl. Analytical grade chemicals and twice-distilled water were used for preparing all solutions.

The following drugs were used: FR 139317, ((**R**)2-[(**R**)-2-[(**S**)-2-[[1-(hexahydro-1H-azepinyl)]-carbonyl]amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1H-indolyl)]propionyl]amino-3-(2-pyridyl)propionic acid), an endothelin ET_A-receptor antagonist, kindly donated by Dr Jo Mori, Fujisawa Pharmaceutical Co, Osaka, Japan; acetylcholine chloride (Sigma Chemical Co., St Louis, MO, U.S.A.), endothelin-1, endothelin-2, endothelin-3 (Peninsula Laboratories, Mountain View, CA, U.S.A.), histamine dihydrochloride (Sigma, Chemical Co., St. Louis, MO, U.S.A.) and noradrenaline hydrochloride (Sigma Chemical Co., St Louis, MO, U.S.A.). All agents were dissolved in, and further diluted in, saline containing 1% bovine serum albumin (Behringwerke, Marburg, Germany) and used in the experiments within 30 min to avoid any possible degradation. The concentrations of the agents are expressed as the final molar concentration in the tissue bath.

Statistics

Statistical differences between means were tested by analysis of variance (ANOVA; Wallenstein *et al.*, 1980). Statistical significance was assumed when $P<0.05$.

Results

Endothelin-1, endothelin-2 and endothelin-3 elicited strong concentration-dependent contractions of the pulmonary artery and the trachea. In the artery, endothelin-1 and endothelin-2 were equipotent, with endothelin-3 being significantly less potent ($P<0.05$). In the trachea, all three isopeptides were equipotent (Table 1). In the pulmonary artery, the endothelin-1- and endothelin-2-induced responses were shifted in parallel to the right by FR 139317 in concentrations between 1×10^{-7} M and 3×10^{-6} M, without any reduction in the maximal response (Figures 1a, 2a; Table 2). An FR 139317 concentration of 1×10^{-6} M caused a rightward shift with a concentration ratio of 9.9 ± 5.3 for endothelin-1 and 10.9 ± 3.2 for endothelin-2. FR 139317 (3×10^{-6} M) caused a further parallel shift to the right with a concentration-ratio of 18.3 ± 9.7 for endothelin-1.

In contrast, FR 139317 (1×10^{-6} M, 3×10^{-6} M or 10^{-5} M) did not affect the endothelin-3 induced concentration-response curve for the pulmonary artery (Figure 2b), not did this antagonist alter the log concentration-response curves induced by endothelin-1, endothelin-2 or endothelin-3 in the trachea (Tables 1 and 2). The maximal responses as well as the pD_2 values were identical both with and without FR 139317 (Figure 1b, Table 1).

The rightward displacement of the endothelin-1 induced

Table 1 Guinea-pig pulmonary artery and trachea: maximal responses and pD_2 values for the endothelins

Pulmonary artery		*Control*			*With FR139317 (10^{-6} M)*	
	n	E_{max}%	pD_2	n	E_{max}%	pD_2
Endothelin-1	20	172 ± 29	8.11 ± 0.28	7	174 ± 42	7.25 ± 0.42*b
Endothelin-2	6	150 ± 34	8.08 ± 0.19	6	137 ± 25	7.10 ± 0.23*c
Endothelin-3	7	78 ± 46	7.24 ± 0.23*a	6	92 ± 49	7.22 ± 0.19
Trachea		*Control*			*With FR139317 (10^{-6} M)*	
	n	E_{max}%	pD_2	n	E_{max}%	pD_2
Endothelin-1	11	78 ± 16	7.66 ± 0.31	8	69 ± 16	7.53 ± 0.15
Endothelin-2	7	81 ± 16	7.86 ± 0.41	7	80 ± 36	7.74 ± 0.35
Endothelin-3	8	71 ± 19	7.89 ± 0.26	6	81 ± 32	7.82 ± 0.11

Maximal responses (E_{max} %) are expressed as a percentage of the contraction induced by 60 mM potassium and sensitivy (pD_2) is expressed as the negative logarithm of the concentration eliciting half the maximum response. The values represent the mean ± s.d. *a$P<0.05$, endothelin-3 vs. endothelin-1/endothelin-2; *b$P<0.05$, endothelin-1 vs. endothelin-1 + FR139317; *c$P<0.05$, endothelin-2 vs. endothelin-2 + FR139317.

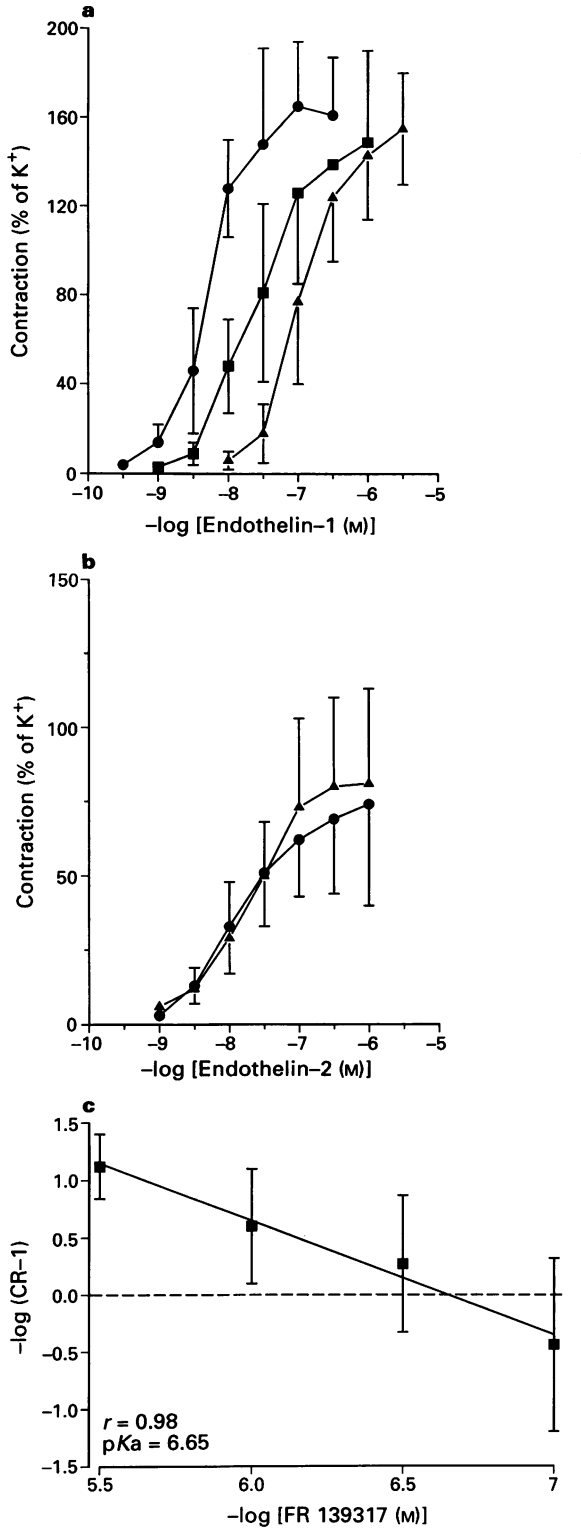

Figure 1 Concentration-response relations for endothelin-induced contractions in the presence of increasing concentrations of the endothelin ET_A receptor antagonist, FR139317. Pulmonary artery (a) and trachea (b): (●) endothelin-1/-2 control; (■) endothelin-1 + FR 139317 (3×10^{-7} M) and (▲) endothelin-1/-2 + FR 139317 (3×10^{-6} M). Responses are expressed as a percentage of the contractions induced by 60 mM potassium and each point is the mean with the s.d. shown by vertical bars ($n = 6-20$). (c) Schild plot for FR 139317 acting at the proposed ET_A-receptor in guinea-pig pulmonary artery. CR is the concentration ratio.

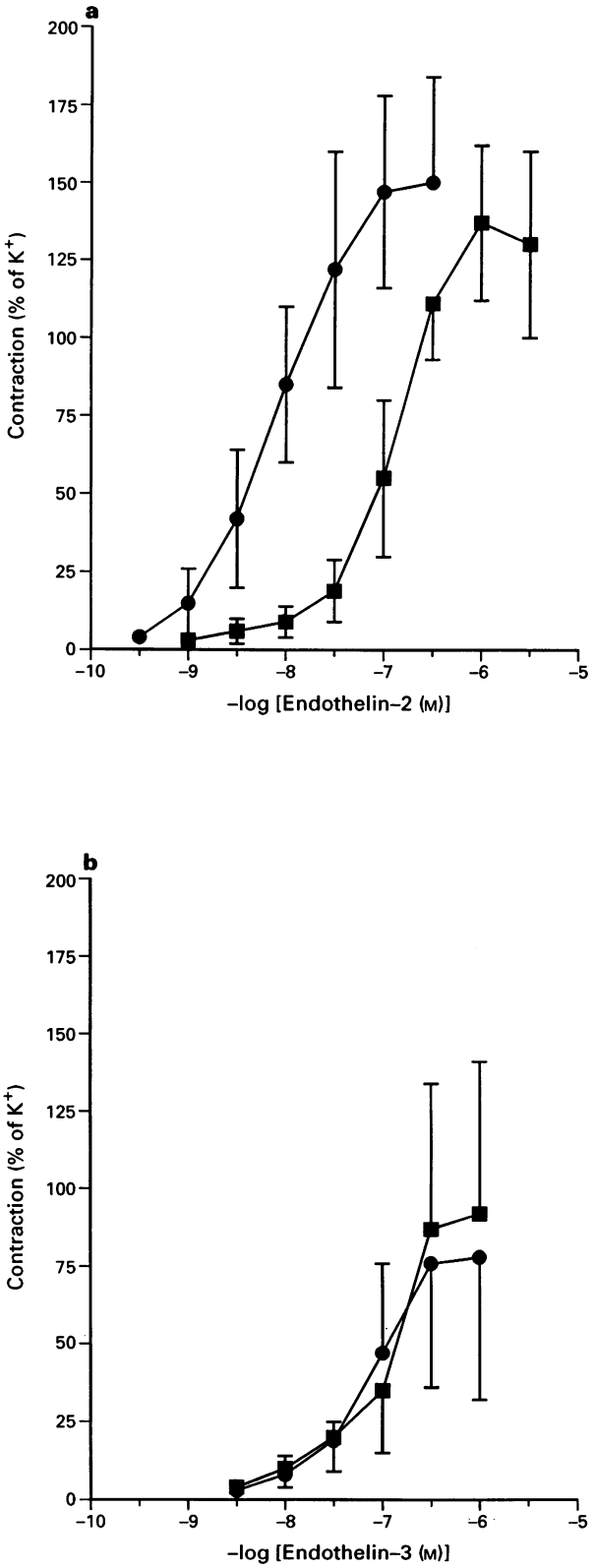

Figure 2 Concentration-response relations for endothelin-2 (a) and endothelin-3 (b) in guinea-pig pulmonary artery in the presence of the endothelin receptor antagonist FR 139317: (●) control and (■) endothelin + FR 139317 (10^{-6} M). Responses are expressed as a percentage of the contraction induced by potassium (60 mM) and each point is the mean with the s.d. shown by vertical bars ($n = 6-7$).

concentration-response curves caused by FR 130317 was used in a Schild analysis. The concentration-ratios for this antagonist yielded a line with the slope of 0.98, suggesting a simple competitive antagonism at one receptor site. The resulting pA_2 for the ET_A-receptor antagonist was 6.65 (Figure 1c).

Table 2 Effects of different concentrations of FR139317 on endothelin-1 induced responses in guinea-pig pulmonary artery

Pulmonary artery		n	K^+	E_{max}%	pD_2
ET	Control	20	3.81 ± 1.79	172 ± 29	8.11 ± 0.28
+ FR 139317	(10^{-7} M)	6	3.97 ± 2.45	150 ± 40	7.87 ± 0.28
+ FR 139317	(3×10^{-7} M)	7	3.08 ± 1.39	154 ± 45	7.58 ± 0.37
+ FR 139317	(10^{-6} M)	7	2.75 ± 0.81	174 ± 42	7.25 ± 0.42
+ FR 139317	(3×10^{-6} M)	6	2.79 ± 0.77	158 ± 29	6.95 ± 0.26
ANOVA			NS	NS	*

Maximal responses (E_{max} %) are expressed as a percentage of the contraction induced by 60 mM potassium and sensitivity (pD_2) is expressed as the negative logarithm of the concentration eliciting half the maximum response. The values represent the mean ± s.d.; *$P < 0.05$; NS, not significant.

Table 3 Guinea-pig pulmonary artery: maximal response to potassium, noradrenaline and histamine

Pulmonary artery		*Control*			*With FR139317 (10^{-6} M)*		
Potassium (60 mM)	6	5.42 ± 3.82 mN			6	5.69 ± 3.66 mN	
	n	E_{max}%	pD_2		n	E_{max}%	pD_2
Noradrenaline	5	139 ± 44	4.69 ± 0.13		5	140 ± 29	4.61 ± 0.14
Histamine	7	119 ± 31	5.39 ± 0.21		7	128 ± 28	5.30 ± 0.14

Maximal responses (E_{max}%) are expressed as a percentage of the contraction induced by 60 mM potassium and sensitivy (pD_2) is expressed as the negative logarithm of the concentration eliciting half the maximum reponse. The values represent the mean ± s.d.

The FR 139317 alone (1×10^{-6} M to 1×10^{-5} M), did not cause any contraction of isolated artery or tracheal segments. Furthermore, this antagonist, even at high concentrations (3×10^{-6} M), did not alter the vascular and tracheal constrictions induced by potassium, noradrenaline or histamine (Table 3).

Discussion

The present study demonstrates that the contractile responses to endothelin-1 and endothelin-2 in guinea-pig isolated pulmonary arteries could be shifted in parallel to the right, in a concentration-related manner, by the endothelin receptor antagonist, FR 139317. Quantitation of the antagonism, using Schild analysis, revealed that this compound consistently exhibited a competitive type of antagonism against endothelin-1 and endothelin-2. Furthermore, FR 139317 did not alter pulmonary artery vasoconstriction induced by endothelin-3, nor did it affect the tracheal smooth muscle contraction induced by endothelin-1, endothelin-2 or endothelin-3. FR 139317 seems to be a specific endothelin antagonist since it did not affect the contractions induced by noradrenaline, histamine and potassium.

Different pharmacological profiles for endothelin-1, endothelin-2 and endothelin-3 resulted in the suggestion that there is more than on receptor subtype for endothelins (Inoue *et al.*, 1989; Yanagisawa & Masaki, 1989). This assumption gained further support from ligand binding studies (Masuda *et al.*, 1989; Watanabe *et al.*, 1989). Subsequent work with cloned receptors revealed the existence of two distinct endothelin receptor subtypes termed ET_A (selective for endothelin-1 relative to endothelin-3) (Arai *et al.*, 1990) and ET_B (non-selective with respect to endothelin-1, endothelin-2 and endothelin-3) (Sakurai *et al.*, 1990). The existence of a third 'endothelin-3-selective' receptor has also been suggested (Webb 1991; Cardell *et al.*, 1992). Definitive pharmacological confirmation of the subtypes of the endothelin receptor has to await the development of selective antagonists.

Recently two pentapeptides (BQ 123 and BQ 153), synthesized by amino acid substitutions of a novel natural endothelin-receptor antagonist BE 18257, were reported to be associated with the ET_A-receptor activity in the porcine coronary artery (Ihara *et al.*, 1991; Atkinson & Pelton, 1992). A small amount of endothelin-1 induced vasoconstriction remained resistant to this compound which led to the suggestion that both ET_A and ET_B receptors were responsible for the endothelin-1-induced vasoconstriction of isolated coronary arteries of the pig. The antagonists reduced the endothelin-1-induced pressure response in a concentration-dependent manner but not the depressor responses and did not affect the blood pressure of rats *in vivo* (Ihara *et al.*, 1991). The acyclic analogue [$Ala^{1,3,11,15}$]endothelin-1, has been reported to be a weak, but selective ET_B-receptor ligand (Saeki *et al.*, 1991). However, other reports state that this tetra-alanyl substituted analogue is equipotent with endothelin-1 at inhibiting the binding of [^{125}I]-endothelin-1 (Hiley *et al.*, 1990). FR 139317 has been shown to inhibit the specific binding of [^{125}I]-endothelin-1 to porcine and human aortic microsomes, but exhibits only a low affinity for endothelin-1 binding sites in porcine brain (Sogabe *et al.*, 1992). In rabbit isolated aorta, FR 132317 shifts the endothelin-1-induced concentration-response to the right and, *in vivo*, this antagonist completely inhibits the pressor response to endothelin-1 in normotensive rats, without any effect on the initial depressor response (Sogabe *et al.*, 1992).

We have previously shown that endothelin-1 and endothelin-2 concentration-dependently contract guinea-pig isolated pulmonary vessels. Endothelin-3 also induces contraction but with less potency. In contrast, endothelin-1, endothelin-2 and endothelin-3 show equal potency in inducing contractions of tracheal segments. By use of a pharmacological desensitization technique, two types of functional endothelin receptors could be demonstrated. In the pulmonary artery an endothelin-1/endothelin-2 receptor was found, while a non-isopeptide-selective type of endothelin receptor was found in the trachea (Cardell *et al.*, 1991; 1992). In the same smooth muscle preparations, FR 139317 strongly inhibited endothelin-1 and endothelin-2 induced vasoconstriction whereas the endothelin-induced contractions of isolated tracheal segments were unaffected. These results are in agreement with previous desensitization experiments (Cardell *et al.*, 1991; 1992) and suggest that FR 139317 is a potent ET_A-receptor selective antagonist. Furthermore, the guinea-pig pulmonary artery is dominated by an ET_A-receptor, while another type of receptor, putatively an ET_B-receptor, is found in the trachea. The possibility of a third endothelin-3 related endothelin receptor in the guinea-pig pulmonary artery cannot be excluded.

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Two distinct cytosolic calcium responses to extracellular ATP in rat parotid acinar cells

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1 Increasing concentrations of ATP (0.5 μM–300 μM) produced a biphasic increase in intracellular calcium concentration $[Ca]_i$ in rat parotid acinar cells, reflecting two distinct Ca_i responses to extracellular ATP.

2 In the absence of Mg^{2+} (with 3 mM $CaCl_2$ in the buffer solution), the more sensitive response was maximal at 3–5 μM and was not further increased by 30 μM ATP. This response to ATP was not well maintained and was blocked by ADP (0.5 mM). A second, much larger increase in Ca_i was observed on addition of 300 μM ATP. This larger effect, which we have described previously, appears to be mediated by ATP^{4-}, and was selectively reversed by 4,4'-di-isothiocyanato-dihydrostilbene-2,2'-disulphonate as well as by high concentrations of α,β-methylene ATP.

3 Among ATP analogues, only the putative P_{2Z} agonist, 3'-0-(4-benzoyl)benzoyl-ATP distinguished between the two responses. This analogue was at least 10 fold more potent than ATP in stimulating the ATP^{4-}-response, but did not evoke the more sensitive response. The agonist potency series for both responses to ATP was identical for other analogues examined (ATP > ATPγS = 2-methylthio ATP (a P_{2Y}-selective agonist) >> ADP, ITP and α,β-methylene ATP (a P_{2X}-selective agonist)).

4 Although the effect of ATP^{4-} could best be characterized as a P_{2Z}-type purinoceptor response, this effect was strongly and selectively blocked by reactive blue 2, a putative P_{2Y}-purinoceptor antagonist. Reactive blue 2 may bind to and block P_{2Z} purinoceptors since [γ^{32}P]-ATP binding to parotid cells was inhibited by this compound.

5 In contrast to the response to ATP^{4-}, the more sensitive response to ATP was potentiated by reactive blue 2 and was less affected by increases in external Mg^{2+} and Ca^{2+}.

6 Parasympathetic denervation selectively increased the more sensitive response, suggesting that it may be physiologically regulated.

Keywords: Purinergic; P_{2Z}-receptor; rat parotid cells; intracellular calcium; nonspecific cation channel; benzoylbenozyl ATP; ATP responses; 4,4'-diisothiocyanato stilbene-2,2'-disulphonate (DIDS); reactive blue 2; parasympathetic denervation

Introduction

ATP is stored and released with biogenic amine neurotransmitters and may itself act as a neurotransmitter or neuromodulator (Burnstock, 1986; Evans *et al.*, 1992). Extracellular ATP elevates the intracellular free calcium concentration $[Ca]_i$ in many tissues, in some cases by activating phospholipase C (Boyer *et al.*, 1989; Boyer & Harden, 1989), and in others by directly gating calcium-permeable ion channels (Benham & Tsien, 1987; Friel & Bean, 1988; Diverse-Pierluissi *et al.*, 1991). ATP-stimulated increases in Ca_i may modulate physiological responses and provide a simple index for characterizing ATP receptors and their mechanisms of action.

Previously we reported that ATP specifically and reversibly elevates Ca_i in rat parotid acinar cells in a manner consistent with activation of P_2-purinoceptors (McMillian *et al.*, 1987a; 1988; Soltoff *et al.*, 1989; 1990a). The effect of ATP on Ca_i is inhibited by increasing the concentration of divalent cations, suggesting that ATP^{4-} is the active species (McMillian *et al.*, 1987a). This purinoceptor effect is reversed on breakdown of ATP to ADP (McMillian *et al.*, 1987a; 1988). We have shown previously that the calcium response in rat parotid acinar cells is not activated by most ATP analogues (McMillian *et al.*, 1987a) and that non-hydrolyzable analogues and ADP are inactive or only weakly active on ATP-stimulated ion fluxes as well (Soltoff *et al.*, 1990b). Pretreatment of cells with 4,4'-diisothiocyanato stilbene-2,2'-disulphonate (DIDS) specifically inhibits the ATP response in parotid cells and also inhibits [α^{32}P]-ATP binding, consistent with an action of DIDS at the parotid ATP receptor complex (McMillian *et al.*, 1988; Soltoff *et al.*, 1990b). ATP elevates Ca_i in parotid cells by a mechanism which differs from that shared by muscarinic, substance P receptors and α-adrenoceptors, all of which activate phospholipase C. ATP has little effect on [^{3}H]-inositol phosphate accumulation, and electrophysiological effects of ATP do not require guanine nucleotide binding proteins, in contrast to muscarinic effects (McMillian *et al.*, 1988; Soltoff *et al.*, 1990a). Effects of ATP on Ca_i in parotid cells are additive with the effects of other calcium-mobilizing agonists, indicating that ATP mobilizes a calcium pool distinct from that mobilized by phospholipase C-linked receptor agonists (McMillian *et al.*, 1988; Soltoff *et al.*, 1990a). A similar phospholipase C-independent increase in Ca_i in response to ATP has been reported in a number of other cell types (Richards *et al.*, 1987; Buisman *et al.*, 1988; El-Moatassim *et al.*, 1989; Sasaki & Gallacher, 1990; Li *et al.*, 1991). ATP has a much weaker effect on amylase release from parotid cells than do muscarinic and α-adrenoceptor agonists and substance P, probably due to its failure to activate protein kinase C, which may play an important role in exocytosis in these cells (McMillian *et al.*, 1988). The mechanism of action of ATP in parotid cells is unknown, but present data are consistent with an ATP-gated non-selective cation channel (Soltoff *et al.*, 1992).

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ATP receptors (P_2-purinoceptors) differ from adenosine receptors (P_1-type), being much less sensitive to blockade by methylxanthines (Burnstock, 1986). There appear to be several subtypes of ATP receptors (Gordon, 1986). Smooth muscle P_2-purinoceptors have been subdivided into P_{2x} and P_{2y} types on the basis of differential sensitivity to the agonists, α,β-methylene ATP and 2-methylthio ATP, and to the antagonists arylazidoaminoproprionyl ATP ($ANAPP_3$) (Fedan et al., 1985) and reactive blue 2 (Burnstock & Warland, 1987; Houston et al., 1987), respectively. P_{2x}-receptors respond with a potency order α,β-methylene ATP > ATP > 2-methylthio ATP; these receptors rapidly desensitize in response to α,β-methylene ATP (and to a lesser extent with ATP) and also appear more sensitive to inactivation by $ANAPP_3$ (Burnstock & Kennedy, 1985). In contrast, P_{2y}-receptors display the reverse potency order and are more sensitive to blockade by reactive blue 2 (Houston et al., 1987). A third type of purinoceptor, designated P_{2z}, requires uncomplexed ATP (ATP^{4-}) for activation; in physiological buffer solutions most of the ATP is complexed with divalent cations and consequently higher concentrations of ATP are required to stimulate this receptor (Cockroft & Gomperts, 1980; Gordon, 1986). The P_{2z}-receptor appears to be coupled to cellular permeabilization to solutes of a restricted size in mast cells (Tatham et al., 1988; Greenberg et al., 1988), and in some cell lines (Weisman et al., 1984; Gonzalez et al., 1989), but this does not appear to be the case in rat parotid acinar cells (McMillian et al., 1988). It has been suggested that benzoylbenzoyl ATP [3'-0-(4-benzoyl)benzoyl-ATP] is a selective P_{2z} agonist (Erb et al., 1990), although recent evidence suggests that this compound also activates P_{2y}-receptors (Boyer & Harden, 1989).

The Ca_i response to ATP in parotid cells is characterized by a broad cumulative concentration-response curve. A small response is apparent at less than 5 μM ATP (regardless of Mg^{2+} concentration) and a larger effect is obtained only at much higher concentrations of ATP (50–300 μM, depending on Mg^{2+} concentrations) (McMillian et al., 1987a; 1988). Recently, with a change in collagenase and trypsin lots, our parotid cell preparations show higher sensitivity to ATP. In the present study we examined the characteristics and pharmacology of the more sensitive Ca_i response to ATP and further characterized the response to ATP^{4-}, enabling us to assign these responses to subtypes of the P_{2z}-purinoceptor class. These results have recently been published as an abstract (McMillian et al., 1990).

The physiological responses to neurotransmitter stimulation of rat parotid acinar cells are altered by denervation of the gland. After parasympathetic denervation of the parotid gland, a heterologous supersensitivity develops to carbachol, noradrenaline and substance P (Ekstrom, 1980; Ekstrom & Wahlestedt, 1982). The effect of parasympathetic denervation on the response to ATP also was investigated to see whether the purinoceptor response is similarly regulated.

Methods

Cell preparations

Parotid glands were removed from male Sprague/Dawley rats (Taconic Farms, Germantown, NY; 150–250 g) and acinar cells were prepared as previously described (McMillian et al., 1988). The small, more sensitive response to ATP became much larger when different dispersing enzyme lots were used (Worthington collagenase CLS1 No. 4177, lot No. 49A095) and trypsin (Sigma, Cat No. T-8253, lot No. 18F-0828). Previous studies were on cells prepared with collagenase (Worthington, CLS Type 2, No. 14174, lot No. 48E295) and trypsin (Sigma); some data obtained with earlier preparations are included since a relatively pure response to ATP^{4-} was obtained. For supersensitivity studies, unilateral parasympathetic postganglionic denervations were performed as previously described (Talamo et al., 1979), and the cells were prepared from denervated and contralateral control parotid gland three to five weeks later.

Ca_i measurements

Cells were loaded with fura-2 acetoxymethyl ester as previously described (McMillian et al., 1987b), and suspended in appropriate medium in a cuvette, with continuous stirring. Ca_i was estimated by determining the ratio of fluorescence emitted at 505 nm when excited at 340 and 380 nm, assuming a K_d of 224 nM for fura-2. Ratios were determined on a Perkin-Elmer LS5 spectrofluorimeter equipped with a computer and a fura-2 programme which allowed ratios to be obtained every 7–8 s. Drug additions were made to the cuvette on-line, with continuous recording. In each run, a correction was made for dye leakage by addition of 250 μM $MnCl_2$ which was then chelated with diethylenetriamine penta acetic acid (DTPA, 1 mM); maximum fluorescence was defined by the addition of digitonin (50 μM) and minimum fluorescence was determined with 100 mM EGTA (McMillian et al., 1987a,b). In some experiments, fluorescence was directly recorded during excitation at 340 nm. In all experiments where Mg^{2+} was added to reverse the effects of ATP, the concentration of Mg^{2+} was 10 mM. All experiments were repeated at least two times with similar results, or more times as indicated.

[$\gamma^{32}P$]-ATP binding

Binding of [$\gamma^{32}P$]-ATP to parotid cells was determined as previously described for [$\alpha^{32}P$]-ATP (McMillian et al., 1988). When present, reactive blue 2 was preincubated with cells for 10 min prior to initiation of the binding reaction with radiolabelled ATP. Binding was carried out for 10 min and cells were then sedimented and washed. Triplicate assays were carried out for each condition in each experiment.

Materials

$ANAPP_3$ was a kind gift from Dr J.S. Fedan; ryanodine, octanol and hexanol were gifts from Dr K. Dunlap. MK801, ketamine and [$\gamma^{32}P$]-ATP (New England Nuclear) were gifts from Drs C. Harrington, J. Kauer & D. Chikaraishi, respectively. 2-Methylthio ATP was purchased from Research Biochemicals Incorporatated; DIDS and 4,4'-diisothiocyanato-dihydrostilbene-2,2'-disulphonate (dihydroDIDS) were from Molecular Probes; ATP and adenosine-5'-O-(3-thiotriphosphate) (ATPγS) were from Boehringer Mannheim. DTPA was obtained from Fluka Chemie AG. Reactive blue 2 (Cibacron blue), benzoylbenzoyl ATP, ATP dialdehyde (adenosine 5'-triphosphate-2',3'-dialdehyde) and α,β-methylene ATP and other drugs were obtained from Sigma, unless otherwise indicated.

Results

Biphasic Ca_i response to ATP

The Ca_i response to ATP in rat parotid cells was characterized by a broad concentration-response curve, even under conditions optimal for eliciting the effect of ATP^{4-} (1 mM $CaCl_2$, 0 $MgCl_2$). At a higher calcium concentration (3 mM $CaCl_2$, 0 $MgCl_2$), a biphasic cumulative concentration-response curve for ATP was clearly evident (Figure 1a). A small, potent ATP-effect (half-maximal at about 1 μM, maximal at about 3–5 μM) could be distinguished from the effect of ATP^{4-} (which became apparent when the concentration of ATP was increased from 60 to 300 μM) (Figure 1a). The rate of increase in Ca_i in cells exposed to concentrations of ATP higher than 300 μM was slower than the response to low concentrations of ATP. While the Ca_i response to low con-

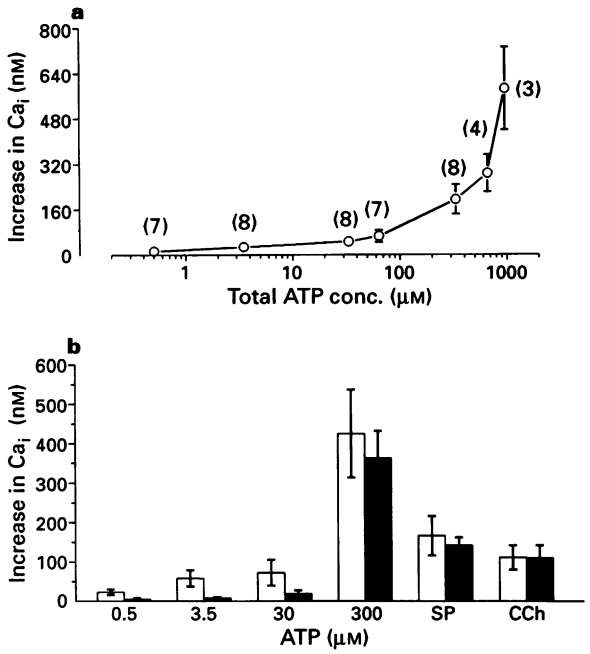

Figure 1 (a) ATP concentration-response curve for Ca_i elevation: The increase in Ca_i over baseline (mean ± s.e.mean) was determined for cumulative ATP concentrations ranging from 0.5–963 μM. At each data point, the number of independent experiments is shown in parentheses. For all experiments, the maximum reached within the first 3 data points (about 22 s) after addition of agonist is taken, although the Ca_i continues to rise slowly for ATP concentrations greater than 300 μM. The calculated concentration of ATP^{4-} (3 mM Ca^{2+}, 0 mM Mg^{2+}) (Fabiato, 1988) is given in parentheses following each value of total added ATP: 0.5 μM ATP (0.023 μM ATP^{4-}), 3.5 μM (0.14 μM), 33.5 μM (1.6 μM), 63 μM (3 μM), 363 μM (19 μM), 663 μM (38 μM), 963 μM (61 μM). Higher concentrations of ATP were not tested because substantial amounts of extracellular calcium are chelated. (b) Effect of ADP on Ca_i responses to ATP, carbachol (CCh) and substance P (SP): The Ca_i response to various agonists was determined in the presence and absence of ADP (0.5 mM). Peak responses are shown for each agonist, $n = 3$ experiments. Ca^{2+} and Mg^{2+} concentrations were 3 and 0 mM respectively. Open columns are controls. Solid columns show responses in the presence of ADP. Note that 30 μM ATP is not significantly more effective than 3.5 μM ATP and that ADP blocks effects of low concentrations of ATP but not effects of 300 μM ATP or of SP (0.1 μM) or CCh (100 μM).

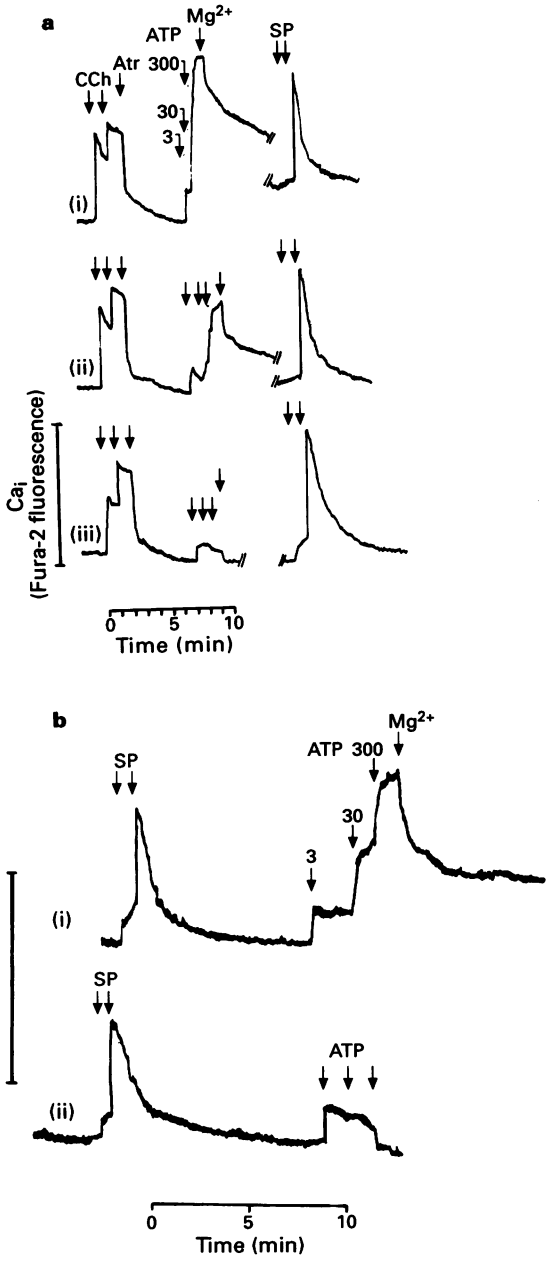

Figure 2 Separation of the two ATP-induced Ca_i responses with (a) Mg^{2+} and (b) 4,4'-diisothiocyanato stilbene-2,2'-disulphonate (DIDS). Ca_i responses to different agonists were tested in the presence of various concentrations of Mg^{2+} or DIDS. Ca^{2+} concentration was 1 mM in each experiment. Where additions are indicated by arrows, the concentrations are: carbachol (CCh) = 3 μM; 100 μM. Atropine (Atr) = 10 μM. Substance P (SP) = 30 pM; 100 nM. Mg^{2+} = 10 mM. ATP concentrations (in μM) are indicated in the figure. Agonists are added in the same sequence in each experiment. These traces are representative of ten or more experiments. (a)i is the control. Mg^{2+} concentrations in the cell suspension medium are: (i) 0; (ii) 1 mM; (iii) 10 mM. Breaks in traces represent time for the ATP response to reverse after high Mg^{2+} addition. This break represents 20 min for (i), 5 min for (ii), and 0 min for (iii). The presumed effect of ATP^{4-} is blocked by increasing concentrations of Mg^{2+}, while the responses to a low concentration of ATP, and CCh and SP responses are unaffected. (b) DIDS selectively blocks the effect of a high concentration of ATP while a low concentration of ATP, and SP, are unaffected. (b)(i) is the control; (b)(ii) Cells were pretreated with 150 μM DIDS for 40 min and washed before assay.

centrations of ATP reached a peak value within 15–23 s, the response to high concentrations continued to increase. We usually added Mg^{2+} within 45–60 s after the addition of concentrations of ATP higher than 300 μM, because reversal was slowed at these high concentrations and because cell damage ensued after prolonged exposure to high Ca_i . The responses in Figure 1a were calculated as the maximal increases in Ca_i within the first 22 s (3 data points) of addition of ATP rather than the maximal elevation in Ca_i after prolonged exposure to the agonist. The maximum peak Ca_i value of the more sensitive response to ATP was typically smaller than the Ca_i responses to maximal concentrations of substance P or the muscarinic agonist, carbachol. These phospholipase C-linked receptor agonists served as useful controls in these studies. Additional manipulations with various agents also demonstrated that the two responses could be distinguished. ADP (0.5 mM), which produced a much smaller Ca_i increase (6 ± 6 nM, $n = 4$) than did 3.5 μM ATP (75 ± 27 nM, $n = 4$) in the same cell preparation, blocked the potent effect of ATP without obviously affecting the Ca_i response to ATP^{4-} (or carbachol or substance P) (Figure 1b). Even when the responses to 3 μM carbachol and 3 μM ATP were of the same magnitude (Figure 4a), ADP blocked only the response to ATP (Figure 4f). The more sensitive response to ATP was not blocked by high concentrations of Mg^{2+} (Figure 2a) (nor Ca^{2+} (Figure 4e)), which lowers the free ATP^{4-} concentration. The concentration of ATP^{4-} available at 3, 30 and 300 μM total ATP in the presence of 1 mM Ca^{2+} and 0, 1 or 10 mM Mg^{2+} was calculated to be (0.36, 3.6,

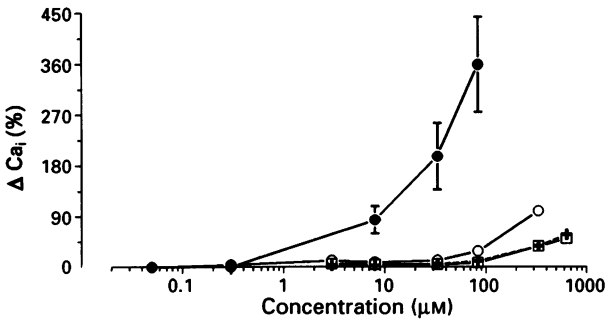

Figure 3 Benzoylbenzoyl ATP is a selective agonist for the ATP^{4-} response. ATP and various analogues were tested at different concentrations for their ability to elevate Ca_i. Each point represents the mean and s.e.mean (vertical bars) of three or more independent experiments in the presence of 1 mM Ca^{2+} and no added Mg^{2+}. Where error bars are not shown, they fall within the borders of the symbol. The increment in Ca_i (Δ Ca_i (%)) is given relative to the response to 300 μM ATP, which is set at 100%. Symbols are: benzoylbenzoyl ATP (●); ATP (○); ATPγS (+); 2-methylthio ATP (□). Benzoylbenzoyl ATP was more potent and more effective at increasing Ca_i than ATP or other ATP analogues (2-methylthio ATP and ATPγS). ATP and other analogues produced a small increase in Ca_i at low concentration, evidenced by the long, shallow concentration-response curve. Benzoylbenzoyl ATP was ineffective in stimulating the more sensitive response and the concentration-response curve was thus much steeper than that of other analogues examined.

43 μM), (0.15, 1.3, 15 μM) or (0.02, 0.19, 2.0 μM) respectively (Fabiato, 1988). Additionally, the more sensitive ATP response was not blocked by pretreatment of cells with DIDS (Figure 2b).

Effects of ATP analogues

Analogues of ATP were examined for effects on Ca_i. Among those analogues tested, only benzoylbenzoyl ATP was more potent than ATP as a stimulus for the response to ATP^{4-}. The maximum effective concentration of benzoylbenzoyl ATP could not be determined in these experiments since concentrations much higher than 30 μM quenched the fura-2 signal. Under conditions that favoured the effect of ATP^{4-} (0 Mg^{2+}, 0.5–1 mM Ca^{2+}), this putative P_{2z}-purinoceptor agonist was at least 30 fold more potent than ATP, which in turn was approximately 10 fold more potent than the P_{2y} agonist, 2-methylthio ATP (Figure 3). Benzoylbenzoyl GTP (0.3 μM–300 μM) was ineffective ($n = 2$). ATPγS was approximately equipotent to 2-methylthio ATP. It was not possible to examine a full range of concentrations for some of the analogues on the ATP^{4-}-response because extracellular calcium was complexed at high concentrations of analogues, but ADP, ITP, GTP and 8-Br ATP had little effect even at 1 mM (<5% of the response to 300 μM ATP; $n = 3$ or more for each compound). α,β-Methylene ATP had no effect on Ca_i in parotid cell suspensions ($n = 7$).

Under conditions selective for the more sensitive response (10 mM Ca^{2+} and 0 Mg^{2+}, or 300 μM DIDS pretreatment), ATP was more effective than any of the analogues. Benzoylbenzoyl ATP (0.3–30 μM) had no stimulatory effect ($n = 4$ cell preparations). Although it was difficult to quantify due to the small magnitude of the more sensitive effect, the potency order for other analogues appeared similar to that found for ATP^{4-}. Concentrations of 30–100 μM ATPγS and 2-methylthio ATP were required to produce effects similar to that obtained with a maximally effective concentration of ATP (3 μM). At 1 mM, ITP and ADP were 10–20% as effective but 8-Br ATP appeared inactive ($n = 3$ preparations for each compound). The non-hydrolyzable analogue adenosine 5'-(β,γ-imido)triphosphate (AppNHp) was approximately 10% as effective as ATP on both Ca_i responses at concentrations 10 fold higher than that required for maximal ATP effects ($n = 3$). Compounds known to block purinoceptor responses in other systems, $ANAPP_3$ (1 mM), 8-azido ATP (1 mM) and 5'-fluorosulphonyl-benzoyladenosine (5 mM), had no effect on either response to ATP in parotid cells even after activation with u.v. light ($n = 3$ or more experiments with each compound).

Reactive blue 2 selectively blocks the ATP^{4-} effect

Reactive blue 2, a putative P_{2y}-type purinoceptor inhibitor, was found to block specifically the response to ATP^{4-} under conditions which allowed the two responses to ATP to be easily separated (3 mM Ca^{2+}, 0 Mg^{2+}) (Table 1). This blocking effect is readily apparent in Figure 4. Reactive blue 2 (Figure 4b,c,d) or high Ca^{2+} (10 mM) (Figure 4e) selectively decreased the effect of high concentrations of ATP while ADP (Figure 4f) selectively blocked the effect of low concentrations of ATP. At the high concentrations of reactive blue 2 (10 and 30 μM) the response to carbachol also was inhibited in this experiment, although the effect is not significant for the pooled data of Table 1. Reactive blue 2 did reduce the

Table 1 Effect of reactive blue 2 on Ca_i response to ATP and other calcium-mobilizing agonists

		Reactive blue 2 concentration [μM]							
		0	*(n)*	3	*(n)*	10	*(n)*	30	*(n)*
ATP	0.5 μM	23 ± 7	(5)	51 ± 15	(4)	50 ± 19	(4)	57 ± 22	(5)
	3.5 μM	58 ± 21	(6)	93 ± 20	(5)	100 ± 25	(5)	97 ± 26	(6)
	30 μM	73 ± 33	(4)	135 ± 17	(3)	131 ± 34	(3)	103 ± 37	(4)
	300 μM	461 ± 110	(6)	306 ± 45	(5)	211 ± 33*	(5)	166 ± 39*	(6)
Δ (300 μM)–(3.5 μM)		403 ± 92		213 ± 45*		111 ± 27*		68 ± 20*	
(% inhibition)		(0)		(17 ± 22)		(48 ± 15)		(75 ± 6)	
Substance P (10^{-7} M)		167 ± 50	(6)	155 ± 39	(5)	207 ± 43	(5)	211 ± 40	(6)
Carbachol (10^{-4} M)		112 ± 31	(6)	107 ± 22	(5)	104 ± 19	(5)	95 ± 14	(6)

Numbers represent mean ± s.emean increase in Ca_i (nM) over basal levels which averaged 252 ± 70 nM, 269 ± 51 nM, 288 ± 44 nM, and 300 ± 48 nM at 0, 3, 10 and 30 μM reactive blue respectively. Δ (300 μM)–(3.5 μM) indicates the increment in Ca_i at 300 μM ATP above the value at 3.5 μM ATP, an indication of the response to ATP^{4-}. (*n*) = number of cell preparations. Extracellular calcium was 3 mM; no Mg^{2+} was added. Ca_i changes were estimated by the ratio method.
*$P < 0.05$ when compared to response in the absence of reactive blue 2 by Newman-Keul's multiple range test. Although reactive blue 2 tended to increase Ca_i responses to low concentrations of ATP (responses to 30 μM ATP or less were increased to 241 ± 44% of the responses observed in the absence of reactive blue 2), no significant potentiation was observed for any one concentration of ATP or reactive blue 2 (Newman-Keul's multiple range test).

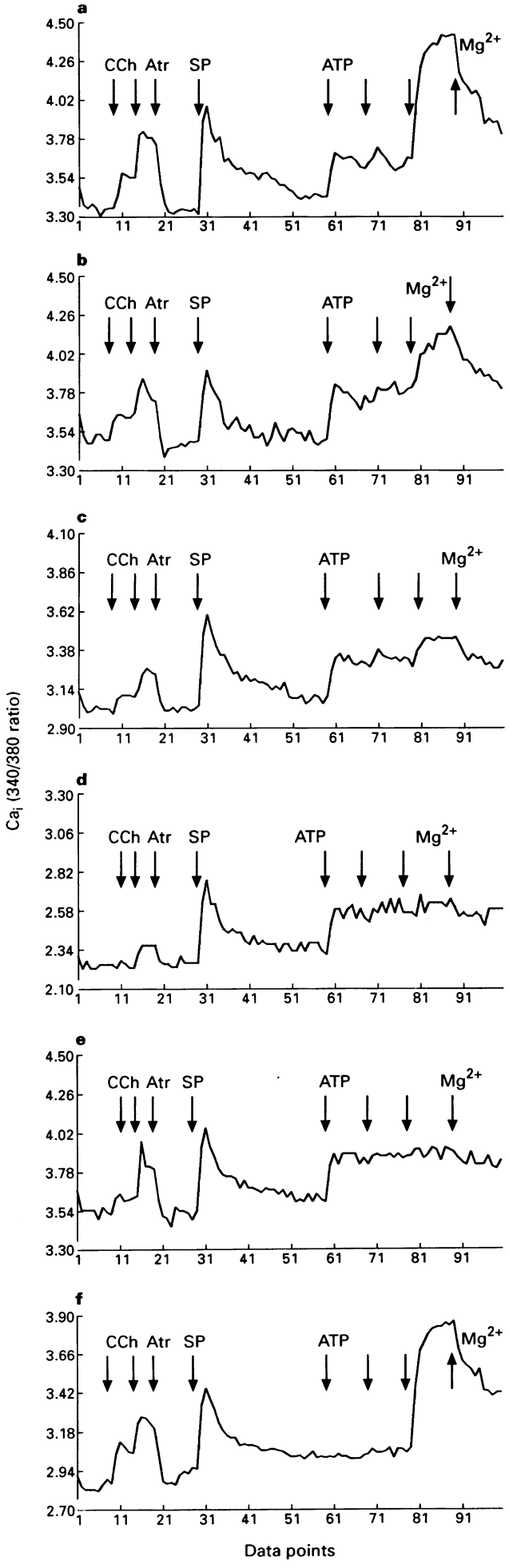

Table 2 Reactive blue 2 displaces [γ^{32}P]-ATP binding to parotid cells.

Reactive blue 2 (μM)	*[γ^{32}P]-ATP bound* (% remaining)
0	100
1	87 ± 10 (4)
3	75 ± 17 (4)
10	78 ± 11 (5)
30	44 ± 11 (7)
100	26 ± 20 (4)

Displacement of [γ^{32}P]-ATP (1–3 μM, usually about 300,000 c.p.m. added) binding by reactive blue 2 was determined after subtracting any binding not displaced by 100 μM ATP (presumably trapped or other non-specific binding: average = 38 ± 5% of total binding, $n = 7$). The mean and s.e.mean are shown for each concentration of reactive blue 2, followed by the number of experiments shown in parentheses. $[Ca^{2+}] = 0$; $[Mg^{2+}] = 0$.

Ca_i response to 10^{-6} M carbachol from 66 ± 19 nM ($n = 3$) to 18 ± 7 nM ($n = 4$) [$P < 0.05$ by Student's 2-tailed unpaired t test]. The more sensitive response to ATP often was potentiated by reactive blue 2 (Table 1).

Reactive blue 2 displaced [γ^{32}P]-ATP binding to parotid cells (Table 2), although this effect varied with different cell preparations. In preparations with pronounced small, potent responses, reactive blue 2 appeared less effective than in preparations which showed a response only to ATP^{4-}. Even so, the antagonism by reactive blue 2 of ATP^{4-} binding (Table 2) showed a similar potency to antagonism of Ca_i responses to ATP^{4-} (Figure 4), and is consistent with blockade of purinoceptors on parotid cells.

A number of compounds known to inhibit effects of ATP and/or calcium mobilization responses in other systems failed to block either of the effects of ATP on Ca_i in parotid cells. All compounds were tested on at least two parotid cell preparations. These included inhibitors of arachidonic acid metabolism, indomethacin (50 μM) and nordihydroguiaretic acid (50 μM), the phospholipase C inhibitor, neomycin (1 mM) and the IP_3 receptor blocker, heparin (1 mg ml^{-1}), three inhibitors of calcium mobilization, ruthenium red (50 μM), caffeine (10 μM) and ryanodine (10 μM), the Ca^{2+}-channel blocker, nifedipine (50 μM), the ATP-sensitive K^+ channel blocker, tolbutamide (1 mM), other K^+-channel blockers [CsCl (15 mM), $BaCl_2$ (2.5 mM), and charybdotoxin (10 nM)], the Cl^--channel blocker picrotoxin (30 μM), glutamate channel blockers [MK801 (50 μM) and ketamine (50 μM)], the Na^+-channel activator veratridine (10 μM), Na_2SO_3 (10 mM) (a shared substituent of DIDS and reactive blue 2), and a putative gap-junction blocker, octanol (150 μM) and an ineffective control alcohol, hexanol (200 μM).

Figure 4 Reactive blue 2 selectively blocks the ATP^{4-} response: (a) control; (b) reactive blue 2, 3 μM; (c) reactive blue 2, 10 μM; (d) reactive blue 2, 30 μM; (e) Ca^{2+}, 10 mM; (f) ADP, 0.5 mM. Data points were recorded every 7–8 s. Drugs were added 10 min prior to addition of agonists, shown by arrows. Additions of the agonists and antagonists were made in the same sequence as designated in the upper panel. The traces from these experiments are representative of those summarized in Table 1. Except as noted, the concentration of Ca^{2+} = 3 mM, Mg^{2+} = 0. Prior addition of reactive blue 2 (3–30 μM) decreased the effect of higher concentrations of ATP without decreasing the response to substance P (SP) or to low concentrations of ATP (b–d). The effect of high concentrations of reactive blue 2 on the response to ATP (d) was similar to that obtained in medium with high Ca^{2+} (e). ADP selectively blocked the more sensitive effect of ATP (f). Additions: carbachol (CCh) = 3 μM, 100 μM; atropine (Atr) = 10 μM; substance P (SP) = 100 nM; ATP = 3 μM, 30 μM, 300 μM.

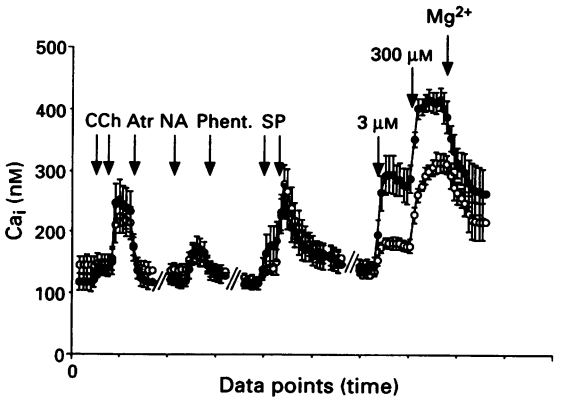

Figure 5 Parasympathetic denervation of rat parotid gland induces selective increases in the more sensitive Ca_i response to ATP: cells were prepared from glands taken from unilaterally denervated rats as described in Methods. The Ca_i responses of preparations from contralateral (unoperated) and denervated glands were compared. Each point represents the average and s.e.mean (vertical bars) of Ca_i values for denervated or contralateral control cell preparations from 4 unilaterally denervated rats; (○) control preparations; (●) denervated preparations. Arrows indicate additions of carbachol (CCh) (3 μM and 100 μM), atropine (Atr) (10 μM), noradrenaline (NA) (30 μM), phentolamine (Phent) (30 μM), substance P (SP) (0.3 and 100 nM), ATP (3 and 300 μM) and $MgCl_2$ (5 mM). Values were determined every 7–8 s; breaks between agonist additions represent 5–10 min pauses (equal for paired denervated and control runs) to allow calcium pools to recover. Note the pronounced increase in the Ca_i response to a low concentration of ATP. This increase also was observed when phospholipase C-coupled receptor agonists were not added prior to stimulation with ATP. The magnitude of the further increase in response to 300 μM ATP was similar in both control and denervated cells (130 and 115 nM respectively). Medium contained Ca^{2+} = 3 mM, Mg^{2+} = 0.

Parasympathetic denervation selectively increases the potent response to ATP

As noted in the introduction, muscarinic receptor, α-adrenoceptor and substance P receptor responses are sensitized following parasympathetic postganglionic denervation of the parotid gland. Sensitivity increases by 3 to 5 fold but there is at best only a small increase in maximal response (Figure 5). The response to 3 μM carbachol was highly variable in cells from control glands of unilaterally denervated animals, but it always was detected in denervated glands. In the course of examining possible supersensitivity to ATP^{4-}, a pronounced increase in the Ca_i response to low concentrations of ATP (3 to 5 μM) was observed (2.8 ± 0.3 fold over contralateral control cell responses, $n = 4$) (Figure 5). Higher concentrations of ATP led to further increases in Ca_i (attributable to ATP^{4-}) which were similar in cells from both denervated and contralateral control glands (Figure 5). The increased response after denervation involved only the more sensitive response, since the increase also was observed in high calcium-containing buffer and in the presence of 30 μM reactive blue 2 ($n = 2$), which eliminated the effect of ATP^{4-}.

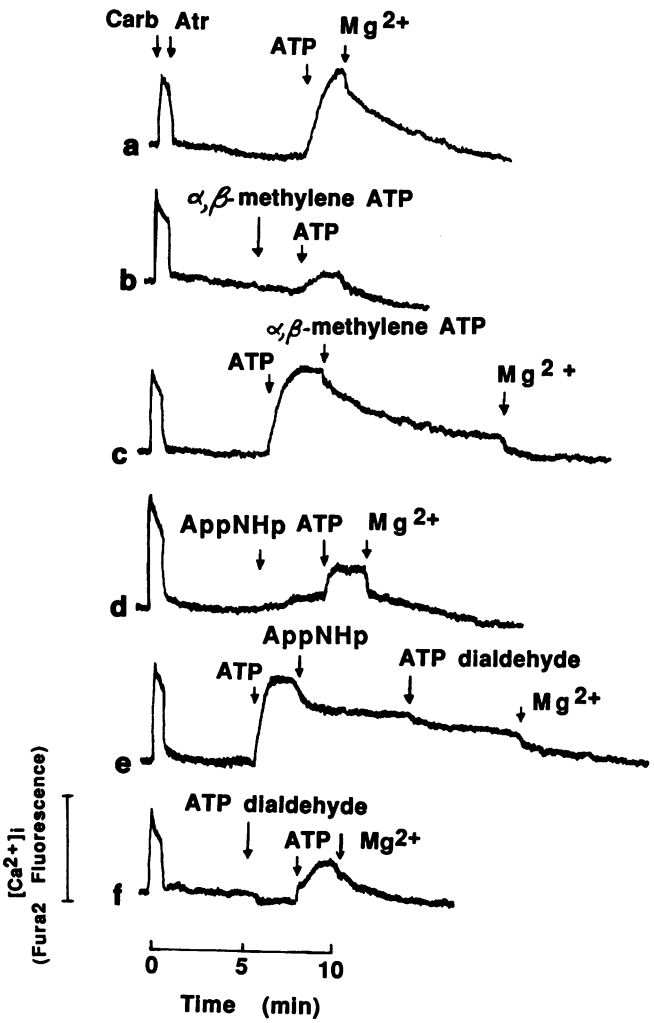

Figure 6 Reversal of the effect of ATP^{4-} by ATP analogues: Ca_i responses to carbachol (CCh) were determined and then various ATP analogues were tested (at 1 mM) for their ability to activate Ca_i responses or to block or reverse the response to ATP (50 μM). Compounds added: (a) ATP; (b) α,β-methylene ATP, then ATP; (c) ATP, then α,β-methylene ATP; (d) 5'-(β,γ-imido)triphosphate (AppNHp), then ATP; (e) ATP, then AppNHp, followed by ATP dialdehyde; (f) ATP dialdehyde, then ATP. Concentrations of other compounds were: $[Ca^{2+}]$ = 1 mM, Mg^{2+} = 0; CCh = 100 μM and atropine (Atr) = 10 μM. Although none of the analogues tested [α,β-methylene ATP ($n = 8$ cell preparations), AppNHp ($n = 6$) and ATP dialdehyde ($n = 3$)] were particularly potent, all partially blocked or reversed the Ca_i response when they were added at 1 mM, a concentration 20 fold higher than that of ATP (50 μM). This particular cell preparation appeared to lack the sensitive response to ATP since Mg^{2+} fully reversed the effect of ATP even in the absence of antagonists. The antagonists did not appear to be acting solely by chelation of extracellular calcium; note that the combination of AppNHp (1 mM) and ATP dialdehyde (1 mM) was no more effective in reversing the response to ATP than pretreatment with AppNHp alone. Note also that AppNHp acted as a weak partial agonist. Antagonism of responses to ATP by analogues, although repeatedly observed, was quite variable, possibly due to inhibition of ATPases by some analogues and potentiation of the more sensitive response to ATP in some preparations.

Reversal of the effect of ATP^{4-}

We hypothesized that inactive compounds or partial agonists that can bind to the ATP^{4-} receptor would reverse the effect of ATP^{4-} on Ca_i. We compared the reversal of the effect of ATP^{4-} by these compounds to reversal by Mg^{2+}, which reduces the concentration of ATP^{4-}.

Under optimal conditions for a response to ATP^{4-} (in the absence of Mg^{2+} and in the presence of 0.5–1.0 mM Ca^{2+}), 50 μM ATP produced a Ca_i response comparable to maximal muscarinic receptor activation (Figure 6a). This effect of ATP was reversed by the addition of Mg^{2+} (Figure 6a). The effects of benzoylbenzoyl ATP, and of high concentrations of ATPγS and 2-methylthio ATP were reversed similarly by Mg^{2+} ($n = 6$ or more experiments for each compound). We show here that 'inactive' analogues of ATP can block and reverse effects of ATP on Ca_i (Figure 6b–f). Three analo-

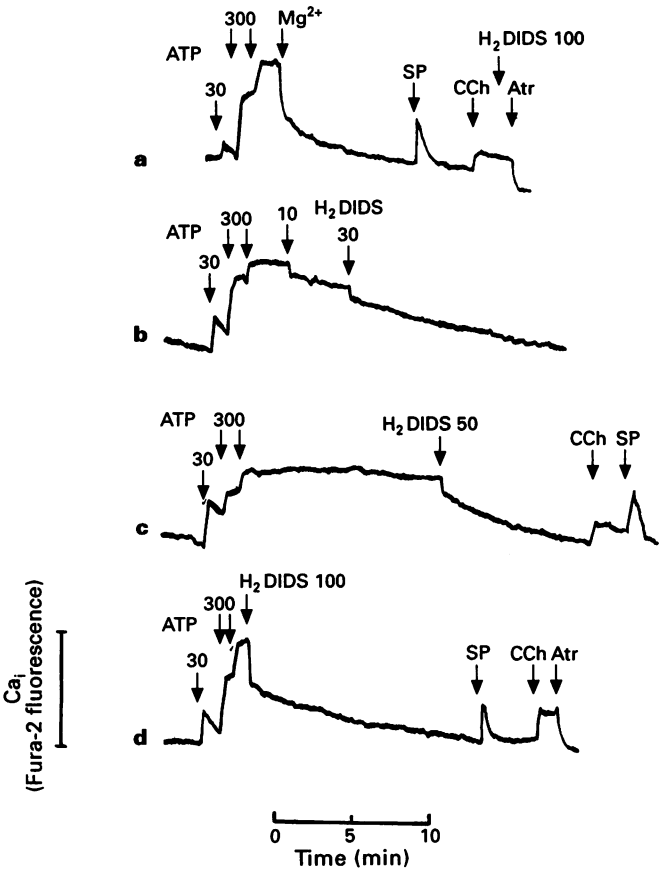

Figure 7 Effect of varying concentrations of 4,4'-diisothiocyanatodihydrostilbene-2,2'-disulphonate (DihydroDIDS) on the Ca_i response to ATP^{4-}: the ability of various concentrations of dihydroDIDS (H_2DIDS) to reverse the response to ATP or to block responses to carbachol (CCh) or substance P (SP) was tested. All concentration values given in the figure are in μM. ATP was added at 30 μM, followed by 300 μM, added twice. SP = 100 nM; CCh = 100 μM; atropine (Atr) = 10 μM. (Note that 100 μM H_2DIDS was as effective as Mg^{2+} in reversing the effect of a high concentration of ATP, without blocking SP or CCh responses). Similar results were obtained on 2 other cell preparations.

gues, α,β-methylene ATP, AppNHp and ATP dialdehyde, which had little or no agonist effects, all partially blocked the response to a subsequent addition of ATP. They also were able to partially reverse the response to a prior addition of ATP. This reversal required a relatively high concentration of the antagonist compound. For example, α,β-methylene ATP was effective only at a concentration of 20:1 relative to ATP and blocked the ATP response by 76 ± 4% ($n = 3$). It seems unlikely that chelation of extracellular calcium accounts for the inhibition by analogues of ATP since benzoylbenzoyl ATP partially overcame the block in the two experiments where it was examined. These experiments illustrate the ability of various ATP analogues to act as antagonists or as partial agonists in these cells.

Another compound which appears to bind to the ATP receptor and which blocks the response to ATP^{4-} is DIDS. Although DIDS pretreatment blocked responses to ATP^{4-} (Figure 2), the fluorescence of this compound limits its utility, and it cannot be used on-line in fura-2 assays. DihydroDIDS, a much less fluorescent derivative of the parent compound, also blocks the response to ATP^{4-} (data not shown). DihydroDIDS specifically reversed the effect of ATP^{4-} on Ca_i (Figure 7b–d), without interfering with the response to carbachol or substance P, consistent with the idea that dihydroDIDS displaces ATP binding to the purinoceptor.

Discussion

The present results provide evidence for two distinct Ca_i responses to extracellular ATP in rat parotid acinar cells. The first response was maximal at low concentrations of ATP (< 5 μM) and was smaller and less well-maintained than the Ca_i response to ATP^{4-} which we have described previously (McMillian *et al.*, 1987a; 1988; Soltoff *et al.*, 1990a). The response to higher concentrations of ATP (ATP^{4-}) does not show a maximum in Figure 1, but in experiments measuring $^{22}Na^+$ influx, maximal rates were reached at 100 μM ATP (with 1.8 mM Ca^{2+} in the absence of Mg^{2+}) (Soltoff *et al.*, 1990a). Rate studies of $^{45}Ca^{2+}$ uptake showed that the response is maximal at 300 μM ATP in the absence of added Mg^{2+}) (Soltoff *et al.*, 1992). ADP selectively blocked the more sensitive response to ATP, while DIDS and reactive blue 2 were selective in inhibiting only the response to ATP^{4-}, as were high concentrations of Mg^{2+} and Ca^{2+} which act by lowering the concentration of ATP^{4-} available on addition of ATP (McMillian *et al.*, 1988; Soltoff *et al.*, 1990a). Benzoylbenzoyl ATP was the only selective agonist and was at least 30 fold more potent than ATP on the ATP^{4-}-specific response, similar to other systems (Gonzalez *et al.*, 1989; Erb *et al.*, 1990). In other experiments which utilized $^{45}Ca^{2+}$ uptake rather than fura-2 fluorescence to assay the response to ATP, dye quenching by benzoylbenzoyl ATP was not a consideration, and higher concentrations of benzoylbenzoyl ATP were tested. Benzoylbenzoyl ATP was equally effective at 30 μM and 100 μM in these studies on rat parotid acinar cells (Soltoff *et al.*, 1992), indicating that maximal stimulation occurs at 30 μM. In fura-2 studies carried out under conditions where the large, low affinity response was blocked, i.e. with 10 mM Ca^{2+} or 300 μM DIDS pretreatment, 30 μM benzoylbenzoyl ATP had no agonist effect on the more sensitive response, which is surprising since this compound binds to ATPases (Williams & Coleman, 1982; Erb *et al.*, 1990) as well as to P_{2y}-purinoceptors (Boyer & Harden, 1989; Boyer *et al.*, 1989). Though generally much weaker, the more sensitive response to ATP may be physiologically important, since this response appears to be neurally regulated, as shown by the increase following parasympathetic denervation.

The response to ATP^{4-} in parotid cells can best be classified as one mediated via P_{2z}-purinoceptors. The potency order for the agonists (benzoylbenzoyl ATP > ATP > 2-methylthio ATP > α,β-methylene ATP) and the strong inhibition by Mg^{2+} and high concentrations of Ca^{2+} are consistent with activation of a P_{2z}-purinoceptor. A similar potency order (benzoylbenzoyl ATP > ATP > 2-methylthio ATP > AppNHp) for the effect on the ATP^{4-}-response was obtained in $^{45}Ca^{2+}$ uptake experiments (Soltoff *et al.*, 1992). It is unclear whether the 'superagonist' effect of benzoylbenzoyl ATP on Ca_i in parotid cells results from covalent interaction with P_{2z}-receptors and ATPases; however in short-term studies much of the effect is reversed by the addition of Mg^{2+}, suggesting that covalent bonds are not required.

Gallacher (1982) previously noted the low potency of ATP in stimulating ion fluxes in mouse parotid cells and suggested that ATP^{4-} might be the active form, but he suggested that the response was of the P_{2y}-type based on the observation that P_{2y} purinoceptors also activate calcium-dependent K^+ channels in other tissues (Gordon, 1986). Our data clearly show that the response to ATP^{4-} in rat parotid cells is of the P_{2z}-type. While its selectivity for the P_{2z}-receptor must be confirmed, the use of benzoylbenzoyl ATP together with P_{2x}- and P_{2y}-selective agonists should prove useful in subtyping P_2-purinoceptors.

Reactive blue 2 was a potent and selective blocker of the parotid response to ATP^{4-}. This antagonist apparently discriminates P_{2y}-receptors from P_{2x}-receptors (Reilly *et al.*, 1987; Burnstock & Warland, 1987; Houston *et al.*, 1987), but also has a strong effect on parotid P_{2z}-receptors (Soltoff *et al.*, 1989). Reactive blue 2 displaced [γ^{32}P]-ATP bound to

parotid cells as did the inactivating compound DIDS, which forms covalent adducts (McMillian et al., 1988). Blockade of ATP-effects by pretreatment of cells with DIDS, a compound structurally unrelated to ATP, appears to result from covalent inhibition of P_2-type purinoceptors (McMillian et al., 1988). While allosteric effects of these compounds on ATP-binding are possible, it seems likely that reactive blue 2 and DIDS inhibit the response to ATP^{4-} by interfering with the binding of the purine rather than by other means, for example by blocking an ATP-dependent channel. In some experiments (as in Figure 4), reactive blue 2 inhibited the muscarinically-activated increase in Ca_i as well as the response to ATP^{4-}, although the responses to substance P were never inhibited. This finding is in agreement with observations that muscarinic effects are inhibited by reactive blue 2 in other ATP-responsive tissues (Choo, 1981). Coomassie brilliant blue may be a more useful antagonist at P_{2z}-receptors (Soltoff et al., 1989) since this compound does not appear to be a potent blocker of P_{2y}-receptors (Inoue et al., 1991; McMillian, unpublished data).

ATP^{4-} increases the membrane permeability to low molecular weight solutes in many cells, and this permeabilization response shares many features in common with the Ca_i response to ATP^{4-} in parotid cells. However, at the concentrations employed in the present study, which are higher than those used for permeabilization studies in other cells, ATP^{4-} has little if any permeabilizing effect (McMillian et al., 1988). As with the Ca_i response reported here, Mg^{2+} halts permeabilization in response to ATP^{4-}, and this reversal on removal of ATP^{4-} has been interpreted as a resealing of ATP^{4-}-induced lesions in the plasma membrane (Tatham et al., 1988). Reversal of the effects of ATP^{4-} in parotid cells by Mg^{2+} (and to a lesser extent by the more weakly chelated Ca^{2+}), as well as by α,β-methylene ATP and dihydro-DIDS, appears to result from displacement of ATP^{4-} from a specific receptor-like site, rather than through resealing of ATP^{4-}-induced lesions.

The more sensitive Ca_i response to ATP does not fit into any P_2-purinoceptor classification. Although pharmacologically distinguishable from the ATP^{4-}-effect, the more sensitive response resembles this P_{2z}-type response much more than it does the P_{2x}- or P_{2y}-purinoceptor responses. Except for benzoylbenzoyl ATP, the agonist potency series for analogues of ATP was identical for the two Ca_i responses, and the P_2-purinoceptors mediating the two responses may be related. The more sensitive response to ATP was variably present in earlier experiments, and it is possible that the receptor mediating the smaller response to ATP is more sensitive to reagents used in our cell preparation procedure and was lost in previous studies. Another possibility is that the different responses to ATP reflect Ca_i increases in different cell populations or that there is only one receptor for ATP on parotid cells that gives rise to a second as a preparation artifact. However, a similar effect of ATP^{4-} has been reported in cells prepared without enzyme treatment (Richards et al., 1987; Buisman et al., 1988; Tatham et al., 1988; Gonzalez et al., 1989). It is also possible that the variability in responses to ATP accurately reflects the situation *in vivo*. Our finding of two P_2-type purinoceptor-mediated responses on a single cell type is not unique. There are at least two effects of ATP which can be distinguished by the use of analogues of ATP in hepatocytes (Okajima et al., 1987), and there appear to be two or more distinct responses in liver (Cobbold et al., 1988), bullfrog atrial cells (Friel & Bean, 1988), in macrophage and pancreatic cell lines (Greenberg et al., 1988; Li et al., 1991), and in adrenal chromaffin cells (Diverse-Pierluissi et al., 1991).

Breakdown of ATP to ADP may contribute to some of the properties of the more sensitive ATP response. ADP selectivity inhibited this response, and production of ADP by metabolism of ATP may limit the size as well as the duration of the Ca_i elevation. The potentiation of the more sensitive response to ATP by reactive blue 2 may reflect blockade of ecto-ATPases (and of ATPase activity from broken cells).

Denervation increased the Ca_i response to low concentrations of ATP much more than to other calcium-mobilizing agonists, suggesting some specificity in the increased purinoceptor response. Further study is required to determine if metabolism of ATP decreases after denervation (perhaps leading to more ATP and less ADP at the putative receptor). Denervation supersensitivity has been cited as evidence for a physiological role for ATP in the vas deferens (Rohde & Huidobro-Toro, 1988) and is consistent with a physiological effect of ATP in the parotid. Postganglionic parasympathetic denervation of the parotid gland is known to lead to supersensitivity to all agonists which mobilize Ca_i through phospholipase C (Ekstrom, 1980; Ekstrom & Wahlestedt, 1982) (including the agonist noradrenaline which is not depleted). It is unclear if the selective increase in the Ca_i response to low concentrations of ATP reflects changes in denervated cells specific to the P_2-purinoceptor response or if the greater effect reflects differences in the handling of Ca_i in response to ATP relative to phospholipase C-linked receptor agonists.

Our data clearly show that two Ca_i responses to ATP can be distinguished in parotid cells. DIDS, reactive blue 2 and, particularly benzoylbenzoyl ATP are offered as useful pharmacological tools to characterize further the response to ATP^{4-} mediated via P_{2z}-purinoceptors. As yet, we have no selective compounds which affect the more sensitive response to ATP, which may be more important physiologically.

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Cyclic AMP and Ca^{2+} interactions affecting epithelial chloride secretion in human cultured colonic epithelia

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1 Chloride secretion in three types of cultured epithelial monolayers derived from a single human colonic adenocarcinoma was measured in terms of short circuit current. The three cell types were designated HCA-7, Colony 3 and Colony 29.

2 Responses of HCA-7 monolayers to basolaterally applied lysylbradykinin (LBK) (10–1000 nM) or carbachol (1–100 μM) were potentiated by pre-exposure to forskolin (10 μM) for 5 min. Forskolin itself increased short circuit current (SCC), so that the total response to forskolin and LBK or carbachol were non-additive.

3 Colony 3 cells did not respond to LBK on either face but did to carbachol on the basolateral side, while Colony 29 epithelia responded to LBK on both sides and to carbachol and histamine basolaterally. Unlike HCA-7 epithelia, responses in Colony 3 and Colony 29 epithelia were not potentiated by forskolin, but were attenuated by piroxicam.

4 In the presence of piroxicam, both forskolin and prostaglandin E_2 were able to potentiate the action of both LBK and carbachol in Colony 29 epithelia.

5 LBK receptor activation in Colony 29 epithelia is transduced into an increase in intracellular Ca^{2+} and cyclic AMP, while in HCA-7 epithelia there is only an increase in intracellular Ca^{2+} (Ca_i). These conclusions are considered to apply to both apical and basolateral kinin receptors.

6 It is shown that forskolin has no effect on the elevation of Ca^{2+} by LBK in either HCA-7 or Colony 29 cells.

7 It is concluded that potentiation of agonist responses occurs when cyclic AMP is raised at the time that intracellular Ca^{2+} increases. No potentiation of LBK or carbachol by forskolin occurs in Colony 29 monolayers as these agonists increase cyclic AMP via eicosanoid production.

Keywords: Lysylbradykinin; carbachol; cyclic AMP; Ca^{2+}; chloride secretion; epithelia

Introduction

Electrogenic epithelial chloride secretion proceeds by moving chloride ions from the basolateral domain into the apical domain. To achieve this, chloride ions move across the basolateral membrane on a Na-K-2Cl co-transporter and down an electrochemical gradient through channels across the apical face (Frizzell *et al.*, 1979), that is, through two barriers in series. This paper describes electrogenic chloride secretion in cultured epithelia derived from a human adenocarcinoma (Kirkland, 1985). Throughout chloride secretion has been measured electrically as short circuit current (SCC) and we have focused on the potentiation of responses to agents which raise intracellular Ca^{2+} by agents which increase intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP). Three different epithelial cell lines, all derived from the same adenocarcinoma, with different phenotypes have been used. The aim of this study arose from two preliminary observations. First, using a single concentration of lysylbradykinin (LBK) (0.1 μM) it was noted its actions were potentiated by preincubation with forskolin (10 μM) in HCA-7 monolayers. Furthermore, potentiation was observed whether LBK was applied apically or basolaterally (Brayden *et al.*, 1989). When Colony 29 epithelia were examined potentiation of LBK by forskolin was not found, but this cell line differed from HCA-7 in that the actions of LBK were suppressed by cyclo-oxygenase inhibition with piroxicam. It is already known that piroxicam does not significantly affect the actions of LBK in HCA-7 cells (Cuthbert *et al.*, 1987). The primary aim was, therefore, to explain the differences in these phenotypes. Cellular phenotypes are not only useful in unravelling the complexities of drug action but can through somatic cell genetics, provide an entrée for the identification of gene products involved in cellular transport mechanisms (Cuthbert, 1990a).

Methods

Epithelial culture

The studies described in this paper have been performed with the cell lines HCA-7, Colony 3 and Colony 29. All were derived from a single colonic adenocarcinoma and described initially by Kirkland (1985). Conditions for culture of the first two are given in detail by Cuthbert *et al.* (1987) while information for handling the latter is given in Brayden *et al.* (1989), and Pickles & Cuthbert (1991, 1992).

Electrical measurement of chloride secretion

Chloride secretion was measured as short circuit current (SCC) in monolayers cultured on permeable supports. Each had an area of 0.2 cm^2. The methodology was that described in recent papers in this journal (Cuthbert *et al.*, 1992) and elsewhere (Pickles & Cuthbert, 1991). Responses are reported either as maximal increases in SCC or in terms of charge transfer during the 8 min following addition of secretagogues. Statistical tests for differences were by the standard Student's *t* test method. Results are given as mean ± s.e.mean.

Measurement of intracellular Ca^{2+} (Ca_i) in epithelial monolayers

Monolayers were grown either on the plastic slips in Leighton tubes or cell suspensions were prepared from flasks. Ca_i was estimated from Fura-2 fluorescence by the dual wave-

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length ratio method. Details of methods of calibration have been described recently (Pickles *et al.*, 1991; Cuthbert *et al.*, 1992; Pickles & Cuthbert, 1992). None of the drugs used in this study affected the fluorescence measurements.

Drugs

Drugs were obtained from the following sources: forskolin and Fura-2 AM were from Calbiochem, La Jolla, CA, U.S.A.; lysylbradykinin, histamine, carbachol, A23187, prostaglandin E_2 and isobutylmethylxanthine were from Sigma Chemicals Co., and piroxicam was obtained from Pfizer, Sandwich, Kent.

Results

Potentiation of the chloride secretory responses to lysylbradykinin by agents increasing cyclic AMP content in HCA-7 monolayers

The potentiating effects of agonists with different modes of action has been described briefly before (Brayden *et al.*, 1989). Here this effect is examined in more detail. Typical data from six paired experiments are illustrated in Figure 1 for HCA-7 monolayers. The upper tracing of each pair shows responses to either LBK or carbachol (CCh), applied basolaterally, in the presence of forskolin, 10 μM, while the lower tracings show paired responses in the absence of forskolin. In these experiments forskolin increased SCC and agonists were added after 5 min when the SCC had stabilized at its new level. The increase in SCC caused by forskolin, 10 μM, in the 24 separate preparations shown in Figure 2 was 2.9 ± 0.2 μA (mean ± s.e.mean). The responses to the agonists were distinctly biphasic, but the two phases became much more distinct after forskolin. Statistical verification of these specimen data is provided in Figure 2, showing that at all concentrations of LBK and CCh examined there was a significant increase in response after forskolin. These data confirm the earlier finding referred to above (Brayden *et al.*, 1989) that basolaterally applied LBK at a concentration of 0.1 μM is potentiated by forskolin. LBK can increase chloride secre-

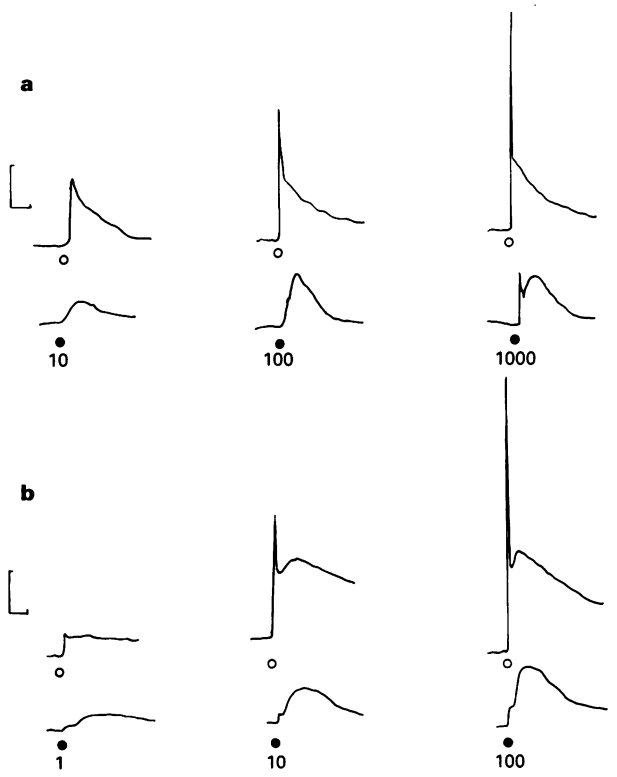

Figure 1 Short circuit current records from HCA-7 monolayers (0.2 cm^2). Responses are to lysylbradykinin (LBK, in nM) in (a) and to carbachol (in μM) in (b). In each pair of traces the lower responses were in untreated preparations (●), while the upper responses were in preparations pretreated with forskolin, 10 μM for 5 min (○). All experiments were made with cultured monolayers from the same batch. LBK and carbachol were added to the basolateral bathing fluid. Calibrations are 5 μA and 2 min.

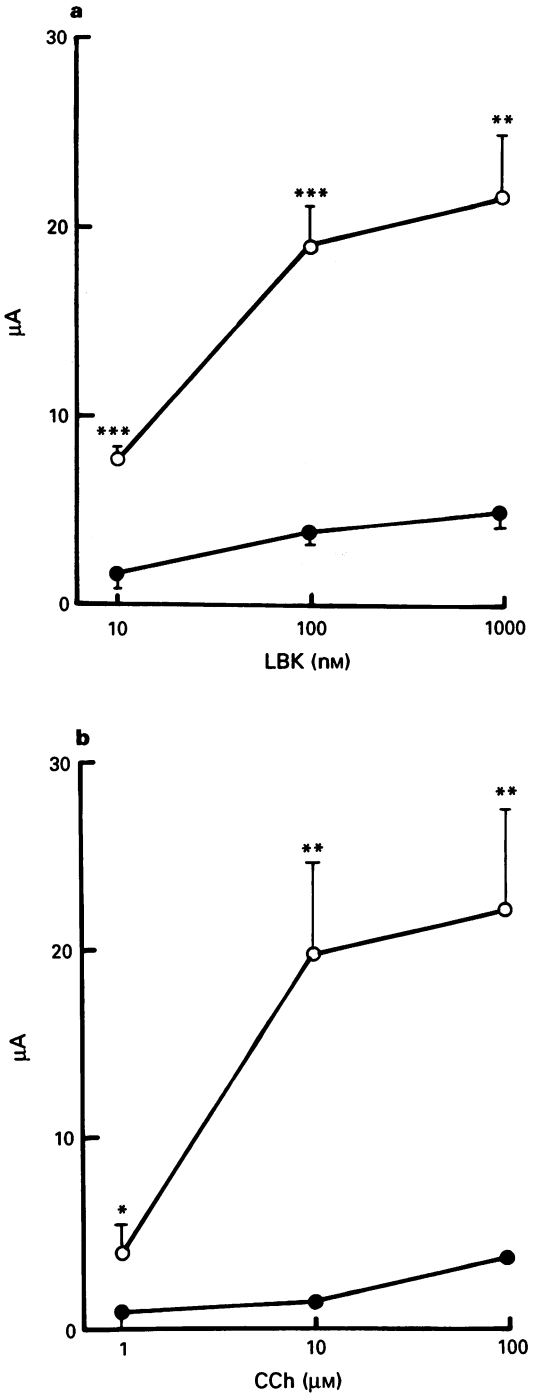

Figure 2 Increases in short circuit current (μA) versus lysylbradykinin (LBK) (a) or carbachol (CCh) (b) concentrations in the absence (●) and presence (○) of forskolin (10 μM) in HCA-7 monolayers (0.2 cm^2). Mean values ± s.e.mean (vertical bars) for 4–5 observations are shown. All responses are to basolaterally applied agonists on HCA-7 monolayers (0.2 cm^2). Asterisks indicate values which are significantly different from controls ($^*P<0.05$; $^{**}P<0.01$, and $^{***}P<0.001$). In preparations pretreated with forskolin, for 5 min, SCC increased by 2.9 ± 0.2 μA (mean ± s.e.mean for 24 preparations) before LBK or CCh was added.

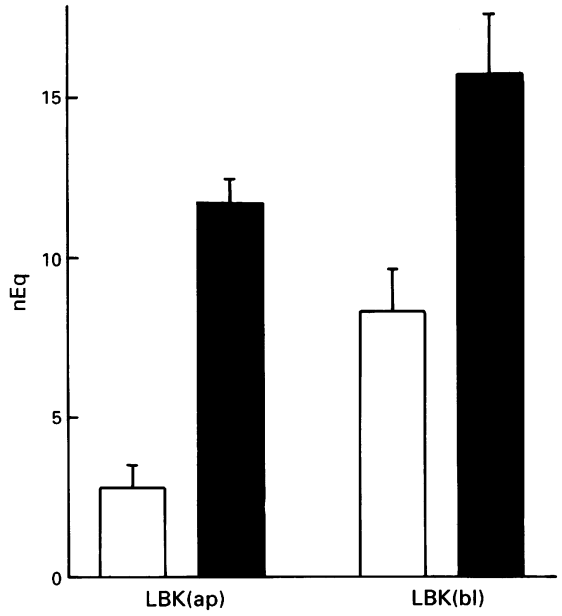

Figure 3 Effects of vasoactive intestinal polypeptide (VIP) (10 nM) applied basolaterally on the responses to lysylbradykinin (LBK, 0.1 μM) applied apically (ap) or basolaterally (bl) in HCA-7 monolayers (0.2 cm^2). Each column shows the mean ± s.e.mean (vertical bars) for 6 observations. LBK responses in the presence of VIP (solid columns) were significantly greater ($P<0.001$) from untreated controls (open columns). Charge transfer (in nEq) for 8 min following LBK is shown.

tion by activating receptors on either the apical or basolateral surface in HCA-7 and Colony 29 monolayers. Most agonists, such as CCh or histamine, have only basolateral receptors in these epithelia. Therefore in this study the major focus will be on potentiation by forskolin, presumably by increasing cyclic AMP content, on basolaterally applied agonists such as LBK which increase intracellular calcium concentration (Pickles & Cuthbert, 1991). Exposure of HCA-7 monolayers to the cyclo-oxygenase inhibitor, piroxicam, 5 μM, did not prevent the potentiating action of forskolin, 10 μM, on the response of basolaterally applied LBK, 0.1 μM. In four paired experiments, basolaterally applied LBK (0.1 μM) caused responses of 5.0 ± 0.9 nEq (measured over 8 min) in the presence of piroxicam and of 13.1 ± 3.7 nEq in the presence of piroxicam and forskolin. The values were significantly different at $P<0.05$. Since it was found earlier that piroxicam did not alter the response to LBK (Cuthbert *et al.*, 1987) it can be presumed that prostaglandin formation plays no part in the response to LBK or its potentiation by forskolin in HCA-7 cells.

Reviewing the form of the responses shown in Figure 1 it is seen that the first rapid phase is considerably potentiated by forskolin yet counts for only a small proportion of the total charge transfer caused by either LBK or CCh. Consequently, the maximal current increase is not necessarily a good indicator of potentiation which is why charge transfer was used in the earlier example. Similarly total charge transfer, measured as area under the response curve for 8 min, was used to examine potentiation of LBK by vasoactive intestinal polypeptide (VIP). Like forskolin, this agent increases cyclic AMP content and activates adenylate cyclase in HCA-7 monolayers (Cuthbert, 1990b). Low concentrations of VIP (10 nM) caused significant potentiation of the responses to LBK, applied either to the apical or basolateral surface (Figure 3). VIP (10 nM) increased the SCC in the 12 preparations of Figure 3 by 2.1 ± 0.4 μA.

Both LBK and CCh increase intracellular Ca^{2+} concentration in our cultured epithelia (Pickles & Cuthbert, 1991). Consequently we have used the calcium ionophore, A23187, to see if it too was potentiated by prior exposure to forskolin. However, since it is known that in some epithelia, A23187 produces prostaglandins (Erlij *et al.*, 1986), preliminary experiments were done to see if piroxicam altered the responses to A23187. In three paired experiments responses to A23187, 1 μM, were 21.7 ± 5.4 μA cm^{-2} ($n=3$) in the absence of piroxicam and 23.3 ± 5.4 μA cm^{-2} ($n=3$) in the presence of 5 μM of the inhibitor. From these few experiments it appears that prostaglandin formation is not a major determinant of the response to the ionophore. In a further series of experiments in which the responses were measured as area under the curve for the first 8 min (note that like forskolin, A23187 produces a sustained response) the response to A23187, 1 μM, was 13.6 ± 0.9 nEq ($n=5$) while in the presence of forskolin, 10 μM, the response was 14.5 ± 2.0 nEq ($n=5$). Clearly these responses are not significantly different.

A further attempt to show potentiation of LBK responses in HCA-7 monolayers was by addition of the peptide after CCh. In a small series of paired experiments LBK, 0.2 μM, applied basolaterally, caused responses of 9.60 ± 0.8 nEq ($n=3$) while after CCh, 1 μM, the responses were 9.85 ± 1.3 nEq ($n=3$), i.e. CCh made no significant difference to the LBK response.

To summarise this section of the results, it is clear that responses to LBK are potentiated by forskolin and VIP.

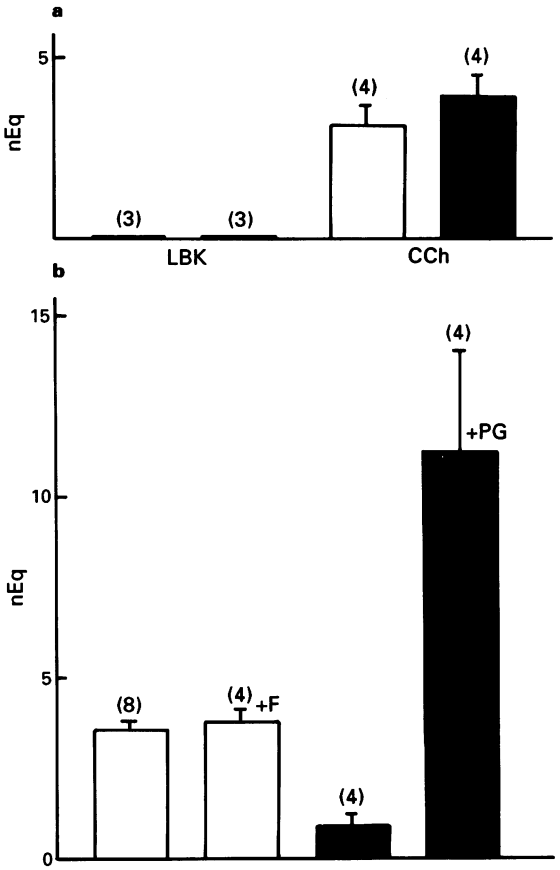

Figure 4 Short circuit current responses to lysylbradykinin (LBK, 0.1 μM) and carbachol (CCh, 10 μM) applied basolaterally in Colony 3 monolayers (each 0.2 cm^2) expressed as charge transfer in 8 mins (in nEq). In (a) responses are to LBK and carbachol in the absence (open columns) and presence of forskolin, 10 μM (solid columns). Forskolin caused no significant changes. In (b) all the responses are to carbachol, 10 μM, applied basolaterally. Solid columns represent experiments carried out in the presence of piroxicam, 5 μM. Addition of forskolin (F), 10 μM, made no significant difference to the responses in the absence of piroxicam. The responses to carbachol were significantly reduced by piroxicam ($P<0.05$) while addition of prostaglandin E_2 (PG), 10 μM, significantly increased the responses ($P<0.001$) even above the untreated controls ($P<0.001$). The number of observations plus s.e. means (vertical bars) are indicated.

However, the potentiation cannot result simply from a Ca^{2+} cyclic AMP interaction intracellularly since A23187 was not potentiated by forskolin. Furthermore potentiation was not seen when two agonists, both of which increase Ca_i, were applied sequentially.

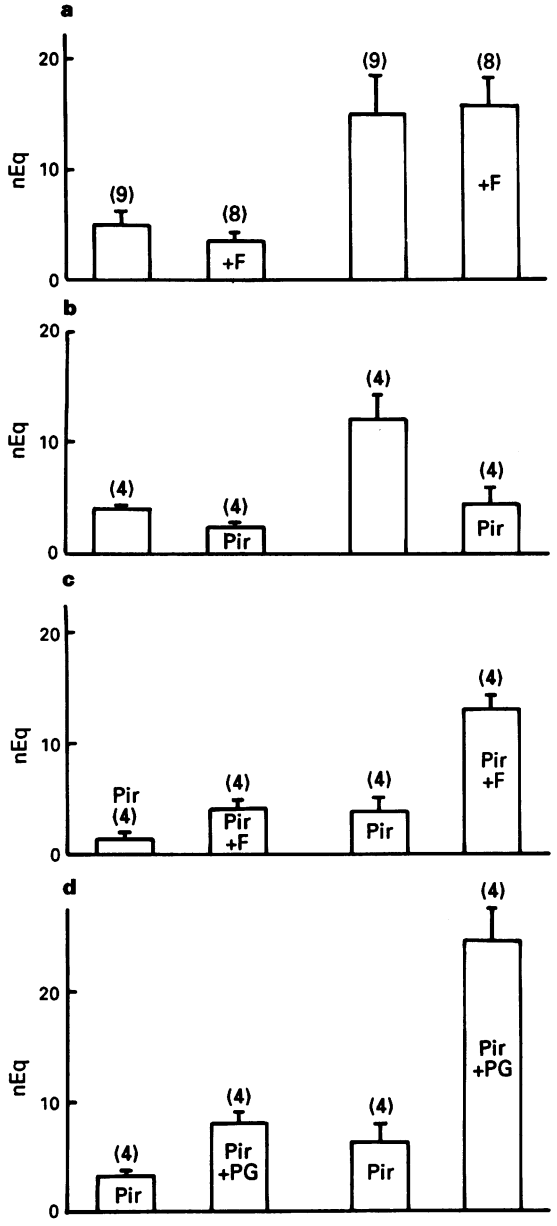

Figure 5 Short circuit current responses to lysylbradykinin (LBK, 0.1 μM) in Colony 29 monolayers (0.2 cm^2) expressed as nEq, integrated over 8 mins. In each line the data represent responses to apical application for the left hand pair of columns, while the right hand pair shows results for basolateral application. The number of observations plus s.e. means (vertical bars) are indicated throughout. Where F, Pir and PG are shown it indicates that tissues were exposed to forskolin, 10 μM; piroxicam, 5 μM, and prostaglandin E_2, 10 μM respectively for 5 min before responses to LBK were measured. All experiments in each line were made using a single batch of cultures. In (a) forskolin did not significantly alter the responses to LBK. In (b) piroxicam significantly reduced the responses to apically ($P<0.01$) and basolaterally ($P<0.05$) applied LBK. In (c) in piroxicam-pretreated tissues forskolin potentiated the responses to apically ($P<0.05$) and basolaterally ($P<0.01$) applied LBK. In (d) in piroxicam-pretreated tissues prostaglandin E_2 significantly increased the responses to apically ($P<0.01$) and basolaterally ($P<0.01$) applied kinin.

Potentiation of chloride secretory responses by agents affecting cyclic AMP in mutant cell lines related to HCA-7

Epithelial monolayers grown from two mutant cell lines related to HCA-7, namely Colony 3 and Colony 29, were used to explore the type of potentiation seen with HCA-7 cells. Colony 3 monolayers show little or no response to forskolin as far as chloride secretion is concerned, yet they accumulate cyclic AMP to a much greater extent than HCA-7 monolayers (Cuthbert, 1990b). They do not respond to LBK but do to CCh and A23187. In Figure 4a it is shown that addition of forskolin neither reveals a response to LBK nor potentiates the response to CCh. In other experiments (not shown) addition of the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX, 100 μM) in the presence of forskolin also failed to potentiate the CCh response. This result makes it unlikely that excessive cyclic AMP degradation is responsible for the failure to see potentiation.

A further set of experiments with Colony 3 cells is shown in Figure 4b. Half of the experiments were performed in the presence of piroxicam, 5 μM, which severely attenuated the responses to CCh, implying that this agent also generated eicosanoids. In the presence of piroxicam, plus prostaglandin E_2 (PGE_2), the responses to CCh were not only restored, but increased to a value far larger than that of the control. In the four experiments illustrated PGE_2 increased the SCC before CCh was added by 4.6 ± 0.7 μA.

Colony 29 monolayers have properties in some ways intermediate between HCA-7 and Colony 3. For example, SCC responses to forskolin, 10 μM, are in the ratio 6.2:1.2:1.0 for HCA-7, Colony 3 and Colony 29 respectively (Cuthbert, 1990b). As the responses in the last two are not maintained, the ratios are even more extreme when charge transfer is

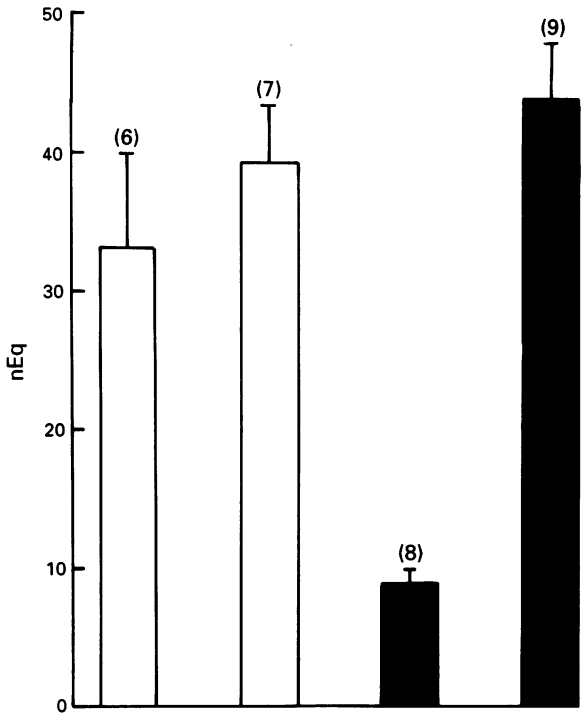

Figure 6 Effects of carbachol (CCh) 10 μM, applied basolaterally to Colony 29 monolayers (each 0.2 cm^2). The number of measurements and s.e.mean (vertical bars) are indicated. The open columns show responses in the absence (left hand) and presence (right hand) of forskolin, 10 μM, for 15 min. Solid columns represent responses obtained in the presence of piroxicam, 5 μM alone (left hand) or piroxicam plus prostaglandin E_2 (PGE_2,, 10 μM) (right hand) added 15 min before CCh. Piroxicam reduced the response to CCh ($P<0.01$) while addition of PGE_2 to piroxicam-treated tissues restored the responses (difference in the solid columns, $P<0.001$).

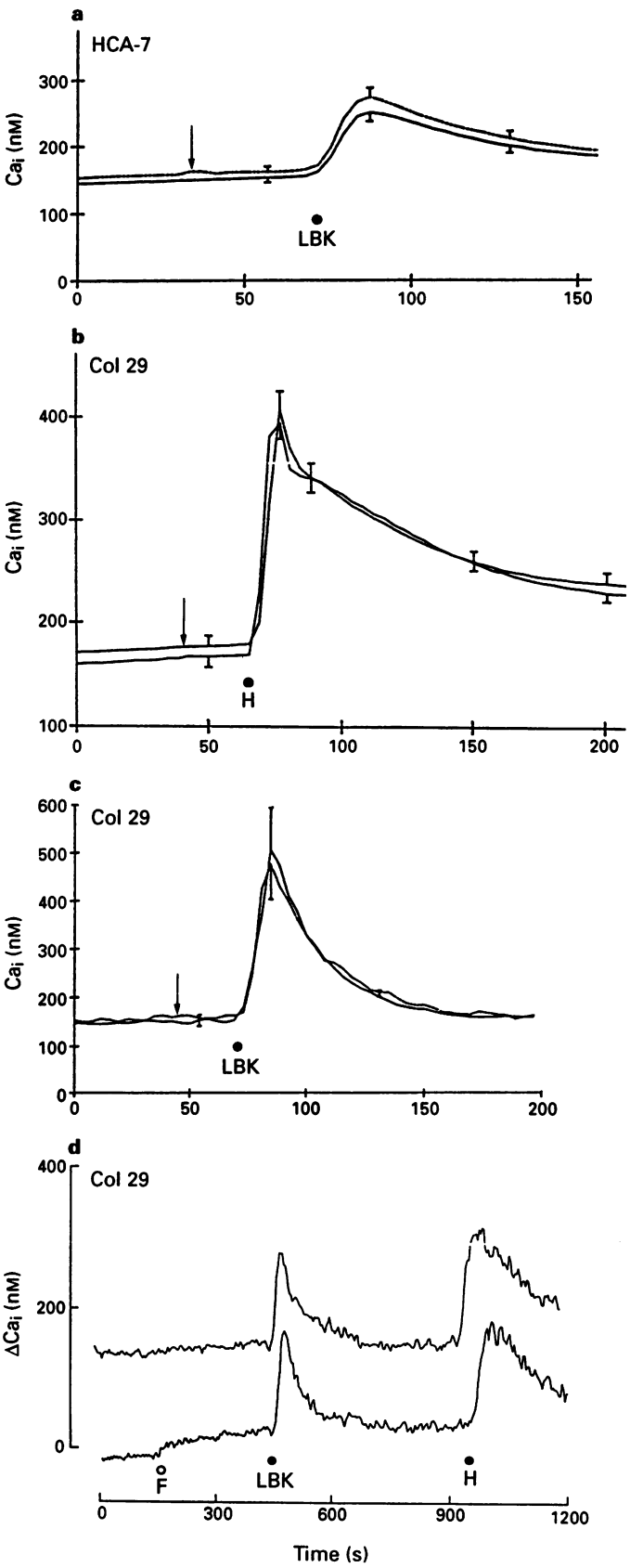

Figure 7 Ca_i measured by Fura-2 fluorescence. Cell suspensions of HCA-7 or Colony 29 cells were used for experiments in (a)–(c). In each experiment one half of a batch of cells was exposed to forskolin, 10 μM, 5 min before the effect of agonists on Ca_i were examined, indicated by arrows on the traces. The other half served as the control suspension. The values of Ca_i at fixed times during the experiments were used to calculate means ± s.e.mean (vertical bars) and are shown on the traces. (a) Shows effect of lysylbradykinin (LBK, 0.1 μM) on HCA-7 cells. Traces are the means of 9 separate experiments; (b) shows effects of histamine (H) (10 μM) on Colony 29 cells. Mean values for 8 experiments are illustrated. (c) Shows effects of LBK (0.1 μM) on Colony 29 cells. Mean values for 9 experiments are given. In (d) single experimental results are shown for a monolayer culture, divided into two. Effects of LBK (0.1 μM) and histamine (H) (10 μM) are shown in the absence and presence of forskolin (F) (10 μM). Note changes in Ca_i rather than actual values are given. Both monolayers had a basal Ca_i of around 100 nM. Composite data for this type of experiment are given in Table 1.

used. Unlike Colony 3 cells, monolayers of Colony 29 cells respond to LBK, as do HCA-7 monolayers. The responses to LBK in Colony 29 cells are severely attenuated by cyclooxygenase inhibition with piroxicam. As with Colony 3 monolayers, forskolin caused a major accumulation of cyclic AMP, significantly greater than with HCA-7 (Cuthbert, 1990b).

Figure 5 illustrates a series of paired experiments in which LBK (0.1 μM) was used throughout, applied either apically or basolaterally to Colony 29 monolayers. In Figure 5a it is shown that forskolin was unable to potentiate the responses to LBK. Figure 5b illustrates that piroxicam produced a significant reduction in the responses to LBK, indicating that part of the kinin response was dependent on prostaglandin formation. When prostaglandin formation was inhibited by piroxicam it was possible to potentiate the responses to LBK by adding forskolin (Figure 5c). The final set of experiments shown in Figure 5d shows that PGE_2 was more effective than forskolin at potentiating the effects of LBK in Colony 29 cells. The above statements apply whether LBK was applied apically or basolaterally. In this series PGE_2 (10 μM) increased SCC in Colony 29 monolayers by 3.7 ± 0.5 μA (mean ± s.e.mean, $n = 8$). A final series of experiments was carried out to examine if the effects of PGE_2 illustrated in Figure 5 were the same when an agonist other than LBK was used. CCh was chosen as agonist and like LBK its effects on chloride secretion were not potentiated by forskolin. Application of piroxicam to inhibit eicosanoid synthesis reduced the responses to CCh to 20% of their normal values, but these could be restored with addition of PGE_2. Thus the limited experiments of Figure 6 essentially recapitulate the data for LBK using CCh as an agonist.

Effect of forskolin on the increase in intracellular Ca^{2+} by agonists

To examine further the mechanism of potentiation of agonists by forskolin, measurements of intracellular Ca^{2+} were made by Fura-2 fluorescence. The aim was to examine the time course and extent of the Ca_i response to LBK both in control conditions and after cyclic AMP had been previously elevated by forskolin. Experiments were carried out both with HCA-7 and Colony 29 cells. Even in Colony 29 cells where it appears agonists generate cyclic AMP via eicosanoid synthesis, there is the possibility that a pre-existing, high cyclic AMP concentration, caused by forskolin, would modify the time course of the response to LBK. In these studies we have used a second agonist, histamine, to see if it too behaved like LBK. Histamine was chosen rather than CCh as it produces much larger Ca_i increases (Pickles & Cuthbert, 1991) so that modification by pre-exposure to forskolin might be more easily detectable. Furthermore the protocols allow examination of whether forskolin alone modifies the Ca_i levels in these epithelial cells. From Figure 7a–c, it is seen that pre-incubation with forskolin (10 μM) for 5 min made no significant change to either the time course or amplitude of the responses to LBK in HCA-7 or Colony 29 cell suspensions, or to histamine in Colony 29 cells. In all three instances the Ca_i values were marginally, but not significantly, greater in the forskolin-treated cells. The change in Ca_i in Colony 29 monolayers is shown in Figure 7d over a greater time period, to show the effect of forskolin addition, 5 min before LBK was added. Again there was a small change in

Table 1 Changes in Ca_i in response to lysylbradykinin (LBK, 1.0 μM) or histamine (100 μM) in Colony 29 epithelia, in the presence and absence of forskolin (10 μM)

		Δ Ca_i (nM)	AUC (nM min)
LBK	Control	112 ± 16	129 ± 18
	Plus forskolin	99 ± 14	124 ± 14
Histamine	Control	120 ± 21	255 ± 45
	Plus forskolin	108 ± 14	213 ± 25

Each value shows mean ± s.e. mean for 5 observations. In each experiment a single monolayer culture was divided into two, and the effects of agonists measured with or without 5 min preincubation with forskolin. Peak changes in Ca_i or area under curve (AUC) for 3 min following agonist addition are given.

Ca_i but this was not always as prominent as that illustrated. Responses to LBK and histamine were not changed by pre-exposure to forskolin and statistical data to support this are given in Table 1, whether peak height or area under curve is measured.

Discussion

In this study attention is focused on the potentiation of response to Ca^{2+} requiring agonists by cyclic AMP in three related epithelial cell lines. Potentiation refers here to an actual functional response, namely electrogenic chloride secretion measured as SCC. As the phenotypic characteristics are very different in these cell lines the mutants may provide starting points for molecular genetic studies to characterize the transport proteins uniquely encoded by structural genes.

The non-additive responses observed relate to a particular type of pairing, that is the interaction between agents known to raise intracellular Ca^{2+}, such as LBK and CCh (Pickles & Cuthbert, 1991) and agents which are known to increase cyclic AMP, such as forskolin, VIP and PGE_2 (Cuthbert *et al.*, 1984; Cuthbert, 1990b). Potentiation was not seen with LBK and CCh and was not seen when a calcium ionophore, A23187, was used with forskolin. This latter finding might indicate that non-additive interactions do not result from a simple Ca^{2+}-cyclic AMP mechanism, and that more complex interactions must be sought. Nevertheless A23187 is an effective secretagogue in HCA-7, Colony 3 and Colony 29 monolayers (Cuthbert, 1990b). Of course, agonists which activate phosphatidyl inositol hydrolysis also produce diacylglycerol (DAG) as well as raise Ca_i via inositol triphosphate. However, pre-incubation of HCA-7 monolayers with phorbol dibutyrate (PDB) inhibited the SCC response to LBK in HCA-7 monolayers, a property not shared by the inactive 4α-PDB isomer (Cuthbert & Pickles, 1991). Thus it is difficult to understand why failure to generate DAG by A23187 should prevent potentiation by forskolin.

Clear differences have been demonstrated between HCA-7 epithelia and those derived from it, i.e. Colony 3 and Colony 29, the latter two failing to show potentiation of LBK by forskolin seen with the former. Another major difference in responses of Colony 3 and Colony 29 monolayers to calcium requiring agonists is their sensitivity to piroxicam. It appears that both LBK and carbachol can cause eicosanoid formation, which is responsible for generating cyclic AMP via prostaglandin receptors linked to adenylate cyclase. We have already shown that these cell lines have cyclic AMP-sensitive chloride channels (Henderson *et al.*, 1992) although it is unclear that these are responsible for chloride secretion. It is possible that very small cyclic AMP-sensitive Cl^- channels, not detected by patching, also contribute to secretion. Further it is known that PGE_2 can increase chloride permeability measured as efflux of $^{125}I^-$ (MacVinish *et al.*, 1993).

It appears that Colony 3 cells do not have functional LBK receptors, although they may be present but not coupled with transduction mechanisms. HCA-7 and Colony 29 cells, on the other hand, have functional LBK receptors both on the apical and basolateral membrane domains. In all three cell lines functional cholinoceptors are present only on the basolateral membranes, yet appear to be coupled to eicosanoid generation in Colony 3 and Colony 29 cells, but not in HCA-7 cells. While it is known that LBK and CCh can activate epithelial receptors linked to phosphatidyl hydrolysis and Ca_i elevation (Smith *et al.*, 1990; Pickles & Cuthbert, 1991; Fischer *et al.*, 1992; Dickinson *et al.*, 1992), it is also known that in other cells receptors can be linked via G proteins to the activation of both phospholipase C and phospholipase A_2, giving both an increase in Ca_i and eicosanoid generation (Burch & Axelrod, 1987).

Once eicosanoid synthesis was prevented with piroxicam it became possible to potentiate the responses to LBK and CCh in Colony 29 cells and to CCh in Colony 3 cells, either by stimulating adenylate cyclase directly with forskolin or indirectly by addition of prostaglandin E_2. Thus a consistent picture emerges in which calcium-dependent agonists are potentiated by cyclic AMP generating agents only when the ability to form prostaglandins is absent, as for example in piroxicam-insensitive HCA-7 monolayers. For epithelia of Colony 3 and Colony 29 cells it is necessary to stop eicosanoid formation before potentiation can be seen. It will be important to investigate why LBK receptors in HCA-7 cells fail to cause prostaglandin formation. Is it because of the absence of the appropriate G-proteins, phospholipase A_2 or both, or the coupling between these units?

A simple explanation of the potentiation phenomenon is that cyclic AMP activates an apical chloride conductance, while Ca^{2+} activates Ca^{2+}-sensitive K channels in the basolateral membrane, the consequent hyperpolarization increasing the electrical gradient for chloride efflux from the cell. It is likely, however, that the mechanisms are even more complex. For example, there may also be calcium-sensitive chloride channels in the apical membrane (see, e.g. Cliff & Frizzell, 1990) and cyclic AMP may regulate or upregulate the Na-K-2Cl co-transporter in the basolateral membrane, responsible for taking chloride into the cell (Pewitt *et al.*, 1990; Paulais & Turner, 1992). Some of these problems are dealt with in the accompanying papers (MacVinish *et al.*, 1993; Henderson & Cuthbert, 1993).

Our results differ in an important way from those for a cultured epithelium derived from dog trachea (Smith *et al.*, 1990). The model proposed was that basolateral kinin receptors were coupled to activation of phospholipase C and A_2, while the mucosal receptors were coupled to phospholipase A_2 only, supporting the idea that Ca^{2+} would be generated close to the basolateral Ca^{2+}-sensitive K channels. No significant effects of piroxicam on either apically or basolaterally applied LBK was found in HCA-7 epithelia (Cuthbert *et al.*, 1987) while in Colony 29 tissues significant effects were recorded at both surfaces (Figure 5). In piroxicam pretreated Colony 29 epithelia, forskolin (or PGE_2) potentiated the responses to LBK, although neither piroxicam (Cuthbert *et al.*, 1992) nor forskolin (Figure 7) affected the Ca^{2+} response. This potentiation occurred both with apical and basolateral addition of LBK, strongly suggesting that in both membrane domains the receptors were coupled similarly to two transduction mechanisms in Colony 29. In a functional sense it makes no difference if the cellular hyperpolarization needed to accelerate chloride exit is mediated by apical or basolateral K channels. This is further addressed in a companion paper (Henderson & Cuthbert, 1993). By the same reasoning, HCA-7 epithelia have LBK receptors at both surfaces, coupled only to a Ca^{2+} response.

Using single cell measurements of chloride while monitoring Ca_i in colon cells in response to neurotensin it was found that the increase in chloride current preceded a measureable rise in Ca_i (Morris *et al.*, 1990). The authors suggested that localized Ca^{2+} release from stores just below the membrane

was sufficient to trigger the chloride conductance before the bulk cellular Ca_i was raised, and indeed the localised Ca_i concentrations could be high. We suggest the failure to potentiate the responses to A23187 by forskolin may similarly be explained by incorrect spatial relationship. Finally the diversity of pharmacology phenotypes expressed by these three cell types indicate how, with relatively few functional proteins but presumably organised in different ways, diversity can be generated. This is especially so when it is remembered these lines were all cloned from a single human source.

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Kinin-induced chloride permeability changes in colony 29 epithelia estimated from $^{125}I^-$ efflux and MEQ fluorescence

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1 The changes in apical Cl^- permeability of Colony 29 human colonic epithelial monolayers were estimated from the rate constant of $^{125}I^-$ efflux from tissues loaded with the isotope.

2 Forskolin was used to increase intracellular concentrations of adenosine 3:5′ cyclic-monophosphate (cyclic AMP), and A23187 to increase intracellular free Ca^{2+} (Ca_i). Both treatments increased the rate constant for $^{125}I^-$ efflux, indicating an increase in apical Cl^- permeability.

3 Lysylbradykinin (LBK) also increased the rate constant for $^{125}I^-$ efflux, sometimes biphasically. Chelation of intracellular Ca^{2+} with BAPTA or prevention of prostaglandin formation with piroxicam, attenuated but did not eliminate the effect of LBK. It is concluded that LBK affects $^{125}I^-$ efflux through the agency of both cyclic AMP and Ca^{2+}.

4 Ba^{2+} attenuated the effect of LBK and A23187 on $^{125}I^-$ efflux, but had no effect on the action of forskolin. It is concluded that Ca^{2+} has a major effect on K^+ channels, the resulting hyperpolarization increasing the driving force for $^{125}I^-$ efflux. A secondary effect on Ca^{2+}-sensitive Cl^- channels is possible. By contrast, cyclic AMP exerts it major effect on apical Cl^- channels.

5 Using a Cl^- sensitive fluorescent dye, MEQ, the intracellular chloride concentration, Cl_i was estimated to be around 30 mM, which was increased to around 50 mM by forskolin, suggesting cyclic AMP could activate the Na-K-2Cl co-transporter.

6 MEQ fluorescence was used to estimate Cl^- influx and efflux rates of epithelial cells. These were increased three fold by forskolin and dibutyryl cyclic AMP and two fold by LBK and histamine.

7 It is concluded that LBK increases electrogenic chloride secretion in Colony 29 epithelia through the generation of second messengers cyclic AMP and Ca^{2+}, each of which may act on both apical and basolateral membranes.

Keywords: Lysylbradykinin; forskolin; A23187; Ca^{2+}; $^{125}I^-$ efflux; epithelia; chloride secretion

Introduction

Data presented in the preceding paper (MacVinish *et al.*, 1993) indicated that lysylbradykinin affects transepithelial chloride secretion by more than one mechanism. In, for example, Colony 29 epithelia it was concluded that efflux of Cl^- across the apical surface was the result of a cyclic AMP-induced activation of chloride channels together with an increase in the electrical gradient for Cl^- efflux, brought about by hyperpolarization consequent on activation of Ca^{2+}-sensitive K^+ channels. The evidence in this earlier paper was rather indirect and based upon transepithelial chloride transport measured as short circuit current.

In this study we have used two methods to examine the Cl^- permeability of the apical membranes of Colony 29 epithelial monolayers. In the first method cells are loaded with $^{125}I^-$ and the washout of the isotope measured. Addition of drugs affecting anion efflux then alters the washout pattern of the radioisotope. $^{125}I^-$ is an ideal marker for Cl^- since it is a poor substrate for the Na-K-2Cl cotransport pathway (O'Grady *et al.*, 1987) or anion exchange mechanisms (Dalmark & Wieth, 1972) while relative conductive permeabilities of I^- and Cl^- rarely vary by more than two fold (Halm *et al.*, 1988). In the third paper of this series (Henderson & Cuthbert, 1993) we have used patch clamping to look for anion channels, yet that technique has its drawbacks. First, relevant anion channels may be too small to resolve by patching and furthermore, run down is not an uncommon phenomenon in isolated patches. The I^- efflux technique measures the collective action of all types of chloride channels including those in which kinetic characteristics are modified by agonists. The I^- efflux technique is similar to that used previously (Venglarik *et al.*, 1990; Clancy *et al.*, 1990; Henderson *et al.*, 1992).

The second method was to use a fluorescence indicator, MEQ, which is sensitive to Cl^- concentration (Biwersi & Verkman, 1991). This technique allows both estimation of intracellular chloride concentration (Cl_i) , and the flux rate for Cl^- movement into and out of the cells. The two methods are essentially complementary and we have confirmed that LBK affects electrogenic chloride secretion by more than one mechanism.

Methods

Cell culture

Colony 29 cells were grown in 6-well (35 mm diameter) plates (Cell Cult, Sterilin) or on the plastic strips in Leighton tubes (Costar). They were maintained in Dulbecco's Modified Eagle's Medium (Gibco, Europe) supplemented with glucose (25 mmol l^{-1}) and 10% foetal calf serum (Gibco), kanamycin (100 μg ml^{-1}) (Bristol Laboratories), and amphotericin B (2.5 μg ml^{-1}) (E.R. Squibb). Cells were grown in an atmosphere of 5% CO_2 at 37°C. T84 cells, another human colonic epithelial cell line, were cultured exactly as for Colony 29. They were used only for verification of intracellular Cl^- measurements by comparison with published values.

^{125}Iodide efflux

The cells were cultured as above for 5–6 days until they were 50–70% confluent. To load with $^{125}I^-$, cells were exposed to

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buffer containing 2.5 μCi ml^{-1} $^{125}I^-$ for 90 min at 37°C. After this, cells were washed three times with 'efflux buffer' (see below). Efflux of $^{125}I^-$ was then measured every 30 s by rapidly removing efflux buffer (3 ml) and replacing it. This procedure was continued for 10 min with buffer maintained at 37°C. At convenient times, efflux buffer containing drugs was substituted for plain buffer. At 10 min, 3 ml 0.1 N HNO_3 was added and left for 30 min to extract residual isotope. All samples were counted for γ-irradiation. The data were processed as described in detail previously (Henderson *et al.*, 1992). In some experiments BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid ester; Molecular Probes, Oregon) was used to chelate intracellular Ca^{2+}. To do this, cells were preincubated with acetylmethoxy ester (5 μM) at the same time as the cells were loaded with $^{125}I^-$. Esterase action liberated BAPTA which was trapped inside the cells.

The efflux buffer used had the following composition (mM): NaCl 135, $CaCl_2$ 1.2, $MgCl_2$ 1.2, KH_2PO_4 0.6, K_2HPO_4 2.4, glucose 10 and HEPES 10 (pH 7.2). In experiments where Ba^{2+} was used, the phosphates were omitted and replaced with KCl, 3 mM, and the HEPES concentration was increased to 15 mM.

MEQ fluorescence

Synthesis of 6-methoxy-N-ethyl-1,2-dihydroquinoline (DiH-MEQ) Synthesis was carried out as described by Biwersi & Verkman (1991). The unstable lipophilic product was stored in small aliquots under N_2 in ampoules at −70°C. DiH-MEQ is insensitive to [Cl^-] but is readily oxidized to MEQ which is [Cl^-]-sensitive. Oxidation of DiH-MEQ by bubbling with O_2 in a phosphate buffer (pH 7.4) gave a compound with an emission spectrum identical to that published. Fluorescence quenching by Cl^- using peak excitation (350 nm) and peak emission (435 nm) wavelengths for MEQ showed that the Stern-Volmer plot was linear over the concentration range 0–50 mM Cl^- with a Stern-Volmer constant, K_{Cl} of 154 M^{-1} and where

$$\frac{F_o}{F} = 1 + K_{Cl}[Cl] \quad (1)$$

with F_o being the fluorescence intensity in the absence of Cl^-, while F is the fluorescence intensity in the presence of Cl. The value of K_{Cl} is close to the value of 145 M^{-1} recorded by Biwersi & Verkman (1991).

Loading Colony 29 monolayers with DiH-MEQ DiH-MEQ was dissolved in a few μl (5–10) of NaOH (pH 9.0) and the volume adjusted to give a solution of around 400 μM in a standard salt solution (solution 1, Table 1). Colony 29 monolayers on plastic slips were exposed to this solution for 10 min at 37°C. Afterwards the monolayers were washed in solution 1 and incubated for a further 15 min at 37°C to allow for oxidation of DiH-MEQ and distribution in the cells.

Table 1 Composition of solutions: concentrations are in mM

	Solution number					
	1	2	3	4	5	6
NaCl	137			25		
$NaNO_3$		137				25
Na isethionate			137			
Na gluconate					25	
KCl	5.4			120		
KNO_3		5.4				120
K acetate			5.4			
K gluconate					120	
$CaCl_2$	1.0			2.0		
$Ca(NO_3)_2$		1.0				4.0
$CaSO_4$			3.0			
Ca gluconate					4.0	
$MgSO_4$	0.3	0.3	0.3	2.0	2.0	2.0
KH_2PO_4	0.4	0.4	0.4	0.4	0.4	0.4
HEPES	10	10	10	10	10	10
Glucose	11	11	11	11	11	11

All solutions are adjusted to pH 7.4

Recording of MEQ fluorescence The plastic strips bearing MEQ loaded Colony 29 cells were cut into two and each held in a vertical plastic block which fitted into a cuvette so that emitted fluorescence could be recorded as described by Pickles & Cuthbert (1991). Cuvettes were mounted in a LS-5B Perkin-Elmer spectrofluorimeter interfaced with a 640 Kbyte IBM compatible PC with a Perkin Elmer Software Package. Monolayers were excited at 350 nm and emission measured at 435 nm. Values of the emission intensity were recorded every 2.5 s and captured on disc, for playback and analysis.

Procedures for calibration and solution changing The cuvette and contents were maintained at 37°C by a heat exchanger and the cuvette could be either perfused with solutions at 37°C (flow rate 8.5 ml min^{-1}) or the solutions changed within 3 s by hand-held syringes connected by tubing to the cuvette for either solution addition or withdrawal.

To calibrate MEQ fluorescence within cells the double ionophore technique was used (Chao *et al.*, 1990). Using high potassium containing solutions (mixtures of solutions 4 and 5 for Colony 29 or mixtures of solutions 4 and 6 for T84 cells) containing nigericin, 5 μM, and tributyltin, 8 μM, the value of F_o/F was measured for a number of Cl^- concentrations. Finally the perfusing solution was changed to potassium thiocyanate solution (170 mM) containing valinomycin, 5 μM, to quench completely fluorescence due to MEQ and define the basal fluorescence unconnected with MEQ (autofluorescence). In the conditions used for calibration, the high K^+ solutions depolarize the cells, nigericin acts as a K^+/H^+ exchanger and clamps the pH and tributyltin acts as a Cl^-/OH^- exchanger to abolish the chloride gradient. In this situation the intracellular and extracellular Cl^- concentrations are equal. Treatment with nigericin and tributyltin increases cellular permeability and dye leakage so experiments were completed in 4 min and calibration at only two Cl^- concentrations was attempted with each preparation.

Measurement of Cl^- efflux rates Flux rates, both influx or efflux, were obtained from the rate of change of fluorescence on changing from zero chloride (solution 2) to high Cl^- (solution 1) or *vice versa*, and finally exposing to KSCN solution with valinomycin. Influx or efflux was calculated as J_{Cl} from

$$J_{Cl} = \frac{F_o}{K_{Cl}\,F^2}\,\frac{dF}{dt} \quad (2)$$

where F and F_o have the same meaning as before, K_{Cl} is the Stern-Volmer constant from the intracellular calibration curve and dF/dt is the initial rate of change of fluoresence. In the experiments in which ionophores were not used the experiments lasted about 15–20 min without great inaccuracy due to MEQ loss. The rate of loss in MEQ monolayers perfused with solution 1 was 25 ± 3% ($n = 4$) per hour.

Drugs and materials

6-Methoxyquinoline, iodoethane, sodium borohydride and diphenylamine carboxylate were from Lancaster Synthesis. Nigericin, tributyltin, valinomycin and 4-bromo A23187 were from Sigma and concentrated stock solutions were made up in ethanol or 95% ethanol. Forskolin was from Calbiochem and again stock solutions were made in 95% ethanol. All other drugs were from Sigma and were dissolved in the appropriate solutions 1 to 6 (Table 1). Hoe 140 was a gift from Dr B. Schölkens of Hoechst AG.

Results are given as mean ± s.e.mean.

Results

Responses to second messengers

As most of this paper is concerned with the effects of LBK on epithelial responses in cultured monolayers and in particular the role of second messengers, typical responses to manoeuvres which increase the concentration of cyclic AMP and Ca^{2+} within cells were investigated. Both forskolin, to increase cyclic AMP levels, and A23187, to increase Ca_i, produced a rapid increase in the rate constant for $^{125}I^-$ efflux underlying a presumed increase in apical chloride permeability (Figure 1). Although the responses appeared rapidly they were not maintained at the initial peak values, but fell to a plateau value which was usually greater than the values before agents were added. The high values of the rate constants seen in the first minute or so of the 10 min experimental period represent washout of residual loading solution. It was preferable not to extend the washing period since the radioactivity released becomes progressively less throughout the experiment. Even with the current format standard errors became somewhat larger in the last minute or two, a consequence of the reducing pool of $^{125}I^-$ left in the tissue.

As with forskolin and A23187, LBK also produced an increase in the $^{125}I^-$ efflux rate constant, as shown in Figure 2a, but the effect was smaller. Figure 2a shows that a secondary increase in rate constant occurs at 6.5 min, a result more clearly understood by examining the individual experiments making up Figure 2a and shown in Figure 2b. Following addition of LBK there was a synchronized rapid increase in rate constants in all monolayers. There was, however, a further secondary increase in rate constants which occurred at variable times after the first peak and which all but disappeared when the data were consolidated (Figure 2a). Nevertheless, this result may indicate that LBK affects apical anion efflux by more than one mechanism, the second being less synchronized than the first.

Diphenylamine carboxylate (DPC), a chloride channel blocker (Distefano *et al.*, 1985), and a B_2 kinin receptor antagonist, Hoe 140 (Hock *et al.*, 1991) reduced the increase in $^{125}I^-$ efflux in Colony 29 monolayers caused by LBK as shown in Figure 3. Notice there is a late onset effect of LBK after Hoe 140, which is discussed later. Therefore it seems reasonable to assume that LBK, acting at a B_2 receptor, increases $^{125}I^-$ efflux through chloride channels which are

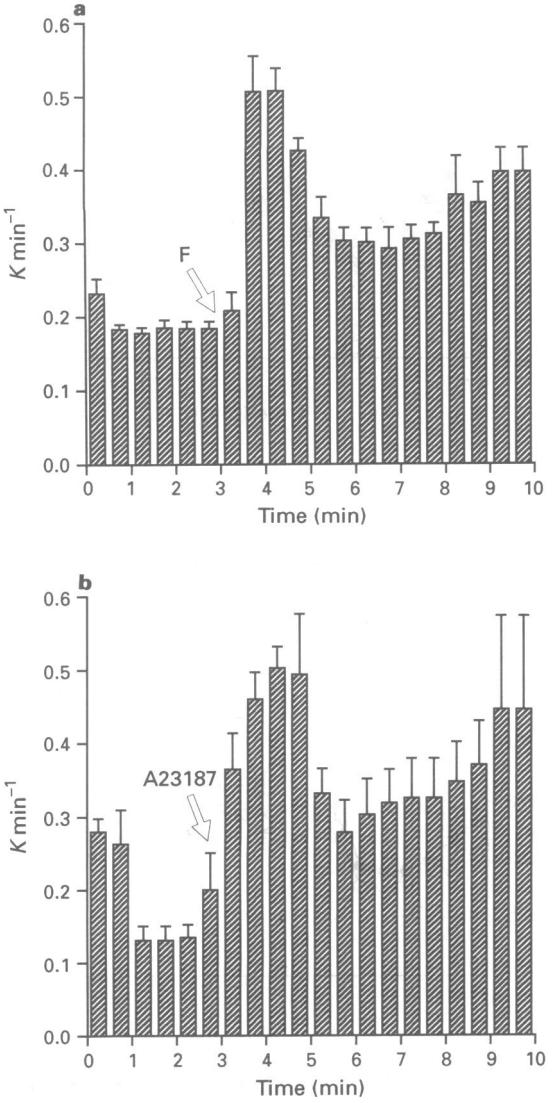

Figure 1 Effect of forskolin (F) (10 μM) (a), and A23187 (1 μM) (b) on the rate constant for $^{125}I^-$ efflux from Colony 29 epithelia. Each column shows the mean ± s.e.mean (vertical bars) for six measurements. Drugs were applied at the arrows.

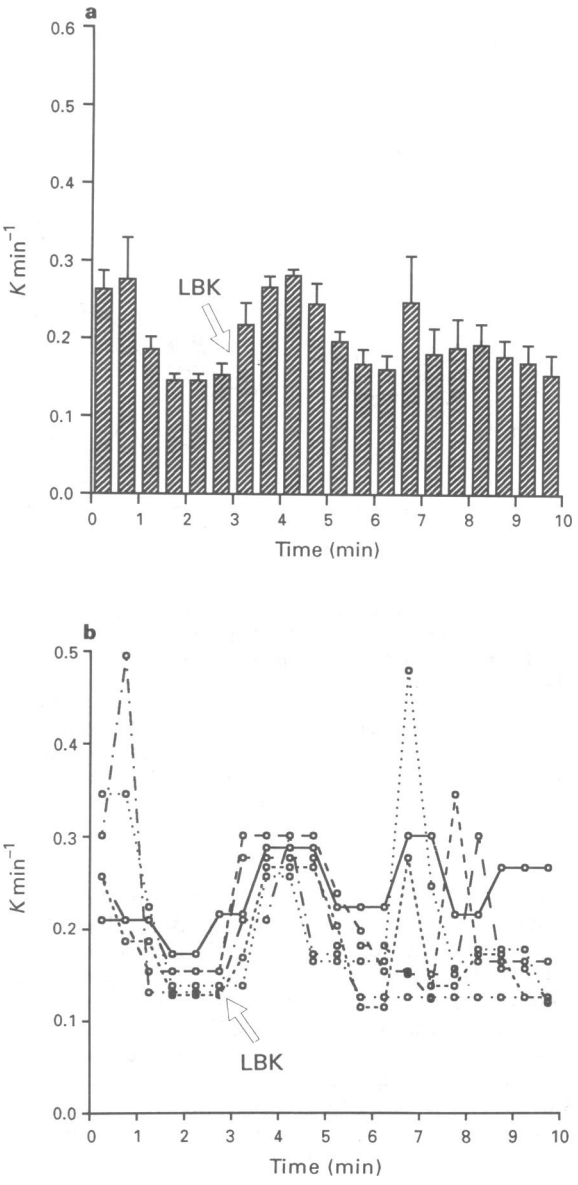

Figure 2 Effect of lysylbradykinin (LBK, 0.1 μM), applied at arrow, on the rate constant for $^{125}I^-$ efflux from Colony 29 epithelia. Each column shows the mean ± s.e.mean (vertical bars) for six measurements. In (b) the individual experiments contributing to the averaged data in (a) are shown.

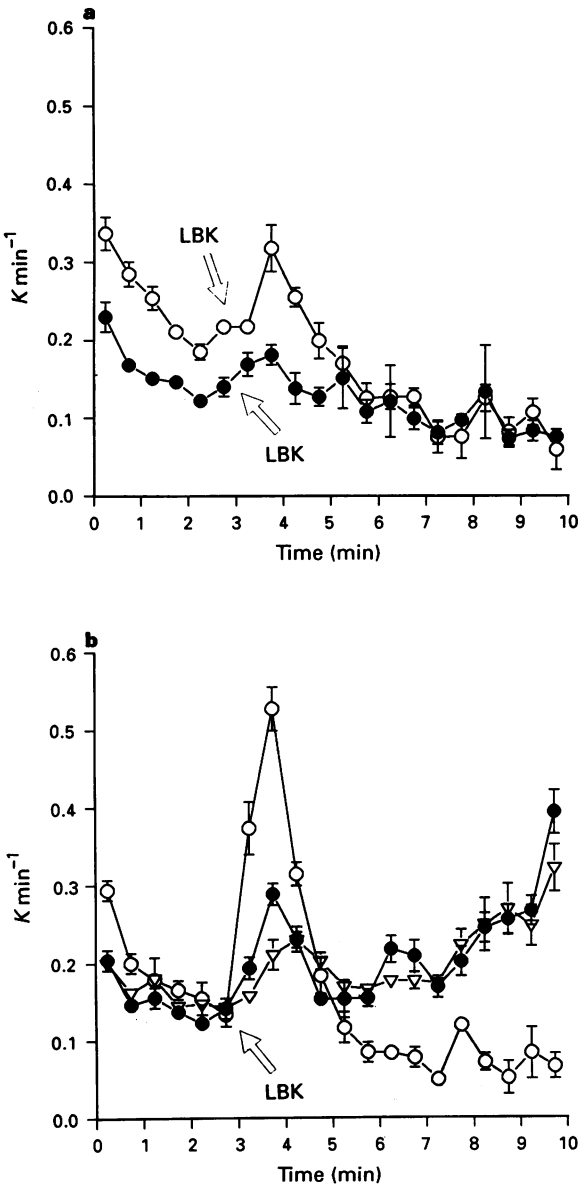

Figure 3 (a) Effects of lysylbradykinin (LBK, 0.1 μM), applied at arrows, on the rate constant for $^{125}I^-$ efflux: (○) are control observations while (●) are for epithelia exposed to diphenylamine carboxylate (DPC) 30 μM. The maximum increase caused by LBK was 0.10 min^{-1} in the control and 0.02 min^{-1} in the presence of DPC. (b) As for (a) with control values shown by (○); (●) and (△) representing tissues pre-exposed to Hoe 140, 3 nM and 10 nM respectively for 30 min. All values are the means ± s.e.mean (vertical bars) for six observations with Colony 29 epithelia.

sensitive to DPC. However, this does not mean the action of the peptide is directly on the channel. Both Hoe 140 and DPC inhibit the SCC responses to LBK, an expected result if the $^{125}I^-$ effluxes report upon apical chloride permeability. Hoe 140 was effective at nM concentrations here and had a K_i of around 5 nM against LBK with SCC measurements (Cuthbert *et al.*, 1992). DPC was much less effective against LBK on $^{125}I^-$ efflux. This agent acts on the apical side of Colony 29 epithelia and is non-selective, blocking Cl^- exit activated by secretagogues. For example, DPC (30 μM) significantly reduced the effect of ATP (30 μM) on Colony 29 monolayers from 14.6 ± 0.9 μA cm^{-2} (mean ± s.e.mean, n = 18) to 8.0 ± 0.8 μA cm^{-2} (mean ± s.e.mean, n = 6). These values were significantly different at $P<0.01$.

Further experiments were performed to examine the mechanism by which LBK brings about the change in $^{125}I^-$ efflux. First, is stimulation of anion efflux related to the known increase in intracellular Ca^{2+} (Pickles & Cuthbert, 1991)? This was investigated by use of BAPTA as an intracellular Ca^{2+} chelator. Inclusion of BAPTA inside the cells reduced the efflux rate significantly ($P<0.001$) during the first minute after addition of LBK. Thereafter, the delayed peak followed the time course of the declining phase of the uninhibited response. This is to be compared with the effects of forskolin which gave identical changes in maximal efflux rate constants whether BAPTA was present or not (Figure 4a,b). Thus it appears that BAPTA can influence the response to LBK but not that to forskolin, suggesting part of the LBK response in $^{125}I^-$ efflux is calcium-dependent.

The role of prostaglandin formation

It was shown in the accompanying paper (MacVinish *et al.*, 1993) that SCC responses to LBK in Colony 29 monolayers are attenuated but not abolished by piroxicam, a cyclo-

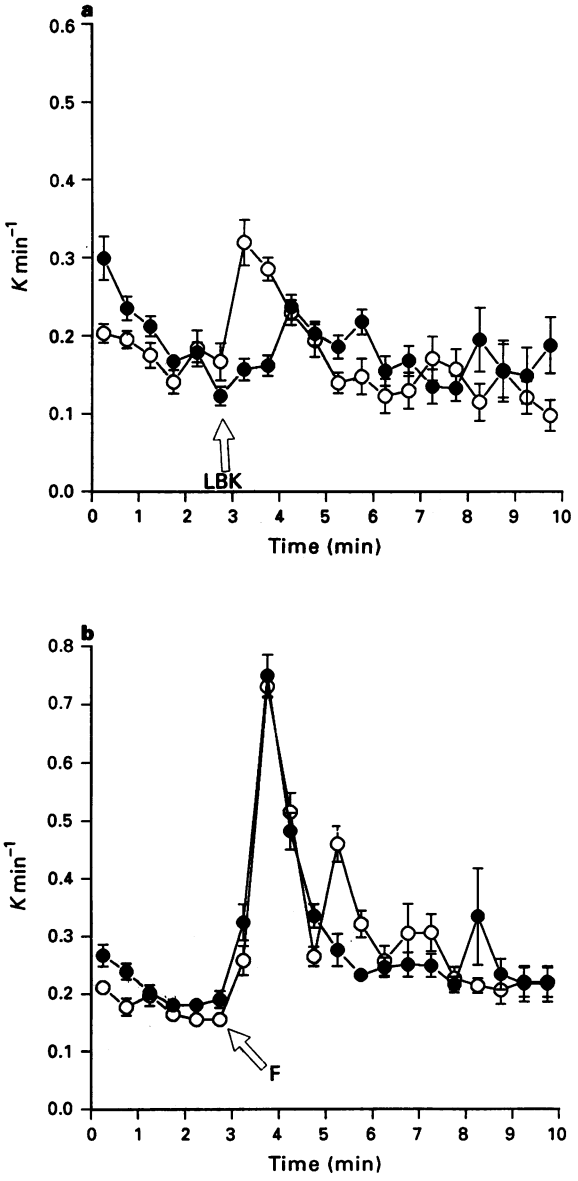

Figure 4 (a) Effect of lysylbradykinin (LBK, 0.1 μM) on the rate constant for $^{125}I^-$ efflux: (○) show control values, while (●) are with tissues containing the intracellular Ca^{2+} chelator, BAPTA. (b) Effect of forskolin (F) (10 μM) on $^{125}I^-$ efflux: (○) are controls and (●) are from tissues containing BAPTA. All measurements are the means ± s.e.mean (vertical bars) for 6 measurements with Colony 29 epithelia.

oxygenase inhibitor. Using $^{125}I^-$ efflux it was shown (Figure 5a) that piroxicam also attenuates the response to LBK. In this example the secondary increase in $^{125}I^-$ efflux in response to LBK is well demonstrated in the controls, but is not so in piroxicam-treated monolayers. It seems likely from these results that prostaglandins are responsible for part of the anion efflux response caused by bradykinin, but additionally there is a non-prostaglandin-dependent component. To obtain data about the first of these, it was necessary to show prostaglandin E_2 (PGE_2) could increase $^{125}I^-$ efflux (Figure 5b). Furthermore the opportunity was taken to examine if the response to LBK could be potentiated in the presence of both piroxicam and PGE_2, as indeed SCC responses were shown to be in the accompanying paper (MacVinish *et al.*, 1993).

In the piroxicam-treated tissues the effects of LBK were only slightly, but not significantly, increased by pre-exposure to PGE_2 (Figure 5c), even though under these conditions SCC responses would have been potentiated. In HCA-7 epithelia where piroxicam has no effect on SCC responses to LBK, the responses to the peptide can be potentiated by forskolin. A further experiment was therefore carried out, but on this occasion with HCA-7 monolayers. As in Figure 5c the $^{125}I^-$ efflux response to LBK was marginally, but not significantly increased by pre-incubation with an agent to increase cyclic AMP content (Figure 5d). Thus in the circumstances which cause potentiation of the transporting responses to LBK there was no significant effect on the increase in efflux rate constant caused by LBK.

Involvement of calcium-sensitive potassium channels

Calcium-sensitive potassium channels are present in epithelia, including Colony 29 monolayers (Henderson & Cuthbert, 1993). Experiments were designed to examine the hypothesis that opening of these channels influences the efflux of $^{125}I^-$ from preloaded cells. The first approach was to use Ba^{2+} ions to block K^+ channels and to examine the effects of LBK. Figure 6a shows that 1 mM Ba^{2+} significantly reduced the effect of LBK on $^{125}I^-$ efflux, but there remained a small

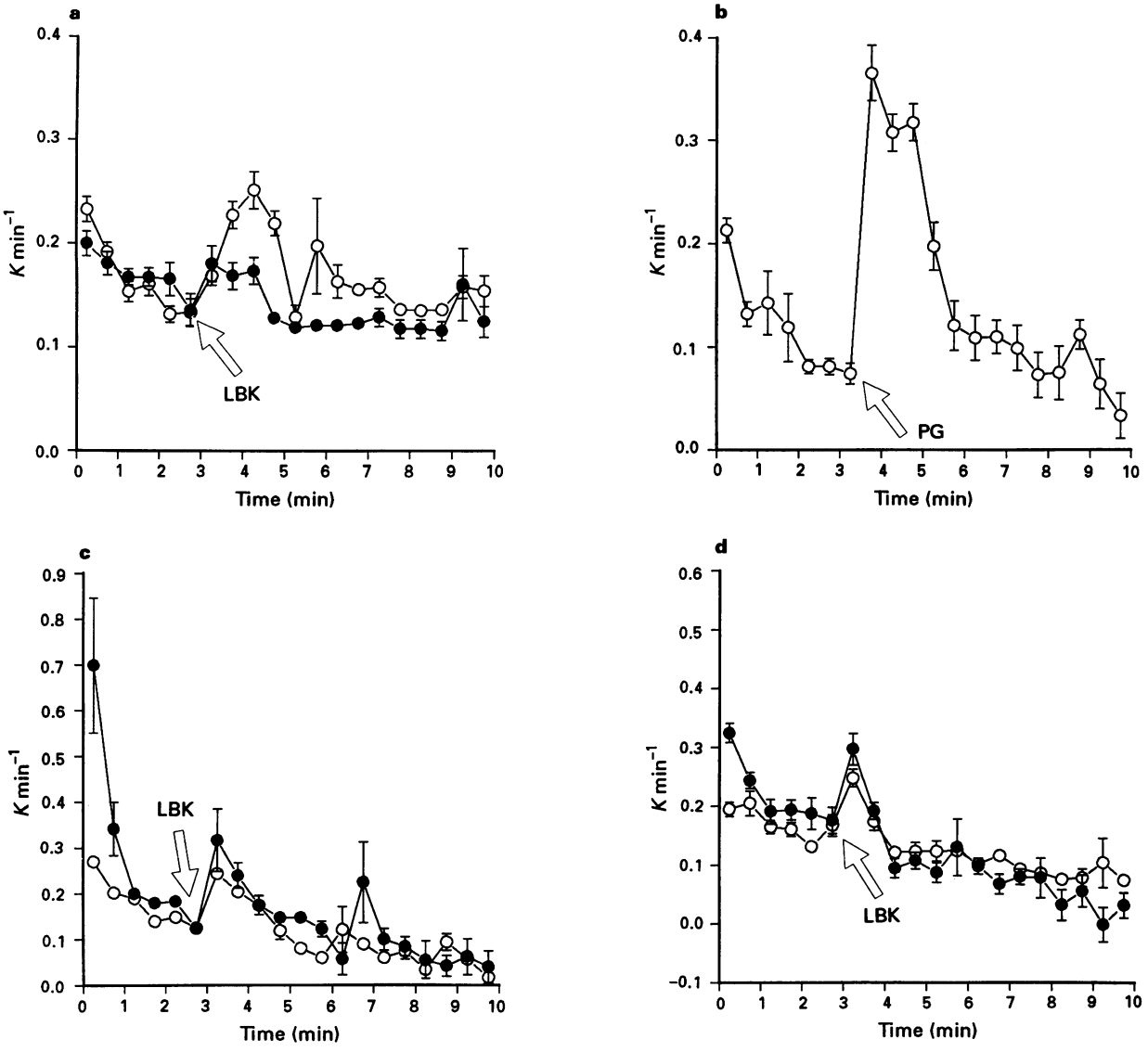

Figure 5 Parts (a–c) illustrate data obtained with Colony 29 epithelia, while (d) are data obtained with HCA-7 monolayers. (a) Effects of lysylbradykinin (LBK, 0.1 μM) in the absence (○), and presence (●) of piroxicam 10 μM. (b) Effect of prostaglandin E_2 (PG) (10 μM) on $^{125}I^-$ efflux. (c) Effect of LBK (0.1 μM) in piroxicam (10 μM)-pretreated tissues in the absence (○), and presence (●) of prostaglandin E_2 (10 μM). (d) Effect of LBK (0.1 μM) on HCA-7 cells in the absence (○), and presence (●) of forskolin, 10 μM. Each value shows the mean ± s.e.mean (vertical bars) for six observations in (b–d) and for 12 observations in (a). Drugs were added at the times indicated by the arrows.

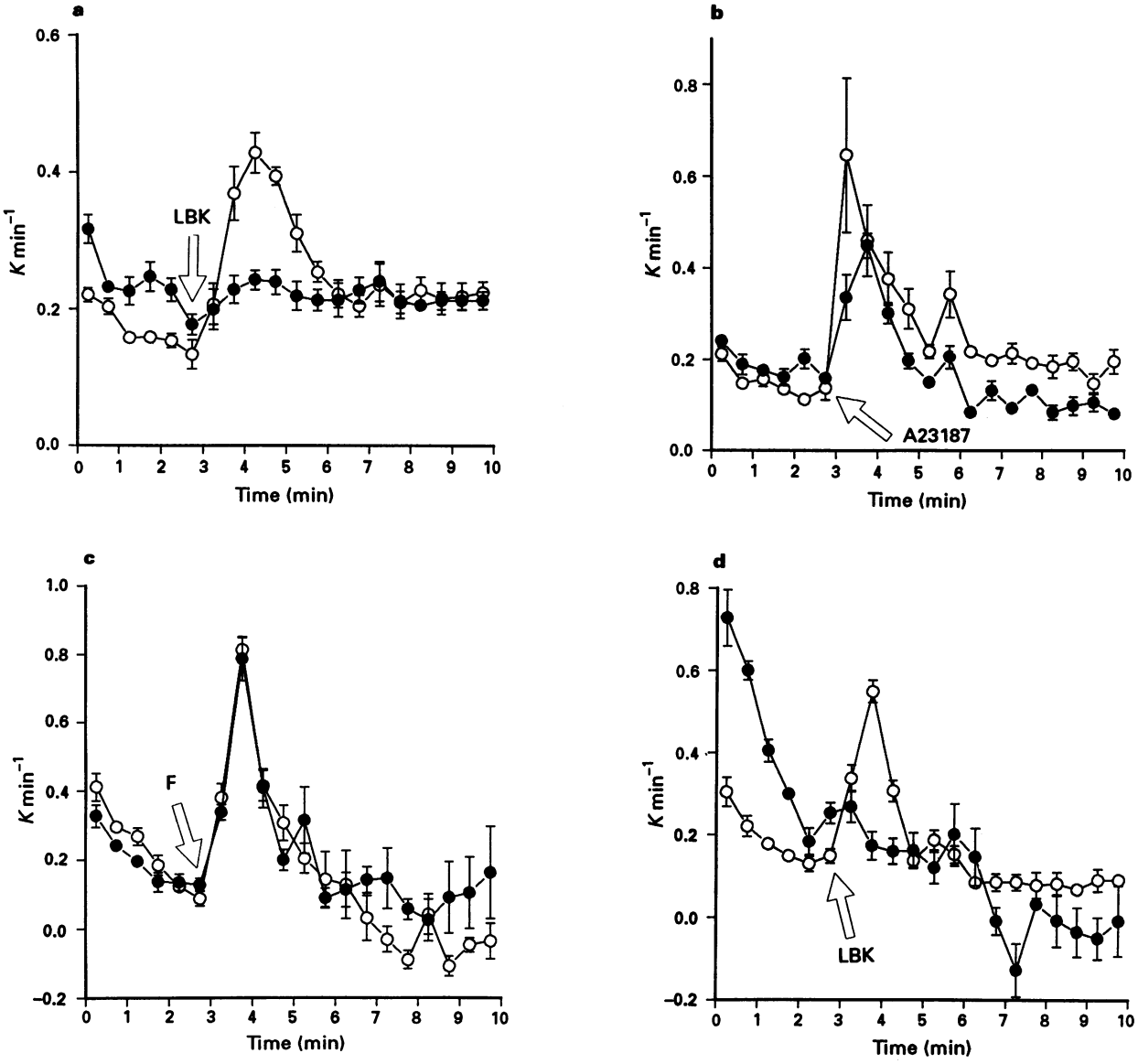

Figure 6 Effect of lysylbradykinin (LBK, 0.1 μM) (a); A23187, 1 μM (b); forskolin (F), 10 μM (c), and LBK, 0.1 μM (d), on $^{125}I^-$ efflux in Colony 29 monolayers. The control results are given by the open symbols. In (a), (b) and (c) Ba^{2+}, 1 mM was present in the test preparations (●), while in (d) test tissues were exposed to high K^+ (145 mM). All values are the means ± s.e. mean (vertical bars) for six observations. Drugs were added as indicated.

increase in the presence of alkaline earth metal ion. Specifically, the peak response to LBK (0.1 μM) in the absence (0.43 ± 0.03 min^{-1}) of Ba^{2+} was significantly greater ($P<0.001$) than the peak response in its presence (0.24 ± 0.01 min^{-1}). Nevertheless the rate constant for efflux in the presence of Ba^{2+} rose from 0.18 ± 0.01 min^{-1} to 0.24 ± 0.01 min^{-1} when LBK was added. These values are significantly different ($P<0.05$) indicating a residual effect of the peptide.

To investigate whether the Ba^{2+}effect was a result of some action other than blocking K^+ channels, such as interfering with the action of LBK at its receptor, the effect of this ion on A23187 effects on $^{125}I^-$ efflux was investigated. In this paradigm the intracellular Ca^{2+} was raised directly by the ionophore without involving surface receptors. While Ba^{2+} delayed and diminished the peak response to A23187 (Figure 6b) significant differences from control ($P<0.05$ to $P<0.01$) only appeared 90 s after addition of the ionophore, indicating that high Ca^{2+} concentrations may have actions other than on K^+ channels. Finally, in Figure 6c, it is shown that the action of forskolin was not affected by Ba^{2+}.

In a final attempt to examine the role of K channels the effects of LBK on $^{125}I^-$ efflux were investigated under high K^+ conditions. It is expected that high K^+ outside the cells would depolarize them and make it impossible to activate Ca^{2+}-sensitive K^+ channels to cause hyperpolarization. This protocol disturbed the normal pattern of $^{125}I^-$ efflux but, nevertheless, there was only a modest increase in efflux after LBK compared to controls (Figure 6d).

Measurements of intracellular [Cl^-] from MEQ fluorescence

Using the double ionophore technique a Stern-Volmer plot for the relationship between F_o/F and [Cl^-] was constructed for Colony 29 monolayers loaded with MEQ. This is shown in Figure 7, and the value of K_{Cl} estimated as 14.4 M^{-1}, from equation 1. To estimate the intracellular Cl^- concentration, MEQ loaded Colony 29 monolayers were exposed first to Cl^--containing and then to zero Cl^- solutions (solutions 1 then 2 or 3), and finally to $KSCN^-$ in the presence of valinomycin. F_o/F ratios were calculated for 12 separate

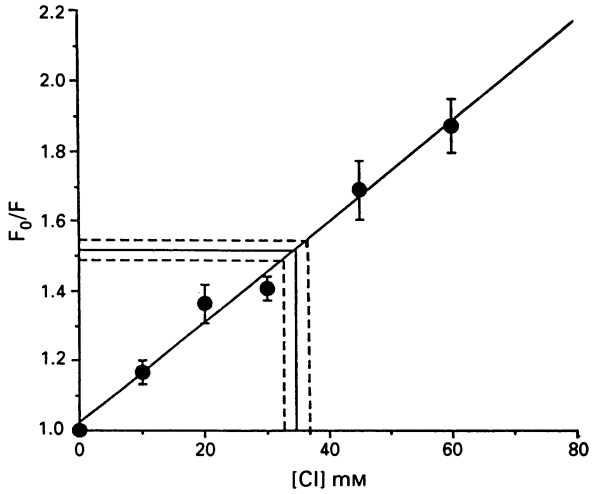

Figure 7 Stern-Volmer plot obtained at a range of Cl^- concentrations in MEQ-loaded Colony 29 monolayers using the double ionophore technique. F_o/F represents the fluorescence ratio as defined in Methods. The F_o/F ratio for 12 untreated monolayers was 1.5 ± 0.02 (mean ± s.e.mean) which translates to a value of Cl_i of 34.1 ± 1.4 mM.

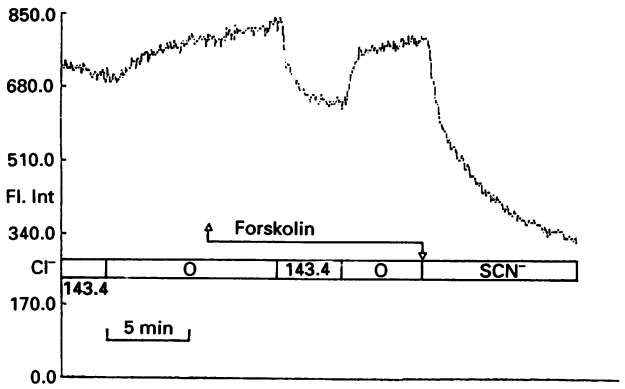

Figure 8 Fluorescence intensity (Fl. Int) from a MEQ-loaded Colony 29 monolayer against time. Measurements were made every 2 s. The Cl^- concentration of the bathing solution (in mM) is as indicated. Finally KSCN (170 mM) plus 10 μM valinomycin was added. Forskolin was present for the period indicated.

monolayers and gave a value of 1.5 ± 0.02. By extrapolation from the Stern-Volmer plot the intracellular Cl^- concentration is 34.1 ± 1.4 mM (i.e. 25.6 mM Cl^- activity). For comparison, Cl^- concentration in T84 epithelial monolayers was also measured and found to be 32.8 mM (24.6 mM activity).

A similar technique was used to measure Cl^- flux by measuring the rate of change of fluorescence when Cl^- containing solutions were added to or removed from the monolayers. However, influx and efflux measurements needed to be performed in different monolayers, in order to limit the experimental protocol to 20 min or less. This precaution was to avoid complexities due to significant dye leakage. An example of an experiment to examine the effects of forskolin on Cl^- efflux is given in Figure 8. By changing from a chloride-containing to a chloride-free solution, the fluorescence signal relaxes to a new and higher steady state. The solution changes are then repeated after forskolin is added to the tissue. In this way the rate of loss of Cl^- from the cells in presence and absence of forskolin can be estimated. Chloride influx can be similarly estimated by measuring the rate of change of fluorescence change in moving from Cl^--free to Cl^--containing solutions.

The initial rate of change of fluorescence was converted to either influx or efflux rate by use of equation 2, and the data are summarized in Table 2. The method also allows estimation of F_o/F values, both in the presence and absence of forskolin. It is seen that forskolin significantly increased both the efflux and influx rates for chloride in Colony 29 monolayers. Furthermore, there was a significant increase in the F_o/F ratio caused by forskolin, corresponding to an average increase in $[Cl_i]$ from 33 mM to 51 mM.

Other experiments with dibutyryl cyclic AMP, LBK, histamine and 4-Br-A23187 were carried out in a similar manner and the data are summarized in Table 3. Significant increases were measured with the cyclic AMP analogue, but with LBK and histamine the increases, while significant, were less than double the basal values. The calcium ionophore, by contrast, produced no change in influx or efflux rates.

Table 2 Effects of forskolin, 10 μM, on the influx and efflux rates for Cl^- estimated from the change in MEQ fluorescence and on the values of F_o/F obtained from steady state fluorescence measurements

	Efflux rate (mM s^{-1})		F_o/F	
	Control	*Forskolin*	*Control*	*Forskolin*
1	0.19	1.08	1.38	1.68
2	0.24	0.77	1.30	1.60
3	0.35	0.81	1.47	1.61
Mean ± s.e.	0.26 ± 0.05	0.89 ± 0.10		
	$P<0.05$			
	Influx rate (mM s^{-1})		F_o/F	
	Control	*Forskolin*	*Control*	*Forskolin*
1	0.42	1.61	1.58	1.80
2	0.53	1.07	1.62	1.73
3	0.54	1.74	1.60	1.77
4	0.62	2.20	1.48	2.00
5	0.65	1.91	1.48	1.87
Mean ± s.e.	0.55 ± 0.04	1.71 ± 0.19	1.49 ± 0.04	1.76 ± 0.05
			32.6 ± 2.7 mM	51.3 ± 3.5 mM
	$P<0.005$			
				$P<0.001$

The mean F_o/F values for all eight experiments were converted to $[Cl_i]$ using equation 1.

Table 3 Effects of drugs on efflux and influx rates estimated from MEQ fluorescence

	Efflux rate (mM s^{-1})	n	P	*Influx rate* (mM s^{-1})	n	P
Control	0.26 ± 0.05	3		0.55 ± 0.04	5	
Forskolin, 10 μM	0.89 ± 0.10	3	<0.05	1.71 ± 0.19	5	<0.005
Control	0.13 ± 0.02	3		0.16 ± 0.04	3	
Dibutyryl cyclic AMP (0.5 mM) plus IBMX (0.1 mM)	0.44 ± 0.02	3	<0.05	0.50 ± 0.11	3	<0.05
Control	0.16 ± 0.04	3		0.31 ± 0.09	4	
LBK, 1 μM	0.22 ± 0.03	3	<0.05[P]	0.54 ± 0.16	4	<0.05[P]
Control	0.14 ± 0.02	5		0.19 ± 0.04	8	
Histamine, 100 μM	0.24 ± 0.04	5	<0.05[P]	0.33 ± 0.06	8	<0.01[P]
Control	0.20 ± 0.07	3		0.22 ± 0.04	3	
4-Bromo A23187, 3 μM	0.19 ± 0.04	3	NS	0.22 ± 0.03	3	NS

Flux rates were calculated from the initial rate of change of fluorescence using equation 2. Student's *t* test was used to test for significance, [P]indicating where it was necessary to use a paired test to obtain significance. Number of observations is indicated by *n*. All values are the mean ± s.e.mean.

Discussion

Unlike the first paper in this series (MacVinish *et al.*, 1993) we have used mainly Colony 29 epithelia in this study, as in this line LBK is considered to affect transepithelial transport by more than one mechanism. The intracellular mediators, cyclic AMP and Ca^{2+}, clearly increase the rate constant for $^{125}I^-$ efflux as shown in Figure 1, but a survey of other figures, for example Figure 3b and Figure 6, shows that these mediators do not necessarily cause a maintained increase in rate constant. At first this may seem unexpected for it implies, for example, that Cl^- channels cease to be sensitive to cyclic AMP. However, it is to be remembered while the $^{125}I^-$ concentration in the cell is falling, the Cl^- content can be replenished. In this circumstance the rate constant for $^{125}I^-$ will only remain constant if the Cl^- concentration is unchanged. Evidence from MEQ fluorscence indicates that the Cl^- concentration actually increases with forskolin so that the specific activity of the $^{125}I^-$ as a marker for Cl^- is depressed further than by a single exponential decline. The conclusion is therefore that while a change in rate constant for $^{125}I^-$ efflux is a useful indicator of a change in chloride permeability, or the driving force for efflux or both, that precise quantitative interpretation is not possible. This would be even more true for agents such as LBK where the action undergoes rapid desensitization. It is shown in Figure 2 that LBK may cause two peaks of increased $^{125}I^-$ efflux, perhaps indicating two separate actions, temporally discrete. The absence of the second phase in the averaged data does not mean the second phase is absent, but simply asynchronous. Again, the method does not allow precise analysis of this and we have relied upon inhibition of the mean overall effect of LBK to unravel mechanisms. From the present data we can conclude that LBK, acting on B_2 receptors, increases anion efflux through DPC-sensitive anion channels. Furthermore the data with monolayers containing BAPTA (Figure 4a) or monolayers pretreated with piroxicam (Figure 5a) allow the conclusion that the well-documented Ca_i signal generated by LBK (Pickles & Cuthbert, 1991) plus prostaglandin formation (Figure 5c) both contribute to the effect of the peptide on anion permeability. Supporting evidence is that PGE_2 itself increases the rate constant for $^{125}I^-$ efflux (Figure 5b) and that there is apparently no Ca^{2+} requirement for the effect of forskolin on anion permeability (Figure 4b).

It was shown in the first paper (MacVinish *et al.*, 1993) that increasing cyclic AMP content in HCA-7 cells dramatically potentiates the response to LBK and that a similar phenomenon can be demonstrated in Colony 29 cells if steps are taken to prevent LBK itself increasing cyclic AMP, i.e. by reducing eicosanoid formation with piroxicam. It was also shown that the extent of the Ca_i increase following LBK is not changed by elevated cyclic AMP levels. In this study it was shown that elevation of cyclic AMP concentration in HCA-7 or Colony 29 monolayers does not significantly affect the size of the $^{125}I^-$ efflux response to LBK. Intuitively this seems contrary to expectations. However as shown in Figure 5c,d the efflux rate constant for $^{125}I^-$ has returned to a low value at the time LBK is added. As discussed earlier this does not mean that the apical Cl^- channels are no longer affected by the elevated cyclic AMP but that a new steady state has been achieved, probably with an elevated Cl^- concentration in the cell. It is then reasonable to expect that the resulting increment in rate constant induced by the Ca_i elevating component of the action of LBK will be constant, as is found. This again speaks for the independence of two distinct mechanisms of LBK action which in Colony 29 epithelia is exerted both on membrane permeability for Cl^- and on the driving force for anion efflux.

Using Ba^{2+}, a general blocker of many different sorts of K^+ channels (Kolb, 1990), there was a severe but incomplete inhibition of increased I^- efflux following LBK, although we have not proven this residue is due to cyclic AMP formation. Furthermore the interplay of increased chloride permeability and increased driving force makes it unpredictable what effect chloride permeability alone would have. It is striking that Ba^{2+} has less inhibitory effect on the action of A23187 (Figure 6b) than might be expected and raises the possibility that Cl^- channels sensitive to Ca^{2+} may occur in the apical membrane (Cliff & Frizzell, 1990). However in the following paper (Henderson & Cuthbert, 1993) we have been unable to detect Ca^{2+} sensitive anion channels or indeed anion channels which are responsible for chloride secretion. The single channel basis of epithelial chloride conductance has proved elusive in many studies. Channel characterization is usually done on inside-out patches yet the picture emerging is often in conflict with whole cell current studies or gross transport measurements. However, many Cl^- channels have very small conductances (Gray *et al.*, 1989; Marunaka & Eaton, 1990) which may be swamped if larger channels are also present in the patch. In addition chloride channels in patches may show run down or Ca^{2+}-dependent regulating proteins may be lost. Only rarely have calcium-dependent chloride currents in the whole cell configuration been reasonably well attributed to very small unitary conductances (Marty *et al.*, 1984; Evans & Marty, 1986). In summary, it seems likely that the Ba^{2+} resistant part of the A23187 response may represent a Ca^{2+} effect on calcium-sensitive chloride channels. Ba^{2+} had no effect on the cyclic AMP-dependent increase in Cl^- permeability (Figure 6c), while depolarization with high potassium solution virtually abolished the response to LBK (Figure 6d).

We have used MEQ fluorescence to provide corroborative evidence for the $^{125}I^-$ efflux data. Because MEQ has no isosbestic point, only a single wavelength technique can be

used. Consequently we have carried out a number of checks on the validity of the method. The intracellular Stern-Volmer constant, K_{Cl}, was 14.4 M^{-1}, in the centre of the range found by others, of 12–18 M^{-1} (Verkman, 1990). The value is much lower than that obtained in phosphate buffer (154 M^{-1}), proably because of interaction with fixed anions within the cells. In Colony 29 monolayers, the value of intracellular Cl^- concentration was 34.1 mM and T84 monolayers, 32.8 mM. These values are comparable to those reported for other epithelial cells; rabbit proximal tubule, 27.5 mM (Krapf *et al.*, 1988); canine tracheal cells, 43 mM (Chao *et al.*, 1990) and T84 cells, 36 mM (Biwersi & Verkman, 1991). The value for Colony 29 cells is some 10 mM above the expected concentration for passive distribution, assuming a membrane potential of −45 mV. Presumably this increase represents activity of the Na-K-Cl cotransporter, which is secondarily powered by the sodium pump. In a further series of experiments the value of Cl_i was 32.6 mM but rose to 51.3 mM in the presence of forskolin (Table 2). This is in spite of the increase apical Cl^- permeability created by cyclic AMP. This provides indirect evidence that cyclic AMP, and hence LBK, can stimulate the cotransporter, as shown for other tissues (Pewitt *et al.*, 1990; Paulais & Turner, 1992).

Table 2 also shows that the Cl^- influx and efflux is increased by forskolin. However, it cannot be assumed that these fluxes are entirely through conductive Cl^- channels. Chloride can enter and leave cells on exchangers and the Na-K-2Cl cotransporter is responsible for Cl^- uptake across the basolateral surface. Ideally it would have been preferable to block the cotransporter with a loop diuretic. Unfortunately, in our hands frusemide severely reduced the fluorescence signal, probably by absorbing at the excitation wavelength and piretanide increased the autofluorescence. It is noticeable that the mean efflux rate in 17 control measurements was 0.15 ± 0.013 mM s^{-1} while the mean influx rate in 23 control measurements was 0.28 ± 0.02 mM s^{-1}, these values being significantly different ($P<0.001$). Both of these must contain a component which represents the conductive flux, plus a component due to the cotransporter when influx is measured. The efflux rate therefore is a truer indication of the rate of Cl^- exchange for NO_3^- through the conductive pathway. Both forskolin and dibutyryl cyclic AMP cause a three fold change in both efflux and influx rates measured from MEQ fluorescence, confirming an effect on apical Cl^- channels. With LBK and histamine significant effects were again observed but the changes were less than two fold. No effect was found with A23187, a finding not in keeping with earlier arguments made for a Ca^{2+}-sensitive Cl^- channel. No explanation for this discrepancy is available.

Finally, a comment is made about the late onset of the effect of LBK on $^{125}I^-$ efflux in the presence of Hoe 140 (Figure 3b). Rapid desensitization occurs when LBK is applied to cultured epithelia. It was demonstrated that cooling can prevent desensitization (Cuthbert *et al.*, 1987) and others showed desensitization is accompanied by receptor internalization (Roscher *et al.*, 1984; Wolsing & Rosenbaum, 1991). Recently, Roscher *et al.* (1991) showed that when Hoe 140 binds to its receptors internalisation is prevented. The late onset effect of LBK may result from freeing of surface receptors when Hoe 140 dissociates, exposing them to activation by the peptide, at a time when all receptors would normally be desensitized. Note that relatively high concentrations of LBK were used (0.1 μM) compared to Hoe 140 (10 nM).

In summary, this study has shown that cyclic AMP and Ca^{2+} increase the rate constant for $^{125}I^-$ efflux indicating an efflux on apical Cl^- permeability or on the electrical driving force for efflux. The Ca^{2+} effect is due to an action on K^+ channels, but some evidence supports a direct action on apical Cl^- channels. Additionally cyclic AMP may have a distinct stimulatory effect on the cotransporter. By separately eliminating the Ca^{2+} component and the prostaglandin generating components of the actions of LBK, it is concluded the peptide uses both second messengers to generate its effects on transepithelial Cl^- secretion.

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Activation of ion channels by lysylbradykinin in the HCA-7 colony 29 human adenocarcinoma cell line

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1 The patch-clamp technique, both cell attached and inside-out patches, was used to examine the effects of lysylbradykinin (LBK) and A23187 on ion channels in cultured Colony 29 epithelial cells derived from a human adenocarcinoma.

2 LBK and A23187 applied directly to the intact cell stimulated the opening of a number of types of ion channel including Ca^{2+}-activated K^+channels.

3 By use of inside-out patches, anion channels could be stimulated to open by application of protein kinase A and ATP to the cytosolic surface. Ca^{2+}-activated K^+ channels were also identified in isolated membrane patches.

4 The results suggest that the anion secretion which is stimulated by LBK is a complex event, involving the activation of a number of different types of ion channel, and that part of the response is the result of hyperpolarization of the cell by activation of Ca^{2+}-activated K^+ channels. From the data presented in this and the accompanying papers it appears that the Ca^{2+}-sensitive K^+ channels would be equally effective in either the apical or basolateral membranes.

Keywords: Ion channels; lysylbradykinin; A23187; colon

Introduction

The occurence of electrogenic chloride secretion in response to kinins has been recognised for some years (Gaginella & Kachur, 1989), from early experiments on guinea-pig ileum epithelium (Manning *et al.*, 1982) and rat colon epithelium (Cuthbert & Margolius, 1982). In these tissues the mechanisms through which kinins increase chloride secretion were investigated by the short ciruit current (SCC) technique. Production of eicosanoids, leading to adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation, plus a requirement for calcium ions was found (Cuthbert *et al.*, 1984b,c). It was suggested that the calcium ions were important in the generation of prostaglandins (Cuthbert *et al.*, 1984b), and might also facilitate secretion by activation of a transporter located on the basolateral pole of the cell (Cuthbert *et al.*, 1984c). Subsequent experiments performed in the presence of the cyclo-oxygenase inhibitor, piroxicam, showed kinin-stimulated Cl^- secretion in rat colon can take place in the absence of eicosanoid synthesis (Cuthbert *et al.*, 1984a), thus reinforcing the probability of a fundamental role for calcium in the response. More recently, lysylbradykinin (LBK) has been shown to stimulate Cl^- secretion, again measured using SCC recordings, in a human colonic cell line, HCA-7 (Cuthbert *et al.*, 1987). In later experiments on Colony 29 cells, a mutant derived from HCA-7, it was shown that LBK increased intracellular Ca^{2+} concentration, Ca_i, but that the relation between Ca_i and SCC was complex (Pickles & Cuthbert, 1991).

In this study the effects on ion channels of LBK, forskolin, an activator of adenylate cyclase, and the calcium ionophore A23187 have been studied in Colony 29 cells. To do this the patch clamp technique has been used, both in the cell-attached and inside-out patch configurations.

Methods

Cell culture

HCA-7 Colony 29 cells, derived from a human colonic adenocarcinoma were cultured as described previously (Cuthbert *et al.*, 1985; Pickles & Cuthbert, 1991). Cells were seeded onto 10 cm^{-2} Petri dishes (Cell Cult) and cultured in Dulbecco's Modified Eagle's Medium supplemented with glucose (25 mM) and 10% foetal calf serum-kanamycin (100 $\mu g\ ml^{-1}$) and amphotericin B (2.5 $\mu g\ ml^{-1}$). Cells were grown at 37°C in an atmosphere of 95% O_2/5% CO_2 as described previously (Cuthbert *et al.*, 1987). All cells were used for experiments 5–10 days after seeding.

Patch-clamp experiments

Experimental arrangements were essentially as described previously (Henderson & Cuthbert, 1991; Henderson *et al.*, 1992; Sheth *et al.*, 1992). Experiments were conducted using the cell-attached and inside-out mode of the patch-clamp technique (Hamill *et al.*, 1981). Cells were studied at 400 × magnification using a Nikon Diaphot microscope equipped with Hoffman Modulation Contrast optics. Recordings of single ion channel activity were made with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA, U.S.A.), filtered at 1500 Hz with an 8 pole Bessell filter (Frequency Devices Model No. 902, Lyons Instruments, Hoddesdon, England) and recorded on digital audio tape (DAT) using a Sony DTC-1000 DAT recorder. Single channel events were viewed during the course of recording on a Gould 400 digital oscilloscpe (Gould, Hainault, England). Patch pipettes were made from 1.2 mm o.d. filamented capillary tubing (GC120F-10; Clark Electromedical Instruments, Pangbourne, England) to give a resistance of 10–25 MΩ. Pipettes mounted on the amplifier head-stage were positioned on cells by use of a Narishige MO-303 low-drift hydraulic micromanipulator (Narishige Instruments, Tokyo, Japan), the head-stage of which was attached to the microscope by a Narishige MN-3 micromanipulator, for coarse positioning. A further MN-3 micromanipulator was attached to the opposite side of the microscope stage and held a puffer-pipette for administration of drugs to the cell surface. Pressure was applied to the back of the puffer-pipette from a WPI PV-800 pneumatic picopump (World Precision Instruments, Sarsota, Florida, U.S.A.). Drugs applied from the puffer-pipette were dissolved in the solution which was currently bathing the cell. Single channel data were analysed on IBM AT-compatible computers using the pClamp system (Version 5.5.1; Axon

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Instruments, Inc.), in combination with a Tecmar Labmaster A/D D/A board. Current amplitudes were determined from single-point histograms and single channel conductances derived from current-voltage relationships obtained by recording amplitude histograms at a number of different holding potential (V_h) values. In contrast to previous studies (Henderson & Cuthbert, 1991; Henderson *et al.*, 1992) the cells were not treated with trypsin to aid seal formation. This was to avoid the possibility of the trypsin damaging receptors or ion channels on the surface of the cell membrane. Data are presented as mean ± s.e. of mean.

Solutions for patch-clamp experiments

The compositions of the solutions used in the experiments are shown in Table 1. The pipette solution is referred to as 'solution P', the bath solution as 'solution B'. Occasionally solution P was used in the bath to depolarize the cells. When ATP was added to solutions total Ca^{2+} and Mg^{2+} were adjusted to allow for complex formation. Solution G contained gluconate substituting for Cl^-. In this case total calcium was elevated by ten fold to allow for chelation by gluconate.

Chemicals

All chemicals used were of the highest commerical grade. LBK, A23187, ATP, dibutyryl cyclic AMP and protein kinase A (PKA) were obtained from Sigma, Poole, England. Forskolin was obtained from Calbiochem (Novabiochem, Nottingham, England). Dulbecco's Modified Eagle's Medium and foetal calf serum were from Gibco Europe, Paisley, Scotland. Kanamycin was from Bristol Laboratories, Langley Slough, England and amphotericin B from E.R. Squibb, Morton, Cheshire, England.

Results

Patch-clamp experiments

In these experiments the aim was to demonstrate single channel events which could be responsible for the secretory activity of the tissue as a whole. Cell-attached and inside-out preparations (Hamill *et al.*, 1981) were used. The strategy of the cell-attached experiments was to make a seal on the cell membrane and to check for any channel activity by applying various holding potentials (V_h, typically from −40 to +40 mV). If no channel activity was evident then LBK (1 μM), A23187 (10 μM) or forskolin (100 μM) was applied to the cell by the picopump with a 100 ms pressure puff. These concentrations are higher than those used for steady state responses measured by other techniques in the accompanying papers. Rapid diffusion of the compounds from the application site served to dilute them to below effective concentrations in a few seconds.

Table 1 Solutions used for patch-clamp experiments (in mmol l^{-1})

	NaCl	*KCl*	*K-gluconate*	*Na-HEPES*[1]	*EGTA*[2]
Solution P	–	145	–	10	1
Solution B	140	5	–	10	1
Solution G	–	5	140	10	1

$MgCl_2$ was added to give a free Mg^{2+} concentration of 2 mmol l^{-1} and $CaCl_2$ to give a free Ca^{2+} concentration of 1 μmol l^{-1} (calculated from equations given by Fabiato & Fabiato, 1979).
[1]*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid adjusted to pH 7.4 with NaOH.
[2]1,2-Di(2-aminoethoxy)ethane-*N,N,N',N'*-tetra-acetic acid.

Effect of lysylbradykinin and A23187

Figure 1a shows that effect of application of LBK to a cell whilst a recording was made in the cell-attached configuration. The cell was bathed in solution B and was therefore at its normal resting potential (given that the experiment is performed at room temperature). The patch was first held at +17 mV relative to the cell membrane potential and then returned to the initial potential for application of the LBK. The peptide resulted in the opening of three channels, two of the same type, with a current amplitude (determined from single-point amplitude histograms) of approximately 1.45 pA, the other smaller one with an amplitude of 0.4 pA. All were carrying inward current.

If LBK is applied whilst the cell is bathed in solution B it is difficult to make any deductions regarding the specificity of any ion channels which might be activated. The cell is at an unknown potential, and it is not possible to be certain of the intracellular activities of K^+, Na^+ or Cl^-. the former of these constraints can be addressed by stimulating the cell while it is bathed in solution P, the high K^+ concentration depolarizing the cell membrane potential. Figure 1b shows the effect of application of LBK under these circumstances. The patch was held first at 0 mV, then at +38 mV and finally at −38 mV. The steps in voltage discount the possibility of there being channels activated directly by voltage changes. Addition of LBK opened two channels of approximately 1.6 pA in amplitude. LBK was applied to Colony 29 cells in the cell-attached mode on 11 occasions in all and channel openings were elicited in 8 of these. The responses shown in Figure 1 are typical of these experiments. On two occasions it was possible to construct current-voltage relationships for channels in the cell-attached mode, immediately after stimulation with LBK and with solution P in the bath, i.e. with the cells depolarized. The reversal potentials on these two occasions were +16 mV and +10 mV which suggests the channels are K^+-selective. If the channels were perfectly K^+ selective and the cells were completely depolarized intracel-

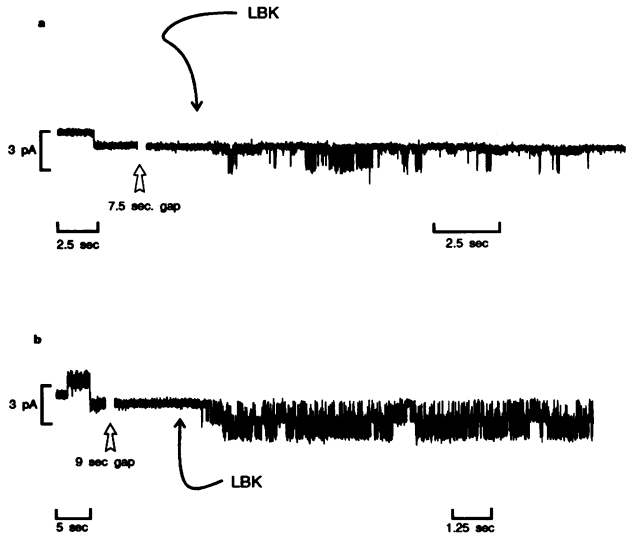

Figure 1 (a) Direct effect of lysylbradykinin (LBK) on channel activity in cell-attached patch on intact cell. The patch is first held at +17 mV with respect to the membrane potential and then returned to the resting value for application of the LBK. The change in base-line with the change in V_h is due to leak current between the membrane and the pipette. No channel activity was visible during the 7.5 s gap and the recording is thus cut at this point. (b) Effect of LBK on cell-attached patch with solution P bathing the cells (i.e. membrane potential = 0). The patch is first held at 0 mV, then at +38 mV and finally at −38 mV when LBK is applied. Note that in this figure and in Figure 2 the time scale is slightly expanded following the gap to clarify the single channel record.

lular K^+ concentrations of 77 mM and 97 mM respectively are predicted.

The effects of A23187 in cell-attached patch-clamp experiments were determined in similar experiments using puffs of a solution containing A23187, 10 μM. In Figure 2a is represented a cell bathed in solution B and thus at its normal membrane potential. Application of A23187 led to channel activation and, as in the LBK experiments, currents were inward under these conditions. The mean amplitude of the channel shown here was 2.00 pA.

Figure 2b shows a representative result obtained when the experiment was repeated with the cell bathed in solution P. The patch was first held at +32 mV then the V_h was stepped to −32 mV, back to 0 mV and then again to −32 mV at which time the A23187 was applied. In common with the LBK experiments, the lack of induction of channel activity during the voltage steps suggests that it is unlikely that there are channels simply activated by changes in voltage in this patch, at least in the range of V_hs examined. Rapid channel activation occurred and again inward currents were evident following application of A23187. The mean current amplitude for this experiment was 1.36 pA.

These results with A23187 are taken from 14 similar experiments of which 12 patches showed A23187 induced channel activity. Many attempts were made to observe channel activity following puffs of forskolin (100 μM). None were seen although forskolin is a powerful secretagogue. It may be that this highly lipophilic drug is taken up into lipid compartments when applied in a few microlitres at high concentration, without significantly affecting cellular adenylate cyclase.

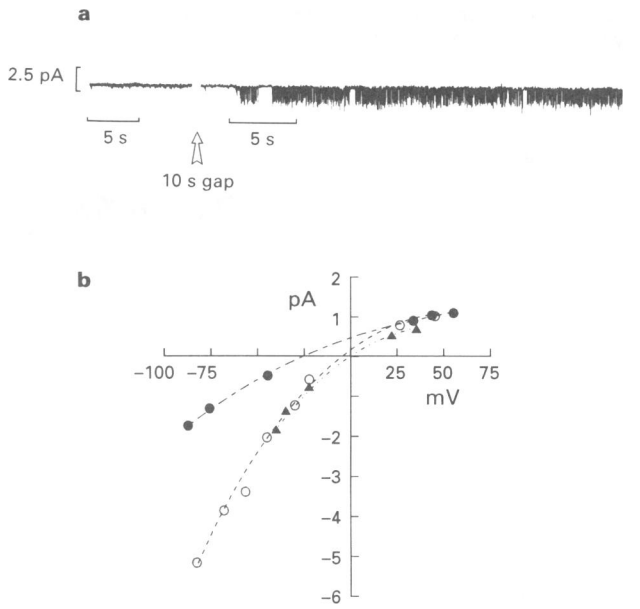

Figure 3 (a) Effect of exposure of inside-out patch to protein kinase A (PKA, 20 units ml^{-1}) and ATP (1 mM), further details in the text. (b) The current-voltage relationship for the channel shown above with symmetrical solution P (▲) and for a channel from a cell pre-stimulated with dibutyryl cyclic AMP (100 μM) bathed in symmetrical solution P (○); (●) show the result with solution G in the bath.

Identification of channel types

Stimulation of channel opening by administration of exogenous agents to the intact cells only gives limited information regarding the identity of channel types involved. In order to clarify the identity and possible mechanisms of regulation of channels, recordings were made from excised inside-out patches. Figure 3a shows the effect of exposure of the intracellular surface of a previously silent patch to solution P, which in addition to its normal constituents contained Ca^{2+} (1 μM), PKA (20 units ml^{-1}) and ATP (1 mM). After 3 min of incubation, channel activity was elicited in the patch, showing predominantly the activity of a single channel type. The current-voltage relationship for this channel is shown in Figure 3b (triangles). Also shown in Figure 3b are the current-voltage relationships for a similar channel which was observed in an inside-out patch from another cell which had been pre-incubated for 10 min before seal formation with dibutyryl cyclic AMP, 100 μM. Relationships are shown for this channel when solution P was in the bath (open circles), and when solution G was present in the bath. Note the current-voltage relationship for the channel activated by PKA/ATP was identical to the one opened by prior exposure of the cell to dibutyryl cyclic AMP. With the latter and with solution G, the reversal potential (E_{rev}) was moved to approximately −25 mV, with outwards current flow at 0 mV. Given the prevailing ion gradients, this corresponds to inward flow of anions. Since the relationship for the PKA/ATP-induced channel overlies that of the dibutyryl cyclic AMP pre-incubated channel (Fig. 3b), the current-voltage relationship showing inward rectification, inward conductance (at −50 mV) is approximately 50 pS, outward conductance (at +50 mV) is approximately 24 pS, it is likely this is a chloride channel.

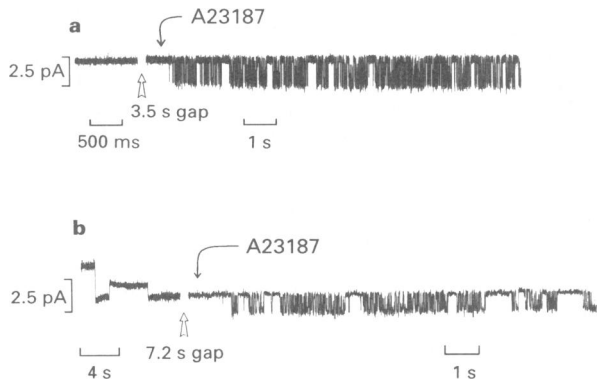

Figure 2 (a) Effect of A23187 on cell-attached patch with low K^+ (solution B) bathing solution. The applied V_h is 0 mV. (b) Effect of A23187 with solution P in the bath. The patch is first held at +32 mV and stepped successively at −32 mV, 0 mV and finally −32 mV, when the ionophore was applied.

Most of the channels seen in isolated patches did not have the characteristics of anion channels. Cation channels were also found which demonstrated calcium sensitivity. Figure 4a shows the single channel recordings for an inside-out patch at a V_h of −11 mV, and Figure 4b the single point current amplitude histogram at a V_h of −48 mV is shown in the absence of bath Ca^{2+}, and with Ca^{2+}, 1 μM, present. No channel openings were discernible in the absence of Ca^{2+}, but channel activity was stimulated in its presence. These channels are therefore sensitive to intra-cellular Ca^{2+}, furthermore as illustrated in Figure 5, they are selective for K^+.

Figure 5 shows the current-voltage relationships obtained from two patches, one from an unstimulated cell, and the other from a cell in which channels had been pre-activated by stimulation with A23187. In symmetrical solution P the channels showed inward rectification with an inward conductance of 19.0 pS and an outward conductance of 12.5 pS at a holding potential of ±40 mV. Replacement of solution P in the bath with solution B (for the patch from the unstimulated cell) produced a shift in the curve, suggesting K^+-selectivity. It was not possible to elicit outward currents under these circumstances. Similar channels were seen on other occasions at a V_h of ±40 mV, the mean inward conductance was 18.9 ± 0.6 pS ($n = 4$) (mean ± s.e.mean and number of observations) and the mean outward conductance was 12.7 ± 0.2 pS ($n = 4$).

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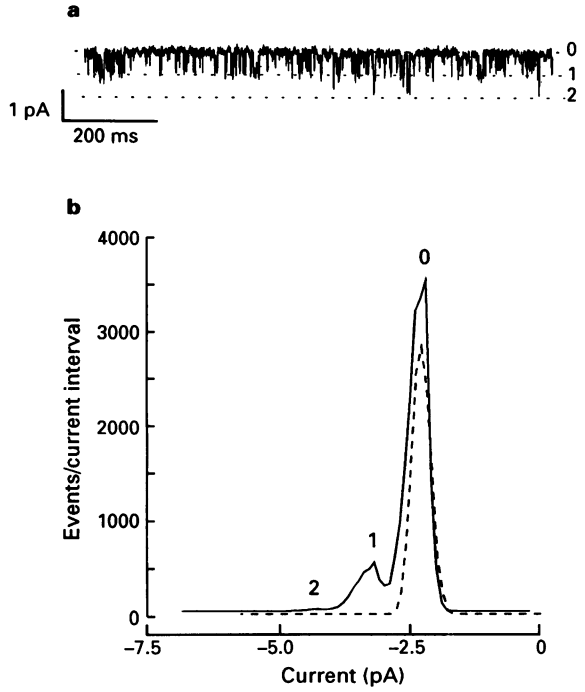

Figure 4 (a) single channel recording from a patch made at V_h of − 11 mV, symmetrical solution P, with Ca^{2+}, 1 μM, in the bath. Two channels are evident and the levels for no channels, one channel or two channels being open are shown by dotted lines and labels. (b) Single point current amplitude histogram for the same channel in the presence of Ca^{2+}, 1 μM, (solid line) and in the absence of Ca^{2+} from the bath (dashed line). The V_h was −48 mV. The relative areas under each peak reflect the total time the patch remains in the corresponding conductance state, i.e. 0, 1 or 2 channels open.

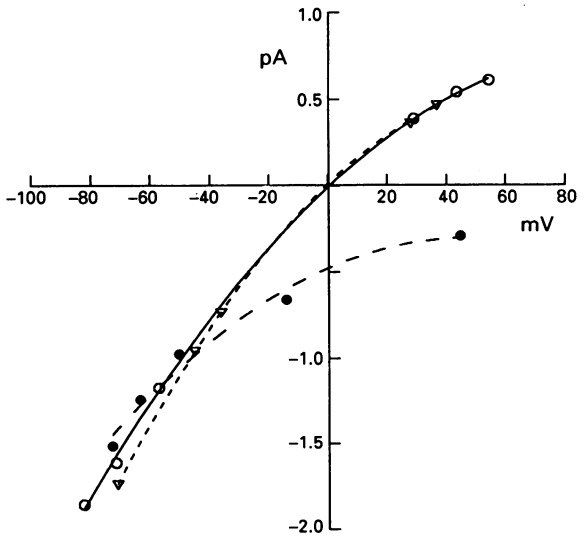

Figure 5 Current-voltage relationship for single Ca^{2+}-activated K^+ channels in inside-out patches: (○) show the relationship for a channel with symmetrical solution P, which was silent in the cell attached mode and in the absence of calcium, but activated by adding calcium (1 μM) to the bath; (●) show the result obtained when the bath solution P is replaced with solution B; (△) show the relationship again with symmetrical solution P, determined from a channel which had been activated in the cell-attached state by treatment of the cell with A23187.

Discussion

In the accompanying papers it was argued that LBK affects chloride secretion in Colony 29 monolayers by more than one mechanism. Indirect evidence for the involvement of cyclic AMP and Ca^{2+} was obtained in the first (MacVinish *et al.*, 1993a), while in the second, direct evidence for an increase in chloride permeability was obtained (MacVinish *et al.*, 1993b). Furthermore, it was argued that part of the increased anion efflux resulted from an increase in the electrical gradient, following activation of Ca^{2+}-sensitive K-channels. The object of this study was to demonstrate the ion channels in Colony 29 cells that are activated by LBK, specifically to see if the data underpin the conclusions from the less direct approaches.

In all the experiments performed in this study the channel openings seen in response to LBK and A23187 in the cell attached configuration always carried an inward current. This was so whether the cells were in their normal polarized state (solution B in bath) or when they were depolarized (solution P in both). It is likely therefore that the currents seen in the cell attached mode in the presence of secretagogues were caused by K^+ ions, as indeed indicated by the current voltage relations shown in Figure 5. However, as shown in Figure 1a, stimulation with LBK can result in the opening of more than one type of channel. This smaller channel is not necessarily K^+-selective and more likely is a non-selective cation channel. The sodium permeability of these channels was masked in these studies by the lack of sodium in the pipette. Small non-selective cation channels with a conductance of 12–13 pS have been seen in isolated patches of Colony 29 cells and the smaller channel in Figure 1a may fall into this group (Sheth *et al.*, 1992).

Epithelial cells always grow with their basolateral surfaces apposed to the substrate and with their apical surfaces uppermost and accessible to the patch pipettes. It is likely, therefore, that the channels induced to open by LBK or A23187, and Ca^{2+}-activated K^+ channels recorded in the inside-out configuration are located on the apical membrane of the colon (for review see Dawson, 1991). It is also possible that the channels which were observed in the present study were from the lateral aspect of the cell since it was easier to form seals on cells at the edge of a group of sub-confluent cells, rather than in the middle of such a group. Current fluctuation analysis has revealed a basolateral K^+ conductance which could be blocked by Ba^{2+} in rabbit colon (Wills & Zweifach, 1987) and a high-conductance Ca^{2+} activated K^+ channel has been isolated from the basolateral membrane of rabbit colon (Turnheim *et al.*, 1989; Salomao *et al.*, 1992). No such high-conductance channels however were seen in the present study.

It is the convention in patch-clamp recording that direction of current flow, and of rectification is always referred with respect to cations. Therefore a current which appears on a current-voltage plot to be carried by cations from the pipette into the cell could also be carried by anions out of the cell. In experiments using the Cl^--sensitive fluorescent dye MEQ, at 37°C, the intracellular Cl^- concentration of Colony 29 cells has been shown to be approximately 35 mM (MacVinish *et al.*, 1993b) which is slightly above the equilibrium value predicted by the Nernst equation (given a membrane potential of = −45 mV the equilibrium Cl^- concentration would be = 25 mM). Under the prevailing conditions produced by stimulation by LBK and A23187, Cl^- currents would appear as outward currents (upward-directed openings on the current records), corresponding to flow of Cl^- ions into the cell. It is perhaps surprising that no single channel currents which could be attributable to anion flow were seen in cell attached patches when the cells were stimulated. The experiments conducted using PKA suggest that anion channels can be stimulated to open in response to second messenger pathways involving cyclic AMP, and indeed that has been documented for a number of anion channels in secretory epithelia includ-

ing the T84 colon cell line (Halm *et al.*, 1988). No direct connection can be made, however, between the PKA-induced anion channels seen in the present study and the forskolin response seen in the efflux experiments (MacVinish *et al.*, 1993b) other than that the forskolin would tend to produce an elevation in cyclic AMP levels and thus put in train events leading to activation of these channels. It was unfortunate that application of forskolin to cell-attached preparations did not yield any results. The reasons for this are unclear, but it maybe that it is not possible to achieve an adequate concentration of the drug at the relevant site because of partitioning into lipophilic sites. However, the single channel Cl^- currents induced in these circumstances might be of such a small amplitude and opening and closings of such high frequency that they fail to be resolved. It would be necessary to study whole cell currents to resolve this. However, the partial inhibition by piroxicam of the effects of LBK on ^{125}I-efflux (MacVinish *et al.*, 1993b) indicates that not all of the peptide effect can be ascribed to the Ca^{2+} elevating effect of LBK and that part of the effect must reside with an action via the prostaglandin/cylic AMP system.

In conclusion, it is shown that Ca^{2+} requiring agonists LBK and A23187 affect Ca^{2+}-sensitive K^+ channels in intact cells. The resulting hyperpolarization increases the gradient for anion efflux, shown in the accompanying papers either as increased $^{125}I^-$ efflux or as an increase in transepithelial chloride transport. While these K^+ channels may exist on either apical or basolateral membranes to achieve this effect it is more likely that those demonstrated here are of apical origin.

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The affinity of betaxolol, a β_1-adrenoceptor-selective blocking agent, for β-adrenoceptors in the bovine trachea and heart

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1 The specificity of betaxolol, a β-adrenoceptor antagonist, for β_1- and β_2-adrenoceptors was compared with that of other β-antagonists, atenolol, ICI-118551, butoxamine and (±)-propranolol, in the bovine trachea and heart by competitive interaction with [^{3}H]-CGP12177 as a radioligand.

2 The radioligand K_d values were 0.75 ± 0.12 and 1.60 ± 0.11 nM in the trachea and heart, respectively, and the B_{max} values were 34.00 ± 4.41 and 21.54 ± 2.94 fmol mg^{-1} protein, respectively.

3 Using ICI-118551, we determined the ratio of β_1:β_2-adrenoceptors in the trachea and heart to be approximately 29:71 and 56:44, respectively.

4 In the trachea, a β_2-predominant tissue, betaxolol and atenolol were more selective for β_1-adrenoceptor binding sites than β_2-adrenoceptor binding sites, whereas ICI-118551 and butoxamine were more selective for β_2-adrenoceptor binding sites.

5 The β_1-selectivity of betaxolol was 2.2 and 2.7 fold higher than that of atenolol in the bovine trachea and heart. These findings suggest that betaxolol may be useful in the treatment of hypertension, cardiac arrhythmia and angina pectoris.

Keywords: β-adrenoceptor antagonists; ^{3}H-CGP12177; binding assay; β_1-selectivity

Introduction

β_1- and β_2-adrenoceptors coexist in various tissues; for example, in the heart, where β_1-adrenoceptors predominate (Hedberg *et al.*, 1980; Heitz *et al.*, 1983; Vago *et al.*, 1984; Tsuchihashi *et al.*, 1989a; Bjørnerheim *et al.*, 1989) and in the trachea, where β_2-adrenoceptors predominate (Barnes *et al.*, 1983; Popovich *et al.*, 1984; Davis *et al.*, 1990; Henry *et al.*, 1990). β-Adrenoceptor antagonists are useful drugs for the treatment of hypertension, cardiac arrhythmias and angina pectoris by blocking β_1-adrenoceptors (Prichard *et al.*, 1980), whereas the β_2-blocking action of these drugs aggravates the condition of asthmatic patients (McNeill, 1964). Because of these side effects, β_1-selective adrenoceptor antagonists, such as atenolol, have been developed in clinical therapeutic use. The β_1-selectivity of β-adrenoceptor antagonists has been mainly determined by comparing the pA_2 value against the effects of β-agonists on cardiac muscle with that on tracheal muscle (Boudot *et al.*, 1979; Pringe *et al.*, 1987; Rimele *et al.*, 1988; Bessho *et al.*, 1990). On the other hand, the β_1-selectivity can also be assessed by comparison of p*Ki* values of β-adrenoceptor antagonists for specific binding of radioligands to β_1- and β_2-adrenoceptors, and various β-adrenoceptor antagonists have been compared in detail (Engel *et al.*, 1981; Tsuchihashi *et al.*, 1989a; 1990).

In order to determine accurately the density of β_1- and β_2-adrenoceptor binding sites and the K_d of radioligands for these subtypes in tissues, quantitative analysis of the selectivity of radioligands for subtypes was required (Neve *et al.*, 1986). We also demonstrated that determination of the selectivity of radioligands was useful for assessment of the selectivity of various unlabelled β-antagonists on the β_1-adrenoceptor predominant tissues, rat myocardium (Tsuchihashi *et al.*, 1989a) and cerebral cortex (Tsuchihashi *et al.*, 1990). To determine the β_1- and β_2-selectivity of β-adrenoceptor antagonists in β_2-predominant tissue such as trachea, we have now examined the effects of five β-antagonists on bovine trachea in comparison with their effects on the bovine heart by the binding assay method.

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Methods

Preparation of the membrane-enriched fractions

Membrane-enriched fractions from bovine trachea and heart were prepared by the following method. Bovine trachea and heart were obtained from a local abattoir. In the laboratory, the tracheae were split longitudinally and the trachealis muscle dissected free. The myocardium was dissected from the heart. The trachealis muscle amd myocardium were frozen in liquid nitrogen, and stored at −80°C until use. The trachealis muscle and myocardium (approx. 2 g) were minced with a small pair of scissors in 20 ml of 10 mM Tris-HCl, 250 mM sucrose buffer (pH 7.4) and then homogenized in a Polytron homogenizer, twice for 10 s at setting 8. The homogenate was filtered through 4 layers of gauze. The filtrate was centrifuged at 40,000 *g* for 30 min, and the resultant pellets were rinsed once; then they were homogenized with a Polytron homogenizer, twice for 10 s at setting 8, in 20 ml of 120 mM Tris-HCl, 40 mM $MgCl_2$ buffer (pH 7.4). The membrane-enriched fraction was frozen in liquid nitrogen, stored at −80°C and diluted to appropriate concentrations immediately before use. Protein concentrations were determined by Lowry's methods (Lowry *et al.*, 1951) with bovine serum albumin as the standard.

Binding assays

(a) Saturation binding assays were carried out in duplicate with [^{3}H]-CGP12177 in the presence (non-specific) and absence (total) of 10 μM (−)-propranolol. In brief, 0.25 ml of membrane suspension (0.15–0.2 mg of protein) was incubated for 45 min at 23°C with various concentrations (0.05–10 nM) of [^{3}H]-CGP12177 in a total volume of 0.5 ml containing 60 mM Tris-HCl and 20 mM $MgCl_2$ (pH 7.4).

(b) Displacement experiments were done in the presence of various concentrations of ICI-118551 in duplicate with various concentrations (trachea: 0.06, 0.6 and 12 nM, heart: 0.26, 1.4 and 20 nM) of [^{3}H]-CGP12177. All displacement experiments except those with ICI-118551 were carried out with a single concentration (trachea: 0.6 nM, heart: 1.4 nM) of [^{3}H]-CGP12177. At the end of the incubation period, the

incubation medium was immediately filtered through a GF/C glass fibre filter by the method described by Tsuchihashi *et al.* (1985). The radioactivity was counted with a scintillation counter (Aloka ALC-500). The difference in mean values between the total and non-specific binding was taken as the specific binding.

Drugs

Betaxolol hydrochloride (Mitsubishi Kasei, Japan) was synthesized. Atenolol hydrochloride, ICI-118551 (erythro-DL-1-(7-methylindan-4-yloxyl)-3-(isopropylaminobutan-2-ol) hydrochloride), (±)-propranolol, (−)-propranolol were kind gifts from ICI Pharma (Japan). Butoxamine (Burroughs Wellcome Co., U.S.A.) and (−)-[^{3}H]-CGP12177 ((−)-4-(3-t-butylamino-2-hydroxypropoxy)-[5,7-^{3}H] benzimidazol-2-one hydrochloride; Amersham, Japan) were purchased from each company. All drugs were dissolved in distilled water.

Kinetic analysis

All kinetic analyses were carried out on an NEC PC-9801 computer system that performs iterative non-linear regression as described previously (Tsuchihashi & Nagatomo, 1987a,b,c; Tsuchihashi *et al.*, 1989b), based on the theory of Munson & Rodbard (1980). Estimates of the dissociation constants (K_d) and maximum binding capacity (B_{max}) of specific [^{3}H]-CGP12177 binding were obtained by Scatchard analysis. In displacement experiments, using various concentrations of the radioligand, parameters describing the competition of ICI-118551 with specific [^{3}H]-CGP12177 binding at two sites (IC_{50} values at β_1- and β_2-adrenoceptors, $\%\beta_1$ and $\%\beta_2$) were estimated by non-linear regression analysis of data which were fitted to a 2-site binding model compared with a 1-site binding model. Then IC_{50} values for various radioligand concentrations (L) were fitted by linear regression using the modified equation of Cheng & Prusoff as follows:

$$IC_{50} = L \cdot K_i/K_d + K_i$$

The K_i values of ICI-118551 for specific [^{3}H]-CGP12177 binding at β_1- and β_2-adrenoceptors were obtained from the intercepts of the line, and K_d values of [^{3}H]-CGP12177 for β_1- and β_2-adrenoceptors were calculated from the slope of the lines. The relative proportion of β_1- and β_2-adrenoceptors within a tissue compartment was derived as $\%\beta_1$- and $\%\beta_2$-adrenoceptors obtained by use of high concentrations ($L/K_d > 10$) of radioligands. An overall estimate of the K_i values of various drugs using displacement analysis was determined by the use of general models with an appropriate concentration of the free radioligand (L) as in the following equation (equation 1):

$$B_1/B_0 = [L \cdot R_1/(L + K_{d_1}(1 + x/K_{i_1})) + L \cdot R_2/(L + K_{d_2}(1 + x/K_{i_2}))]/ (L \cdot R_1/(L + K_{d_1}) + L \cdot R_2/(L + K_{d_2}))$$

where either B_1 or B_0 is the concentration of the radioligand bound with or without the cold ligand, x is the cold ligand concentration; L is the concentration of free radioligand used. R_1 and R_2 are the proportional ratio of receptors 1 and 2 ($R_1 + R_2 = 1$), and K_{d_1} and K_{d_2} are the dissociation constants between a radioligand and receptors 1 and 2. The values of R_1, R_2, K_{d_1} and K_{d_2} were preliminarily determined by the above-mentioned methods and the determined values were substituted in equation 1. By means of these substitutions, the K_i values for two receptor sites can be directly determined by equation 1. In these non-linear or linear regression analyses, the parameter fitting method, termination of iteration, and justification of the models were carried out by previously described methods (Tsuchihashi & Nagatomo, 1987a,c).

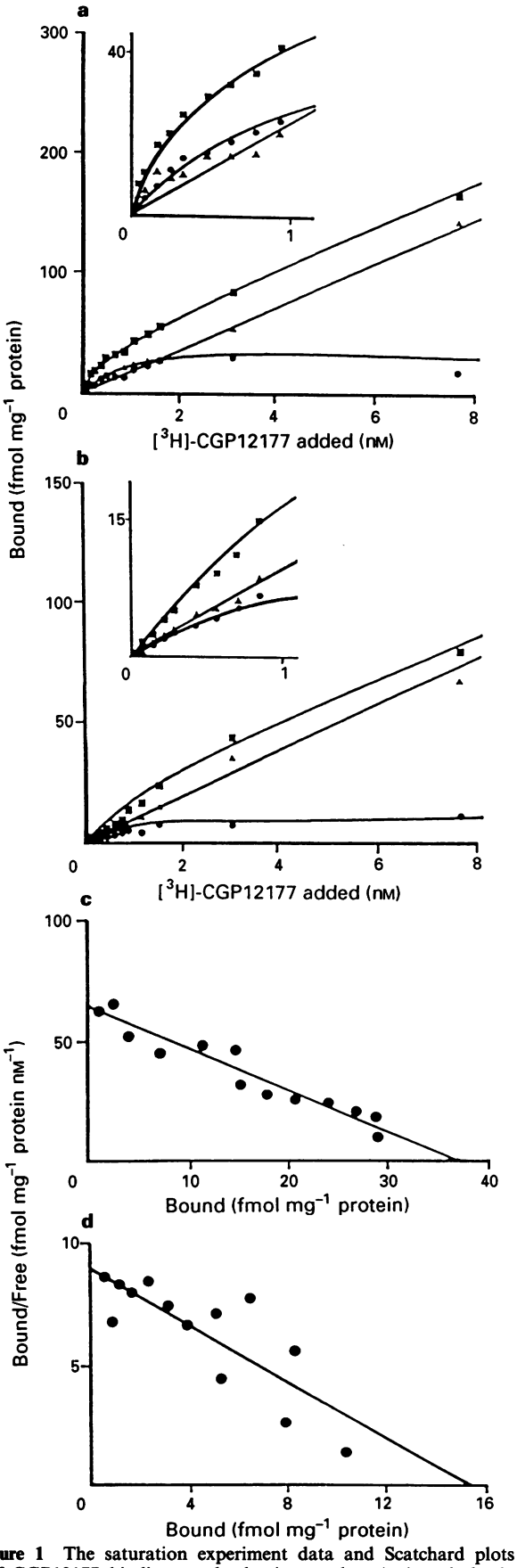

Figure 1 The saturation experiment data and Scatchard plots of [^{3}H]-CGP12177 binding to the bovine trachea (a,c) and the heart (b,d). Specific binding (●) is the difference between the total binding (■) and the binding (▲) in the presence of 10 μM (−)-propranolol (non-specific binding) at [^{3}H]-CGP12177 concentrations between 0.05 and 10 nM. The insets in (a) and (b) show the data points around the K_d values. The data shown are those from a single experiment which is representative of six such experiments in the bovine trachea and the heart, respectively.

Results

Yields of membrane protein per g wet weight of bovine trachea and heart were 6.94 ± 2.45 ($n = 6$) and 7.56 ± 0.58 ($n = 6$) mg protein g^{-1} tissues, respectively. Figures 1a and b show saturation experiments for [^{3}H]-CGP12177 binding to β-adrenoceptors in bovine trachea and heart, respectively. When Scatchard analyses were carried out in the absence (total) and presence (non-specific binding) of 10 μM (−)-propranolol, the curves for specific binding were uniphasic in character (Figure 1c and d). Table 1 summarizes the K_d and B_{max} values in the trachea and heart.

Figure 2 shows the biphasic displacement curves of specific [^{3}H]-CGP12177 binding to the bovine trachea and heart by the β_2-selective antagonist, ICI-118551, using three different concentrations of radioligand. From these results, the values of K_d and B_{max} (%) for [^{3}H]-CGP12177 to the β_1- and β_2-adrenoceptor sites in the trachea and heart were obtained from the modified equation of Cheng & Prusoff (Table 2). [^{3}H]-CGP12177 was 1.5 fold more selective for β_2-adrenoceptors than β_1-adrenoceptors in both tissues, while the proportional percentage of B_{max} of these two binding sites (β_1- and β_2-adrenoceptors) for [^{3}H]-CGP12177 in the trachea and heart were approximately 29:71 and 56:44, respectively.

Displacement curves for five unlabelled ligands, atenolol, betaxolol, butoxamine, (±)-propranolol and ICI-118551 against the radioligand at a single concentration were examined by the fitting method of comparison between a 1-site and a 2-site model in both trachea and heart (Figure 3). The displacement curves for atenolol, betaxolol and ICI-118551 all appeared to fit a 2-site model, while those for butoxamine and (±)-propranolol fitted a 1-site model. The K_i values of these ligands were directly determined by equation 1, and these pK_i values at β_1- and β_2-adrenoceptors against [^{3}H]-CGP12177 binding in the trachea and heart, are summarized in Tables 3 and 4. In both tissues, the β_1-selective antagonists (betaxolol and atenolol) had higher affinity for the β_1- than the β_2-adrenoceptor binding sites, whereas β_2-selective antagonists (ICI-118551 and butoxamine) had higher affinity for the β_2-adrenoceptor binding sites. There was no significant difference between the pK_i values of (±)-propranolol for β_1- and for β_2-binding sites. In the trachea, betaxolol had an approximately 37 fold higher affinity for β_1-adrenoceptors than β_2-adrenoceptors, and it was about 2.2 fold more selective for β_1-adrenoceptors than was atenolol. Similarly in the heart, betaxolol was approximately 2.7 fold more β_1-adrenoceptor-selective than was atenolol.

Table 1 K_d and B_{max} values of [^{3}H]-CGP12177 binding to bovine trachea and heart

	Trachea ($n = 6$)	*Heart* ($n = 6$)
K_d (nM)	0.75 ± 0.12	1.60 ± 0.11
B_{max} (fmol mg^{-1} protein)	34.00 ± 4.41	21.54 ± 2.94

Data are the means ± s.e.

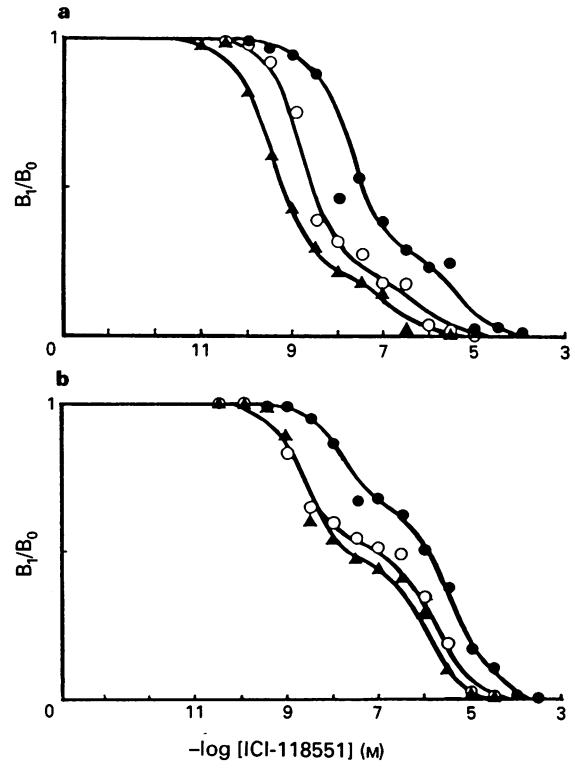

Figure 2 Displacement curves of ICI-118551 for specific [^{3}H]-CGP12177 binding to bovine trachea (a) and heart (b) using three different concentrations of radioligand: 0.06 (▲, slope factor (n_H) = 0.62), 0.6 (○, $n_H = 0.67$) and 12 nM (●, $n_H = 0.66$) in the trachea and 0.26 (▲, $n_H = 0.67$), 1.4 (○, $n_H = 0.65$) and 20 nM (●, $n_H = 0.69$) in the heart. The typical data shown are those from single experiments performed in duplicate and represent the results of three to six such experiments at each radioligand concentration.

Table 2 The K_d and B_{max} of the β_1- and β_2-adrenoceptor binding site of [^{3}H]-CGP12177 in the bovine trachea and heart by displacement experiments using ICI-118551

	Trachea ($n = 3-6$)	*Heart* ($n = 3-6$)
β_1-Adrenoceptor binding sites		
K_d (nM)	1.286	1.866
B_{max} (%)	29.18 ± 3.22	56.20 ± 2.16
β_2-Adrenoceptor binding sites		
K_d (nM)	0.792	1.315
B_{max} (%)	70.82 ± 3.22	43.80 ± 2.16

K_d values were calculated by the modified method of Cheng & Prusoff, $IC_{50} = K_i \times L/K_d \times K_i$, using linear regression analysis in which IC_{50} values and free radioligand (L) at three concentrations were used (trachea; $K_i\beta_1 = 66.67$ nM, $K_i\beta_2 = 0.792$ nM, and heart; $K_i\beta_1 = 681.4$ nM, $K_i\beta_2 = 2.103$ nM). B_{max} values were obtained a high concentration of radioligand (trachea; 12 nM, heart; 20 nM).

Discussion

The coexistence of β_1- and β_2-adrenoceptors in trachea has been reported in the dog (Barnes *et al.*, 1983), pig (Popovich *et al.*, 1984), human (Davis *et al.*, 1990) and mouse (Henry *et al.*, 1990), where the proportions of β_2- relative to β_1-adrenoceptors is between 60 and 90%. In the present study we have also shown that β_1- and β_2-adrenoceptors coexist in the bovine trachea at a β_1- to β_2-adrenoceptor ratio of approximately 30:70. Thus, we found that bovine trachea is also a β_2-adrenoceptor predominant tissue.

In previous studies (Tsuchihashi *et al.*, 1989a; 1990), we reported the pK_i values of several β-antagonists for β_1- and β_2-sites by use of a radioligand binding method. These values correlated closely with the antagonist potencies (pA_2 values) of these β-antagonists against the positive inotropic, chronotropic (β_1) and tracheal relaxant actions (β_2) of isoprenaline (Tsuchihashi *et al.*, 1989a). In the present study, we obtained pK_i values for β-adrenoceptor antagonists at β_1- and β_2-sites by the same method. In the bovine trachea, the order of affinity for β_1-adrenoceptors was (±)-propranolol >> betaxolol, ICI-118551 >> atenolol > butoxamine, whereas that for β_2-receptors was ICI-118551 = (±)-propranolol >> betaxolol > butoxamine >> atenolol. Similar findings were obtained in the bovine heart (present study), rat heart (Tsuchihashi *et al.*, 1989a) and cerebral cortex (Tsuchihashi *et al.*, 1990). However, the relationship between the affinities of [^{3}H]-CGP12177 (K_d value) for β_1-adrenoceptors and for

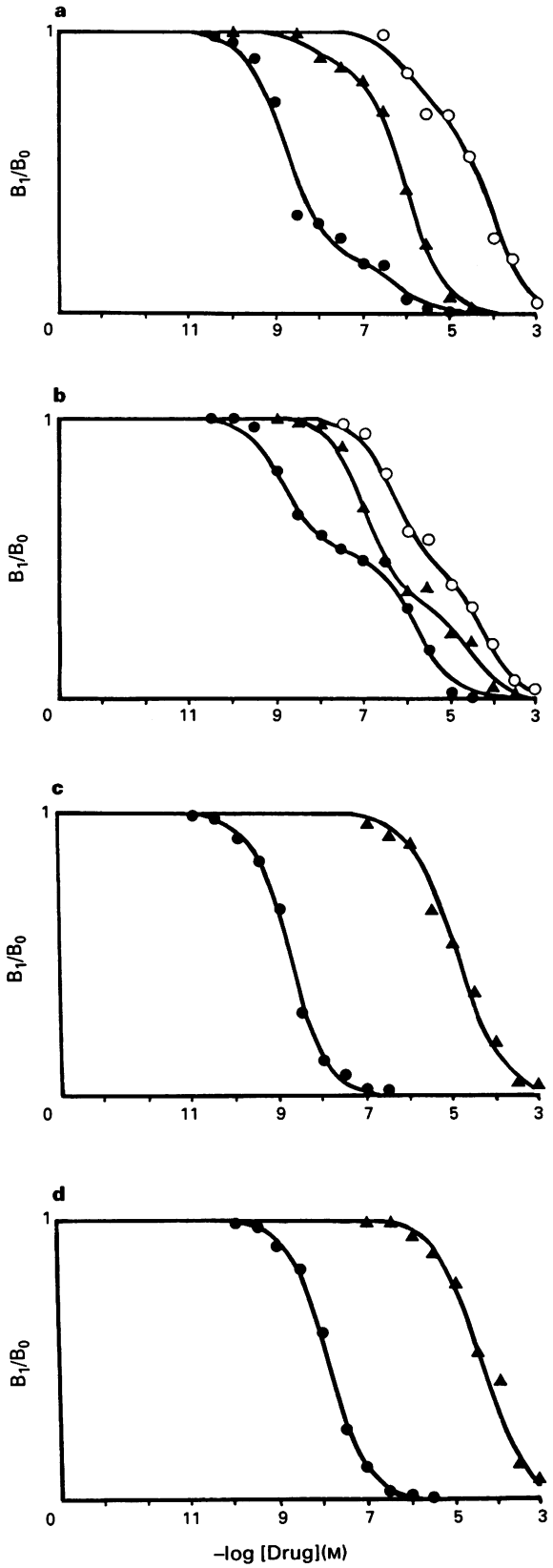

Figure 3 Displacement curves of specific [^{3}H]-CGP12177 (0.6 and 1.4 nM) binding to bovine trachea (a,c) and heart (b,d). (a and b) Betaxolol (▲), atenolol (○) and ICI-118551 (●); (c and d) (±)-propranolol (●) and butoxamine (▲). The slope factors of the plots of β-antagonists were: betaxolol (0.73), atenolol (0.71), ICI-118551 (0.62), (±)-propranolol (1.06) and butoxamine (0.93) in the trachea, and betaxolol (0.69), atenolol (0.75), ICI-118551 (0.71), (±)-propranolol (1.01) and butoxamine (0.92) in the heart. The typical data shown are those from single experiments performed in duplicate and represent the results of six to eight such experiments.

Table 3 p*Ki* values of β-antagonists in the bovine trachea

	pKi values		$K_i\beta_1/K_i\beta_2$
	β_1-sites	*β_2-sites*	*ratio*
Betaxolol ($n = 8$)	7.70 ± 0.21	6.13 ± 0.12	37.2**
Atenolol ($n = 7$)	5.64 ± 0.27	4.41 ± 0.06	17.0**
ICI-118551 ($n = 6$)	7.49 ± 0.07	9.19 ± 0.06	0.02***
Butoxamine ($n = 6$)	4.82 ± 0.18	5.43 ± 0.06	0.25**
(±)-Propranolol ($n = 6$)	9.18 ± 0.06	8.95 ± 0.07	1.70^{NS}

Data are the mean values ± s.e. These data were obtained by the displacement analysis 0.6 nM [^{3}H]-CGP12177 and calculated from equation 1.
Significance of difference between values of p*Ki* for β_1- and β_2-sites was determined by Student's *t* test: $^{**}P<0.01$; $^{***}P<0.001$ and NS: not significant.

Table 4 p*Ki* values of β-antagonists in the bovine heart

	pKi values		$K_i\beta_1/K_i\beta_2$
	β_1-sites	*β_2-sites*	*ratio*
Betaxolol ($n = 6$)	7.56 ± 0.14	5.82 ± 0.21	55.0***
Atenolol ($n = 6$)	5.94 ± 0.14	4.63 ± 0.12	20.4**
ICI-118551 ($n = 5$)	6.46 ± 0.21	9.05 ± 0.25	0.003***
Butoxamine ($n = 6$)	4.29 ± 0.09	4.65 ± 0.13	0.44*
(±)-Propranolol ($n = 7$)	8.32 ± 0.12	8.03 ± 0.18	1.95^{NS}

Data are the mean values ± s.e. These data were obtained by the displacement analysis 1.4 nM [^{3}H]-CGP12177 and calculated from equation 1.
Significance of difference between values of p*Ki* for β_1- and β_2-sites was determined by Student's *t* test: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ and NS: not significant.

β_2-adrenoceptors differed between these studies. Thus the affinity of [^{3}H]-CGP12177 for β_1-sites was higher than that for β_2-sites in rat heart, which was in apparent contrast to that obtained in the bovine trachea and heart, where the affinity was higher at β_2-adrenoceptors. Similarly, the p*Ki* values for (±)-propranolol at β_1- and β_2-adrenoceptors were different. Furthermore, there were inconsistencies in the relative affinities of atenolol and ICI-118551 for β_1-adrenoceptor in various tissues: thus in bovine trachea, ICI-118551 >> atenolol (present study), bovine heart (present study) and rat heart (Tsuchihashi *et al.*, 1989a), ICI-118551 > atenolol; and rat brain, ICI-118551 = atenolol (Tsuchihashi *et al.*, 1990). Furthermore, in a comparison between the *Ki* values for β_1- (rat salivary gland) and β_2-adrenoceptor predominant tissues (rat reticulocyte) (Wellstein *et al.*, 1986) using radioreceptor assay, and between antagonistic potencies for the positive inotropic and chronotropic effects on left and right atria of the guinea-pig, ICI-118551 = atenolol (Tsuchihashi *et al.*, 1989a). These findings could suggest that the β-adrenoceptor conformation and/or the receptor environment differed between species and tissues. We have previously demonstrated that the environment of the receptor site could indeed have a crucial role in ligand-receptor interactions (Tsuchihashi & Nagatomo, 1985a,b,c).

The displacement curves for butoxamine were found to be monophasic when the data were fitted to 1-site and 2-site models in the present study, and the results showed that butoxamine had β_2-adrenoceptor selectivity as assessed by equation 1. We have previously demonstrated that alprenolol has an approximately 7 fold higher affinity for β_2-adrenoceptors than for β_1-adrenoceptors in both rat heart and brain, although it is generally known as a non-selective antagonist, suggesting that the use of equation 1 to directly determine K_i values of ligands is useful for detecting the selectivities of ligands with low affinity differences between β_1- and β_2-adrenoceptors (Tsuchihashi *et al.*, 1989a; 1990).

β_2-Adrenoceptor blockade has been reported to aggravate asthma, whereas β_1-adrenoceptor blockade appears to be less

associated with this side-effect (McDevitt, 1983). In fact, it has been reported that β_1-selective agents are less likely to worsen the condition of asthmatic patients than is propranolol, a non-selective β-antagonist (Johnsson *et al.*, 1975; Singh *et al.*, 1976; Palmiteri & Kaik, 1983). Therefore, β_1-selectivity is believed to be important in a β-adrenoceptor antagonist used clinically for treating cardiovascular disease. Betaxolol, like atenolol, exhibits a high β_1-adrenoceptor selectivity in isolated tissues (Boudot *et al.*, 1979; Pringe *et al.*, 1987; Rimele *et al.*, 1988; Bessho *et al.*, 1990). However, these tissues are thought to contain both β_1- and β_2-adrenoceptors, and therefore these results may not reflect the true β_1-selectivity of the β-antagonists. The net β_1-selectivity of β-antagonists in various tissues has been examined by the receptor binding assay method (Engel *et al.*, 1981; Tsuchihashi *et al.*, 1989a; 1990). We previously reported that betaxolol was a β_1-selective antagonist in rat heart (Tsuchihashi *et al.*, 1989a) and cerebral cortex (Tsuchihashi *et al.*, 1990) and that the affinity for β_1-adrenoceptors was 23 and 170 fold higher than that for β_2-adrenoceptors, respectively. In the guinea-pig lung (Engel *et al.*, 1981), a β_2-predominant tissue, the affinity of β_1-sites was 200 fold higher than that for β_2-sites. In the present study, betaxolol had a 37 fold higher affinity for β_1-adrenoceptors than for β_2-adrenoceptors in the trachea, and 55 fold higher affinity in the heart. Similarly, atenolol was shown to be β_1-selective in both bovine trachea and the heart, but the selectivity was about 2 to 3 fold lower than that of betaxolol. These findings are consistent with our previous data (Tuschihashi *et al.*, 1989a) and those obtained in isolated tissues (Bessho *et al.*, 1990). These findings indicate that betaxolol and atenolol also have a β_1-selective profile in the trachea (β_2-predominance) as well as heart (β_1-predominance).

In conclusion, the results of the present study using a radioligand binding method indicate that β_1- and β_2-adrenoceptors coexist in the bovine trachea and heart, and that not only does betaxolol have a β_1-selective profile in both preparations, but it is more β_1-selective than atenolol. These findings suggest that betaxolol may be a useful drug for the treatment of hypertension, cardiac arrhythmia and angina pectoris.

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Quantitative analysis of the agonist and antagonist actions of some ATP analogues at P_{2X}-purinoceptors in the rabbit ear artery

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1 The agonist and antagonist effects of a series of β,γ-methylene dihalo- and 2-methylthio-substituted analogues of ATP at P_{2x}-purinoceptors have been analysed on the rabbit isolated ear artery preparation. Cumulative and sequential dosing experimental protocols were employed in the construction of agonist concentration-effect curves in order to address the possible influence of acute receptor desensitization on subsequent analyses.

2 Using the cumulative curve design the following results were obtained: D-AMP-$PCBr_2P$, 2-methylthio-D-AMP-$PCCl_2P$, L-AMP-PCF_2P, L-AMP-$PCCl_2P$ and LAMP-$PCBr_2P$ each behaved as partial agonists. D-AMP-CPP was used as a reference full agonist and these analogues were analysed by the comparative method of Barlow *et al.* (1967), to provide estimates of affinity and efficacy. 2-Methylthio-L-AMP-$PCBr_2P$ was virtually silent as an agonist and was analysed as a competitive antagonist by Schild analysis.

3 Two agonists, L-AMP-$PCCl_2P$ and L-AMP-$PCBr_2P$, were analysed by the sequential curve design, and the antagonist effects of one of the agonists, L-AMP-$PCBr_2P$ were also analysed using this protocol. The resulting estimates of affinity and efficacy, while similar to those obtained with the cumulative design, indicated that acute desensitization may affect curve definition and estimation of these quantities.

4 The following structure-activity trends emerged: D-analogues tended to have higher efficacy but lower affinity than L-analogues; efficacy varied markedly and inversely with the atomic weight of the halogen while affinity was only minimally affected; 2-methylthio- substitution also reduced efficacy with minimal effect on affinity.

5 The results of this analysis are discussed in terms of the utility of affinity and efficacy information in the classification of purinoceptors and the design of chemical probes for them.

Keywords: P_{2x}-purinoceptors; ATP analogues; partial agonist; antagonist; affinity; efficacy; rabbit ear artery; receptor classification; medicinal chemistry

Introduction

The absence of selective and competitive antagonists for P_2-purinoceptors has meant that their pharmacological classification has depended primarily on the use of agonists (Burnstock & Kennedy, 1985; Kennedy, 1990). In turn, the use of agonists has been limited to the provision of potency order information. No attempt appears to have been made to analyse agonist action quantitatively, that is, to provide estimates of affinity and efficacy, despite the potential impact on and refinement to receptor classification that such information can provide. The purpose of the present study was to determine whether such data could be obtained for P_{2x}-purinoceptors.

Affinity and efficacy estimates can, in principle, be obtained for full agonists by the method of receptor inactivation (Furchgott, 1966), and for partial agonists by the comparative (Barlow *et al.*, 1967) or interaction (Stephenson, 1956) methods. The former method relies on the availability of an irreversible receptor antagonist. Unfortunately, in the case of P_{2x}-purinoceptors such an agent has not been discovered, meaning in the present study that full agonists could not be used to provide affinity and efficacy information. Such estimates would only be possible for partial agonists.

Few reports of partial agonism have appeared in the purinoceptor literature. However, in a study by Cusack and colleagues (1987), data were presented suggestive of such behaviour at P_{2x}-purinoceptors. Those workers investigated the effects of a series of phosphonate ATP analogues in the guinea-pig taenia coli and urinary bladder. In the latter tissue, which was classified as a P_{2x}-purinoceptor system, some β,γ-methylene dihalo- and 2-methylthio-substituted compounds appeared to produce lower maximal effects than other analogues. This feature of the data was not remarked upon but served to stimulate us to reinvestigate this series of compounds.

We chose to use the rabbit isolated ear artery preparation for this study. This tissue has previously been shown to contain P_{2x}-purinoceptors and, unlike other smooth muscle preparations, to be amenable to precise agonist-concentration effect curve shape definition (O'Connor *et al.*, 1990), the latter being particularly important for affinity and efficacy analysis. The results of this study are discussed in terms of the confidence with which affinity and efficacy estimates for P_{2x}-purinoceptor agonists can be provided, and the utility of this information in the definition of P_{2x}-purinoceptors.

Methods

Rabbit ear artery

Male New-Zealand White rabbits (2.5–3.5 kg) were killed by an overdose of pentobarbitone sodium (300 mg, i.v.). The ears were removed and following insertion of a polythene cannula (0.75 mm o.d.) the central artery ear was dissected free; 5–10 mm rings of tissue were mounted on fine tungsten wire hooks and suspended horizontally in 20 ml organ baths containing Krebs solution of the following composition

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(mM): NaCl 117.56, NaH_2PO_4 0.89, $NaHCO_3$ 25.0, $MgSO_4$ 1.18, glucose 11.1, KCl 5.36 and $CaCl_2$ 2.55. The solution routinely contained the P_1-purinoceptor antagonist 8-sulphophenyltheophylline (8-SPT) (3×10^{-4} M) (Collis *et al.*, 1987) to exclude possible direct or indirect effects mediated through P_1-receptors, and indomethacin (2.8×10^{-6} M) to eliminate the influence of products of cyclo-oxygenase. The tissues were maintained at 37°C and gassed continually with 95% O_2/5% CO_2.

Each ring was subjected to an initial force of 1.0 g and allowed to equilibrate for 1 h before any further intervention. Changes in the contractile state of the tissues were recorded isometrically with Ormed force transducers and displayed on Advance Bryans chart recorders.

Experimental protocols

In all experiments tissues were initially contracted with KCl (80 mM) in order to establish viability. Once the response had reached steady state, acetylcholine (10^{-6} M) was added to confirm endothelium denudation. Following washing, tissues were allowed to equilibrate for 45 min then a cumulative agonist-concentration effect (E/[A]) curve was constructed with D-α,β-methylene ATP (D-AMP-CPP). Following washing, tissues were again allowed to settle for 70 min before being subjected to one of the following protocols.

Cumulative curve design In this protocol a second cumulative E/[A] curve was then constructed to D-AMP-CPP or a test agonist. Following subsequent washing, D-AMP-CPP (3×10^{-5} M) was applied and its contractile response allowed to fade (15 min). Cumulative applications of the test agonist were then repeated in the continued presence of D-AMP-CPP.

One compound, which was virtually inactive as an agonist was examined for antagonist properties. It replaced the test agonist in the protocol and was incubated for 45 min prior to the construction of a second E/[A] curve to D-AMP-CPP.

When histamine, phenylephrine or KCl were employed, they replaced D-AMP-CPP in the protocol.

The reproducibility of paired curves was analysed by comparing first and second D-AMP-CPP curves. The following mean differences in parameter estimates were computed: $\Delta p[A_{50}] = 0.01 \pm 0.01$; $\Delta\alpha = -3.18 \pm 2.10$; $\Delta p = 0.06 \pm 0.03$ ($n = 29$) (see Data analysis). These differences were not significant.

Sequential curve design In this protocol, a second E/[A] curve was obtained either to D-AMP-CPP or to a test agonist by making individual applications of ascending concentrations with a 40 min re-equilibration period between each application. Reproducibility of individual applications was assessed by repeated administration (6 times) of an $[A_{50}]$ and a maximal concentration of a single agonist. Variation, expressed as standard error on the measured effects across time, was in the order of only 1%. Reproducibility between the first cumulative and second sequential D-AMP-CPP curve was tested giving the following mean differences in parameter estimates: $\Delta p[A_{50}] = -0.06 \pm 0.02$; $\Delta,\alpha = -5.22\% \pm 3.39\%$; $\Delta p = 0.22 \pm 0.06$ ($n = 18$) (see Data analysis). Although the differences in $p[A_{50}]$ and p were significant it was calculated that changes of this magnitude would have trivial effects on subsequent parameter estimation.

In experiments involving interactions between two agonists, application of the second agonist was made immediately after maximal response had been attained to the first.

In all experiments contractile effects of agonists were expressed as percentages of the maximum of the first curve to D-AMP-CPP obtained in the tissue.

Data analysis

General curve fitting In order to obtain objective estimates of E/[A] curve parameters, data sets were fitted using the logistic equation

$$E = \frac{\alpha\,[A]^p}{[A_{50}]^p + [A]^p} \qquad \text{(i)}$$

in which α, $[A_{50}]$ and p are respectively the asympote, location and slope parameters. Location parameter estimates are quoted in the text as $p[A_{50}]$ ($-\log_{10}[A_{50}]$) values.

Each individual curve, whether obtained cumulatively or sequentially, was fitted separately.

When the interaction between a partial agonist and D-AMP-CPP was studied the effects of the full agonist were measured from the response level produced by the partial agonist alone.

Paired Student's *t* test was used to compare parameter estimates for test agonists with those for D-AMP-CPP.

Affinity and efficacy estimation Analogues which behaved as partial agonists were analysed by the comparative method (Barlow *et al.*, 1967) using operational model-fitting procedures described elsewhere (Leff *et al.*, 1990). The comparative method involved the comparison of the partial agonist with a reference full agonist. D-AMP-CPP was adopted as the reference full agonist in the present study. This agonist has been shown elsewhere to have efficacy corresponding to full agonism in the ear artery (Wood *et al.*, 1992).

The operational model has the following algebraic form (Black & Leff, 1983):

$$E = \frac{E_m\,\tau^n\,[A]^n}{(K_A + [A])^n + \tau^n\,[A]^n} \qquad \text{(ii)}$$

K_A is the equilibrium dissociation constant for the agonist and is a reciprocal measure of affinity. For convenience, pK_A values are quoted in the test. τ is the efficacy of the agonist. E_m is the maximum possible effect and n is the slope parameter in the receptor system.

For agonists of high efficacy, equation (ii) reduces to an equation of the logistic form

$$E = \frac{E_m\,[A]^n}{[A_{50}]^n + [A]^n} \qquad \text{(iii)}$$

E_m and n are estimated by fitting this equation to the full agonist E/[A] curve data. Once these two parameters are known K_A and τ can be obtained by fitting the partial agonist data with equation (ii). In practice, both steps are carried out in a single computer fit.

Each piece of tissue provided a reference full agonist and a partial agonist E/[A] curve and, therefore, an estimate of pK_A and τ.

Analysis of antagonism Paired E/[A] curves to D-AMP-CPP obtained in the absence and presence of an antagonist were fitted to equation (i). Parallelism was tested by one-way analysis of variance on the paired differences in computed asymptote, α, and slope, p, values. Paired differences in $p[A_{50}]$ estimates were used to calculate dose-ratios (r) and analysed according to Arunlakshana & Schild (1959):

$$\log_{10}(r-1) = n\log_{10}[B] + pK_B \qquad \text{(iv)}$$

in which n is the Schild plot slope and pK_B is the antagonist affinity constant. If n was found not to be significantly different from unity the pK_B was estimated with n constrained to that value.

Data on the antagonistic interaction between a test agonist and D-AMP-CPP were also analysed using this equation.

Data are presented as mean ± s.e.mean. On occasions when weighted averages were used, the reciprocal of the variance was used as the weighting factor. All fitting procedures were carried out with a VAX Mainframe computer employing the BMDP Statistical Software package.

Drugs and solutions

Drugs were obtained from the following sources: acetylcholine bromide, histamine dihydrochloride, D-adenosine 5'-(α,β-methylene)triphosphonate (D-AMP-CPP) dilithium salt, D-adenosine 5'-(β,γ-methylene)triphosphonate (D-AMP-PCP) disodium salt, phenylephrine hydrochloride and indomethacin, Sigma Chemical Co., Poole, U.K.; 8-sulphophenyltheophylline (8-SPT), Research Biochemicals Inc., St Albans, U.K.; pentobarbitone sodium (Euthatal), RMB Animal Health Ltd., Dagenham, UK; (L-adenosine 5'-(β,γ-methylene) triphosphonate (L-AMP-PCP), L-adenosine 5'-(β,γ-difluoromethylene)triphosphonate (L-AMP-PCF_2P), L-adenosine 5'-(β,γ-dichloromethylene)triphosphonate (L-AMP-$PCCl_2P$), L-adenosine 5'-(β,γ-dibromomethylene)triphosphonate (L-AMP-$PCBr_2P$), D-adenosine 5'-(β,γ-difluoromethylene)triphosphonate (D-AMP-PCF_2P), D-adenosine 5'-(β,γ-dichloromethylene) triphosphonate (D-AMP-$PCCl_2P$), D-adenosine 5'-(β,γ-dibromomethylene)triphosphonate (D-AMP-$PCBr_2P$), 2-methylthioadenosine 5'-(β,γ-dichloromethylene) triphosphonate (2-methylthio-D-AMP-$PCCl_2P$) and (2-methylthio-L-adenosine 5'-(β,γ-dibromomethylene)triphosphonate (2-methylthio-L-AMP-$PCBr_2P$) [all tetrasodium salts] were synthesized by P.A. Cage, D. Cox and A.H. Ingall, Department of Medicinal Chemistry, Fisons R&D Labs, Loughborough, U.K.

Indomethacin was dissolved in 10% w/v Na_2CO_3 at 10 mg ml^{-1} and subsequently diluted in Krebs buffer. Histamine was dissolved in distilled water at 0.2 M, the solution was neutralised with 10 M NaOH (10 μl to 2 ml). All other drugs were dissolved in distilled water.

Results

Figure 1 illustrates mean cumulative E/[A] curves for a series of D- and L-β,γ-dihalomethylene ATP analogues and two compounds with an additional 2-methylthio-substituent. Paired *t* tests on the computed asymptotes of the curves showed that five of the compounds produced maximal effects which were significantly lower than that of D-AMP-CPP. They were: D-AMP-$PCBr_2P$; 2-methylthio-D-AMP-$PCCl_2P$; L-AMP-PCF_2P; L-AMP-$PCCl_2P$; L-AMP-$PCBr_2P$. A sixth

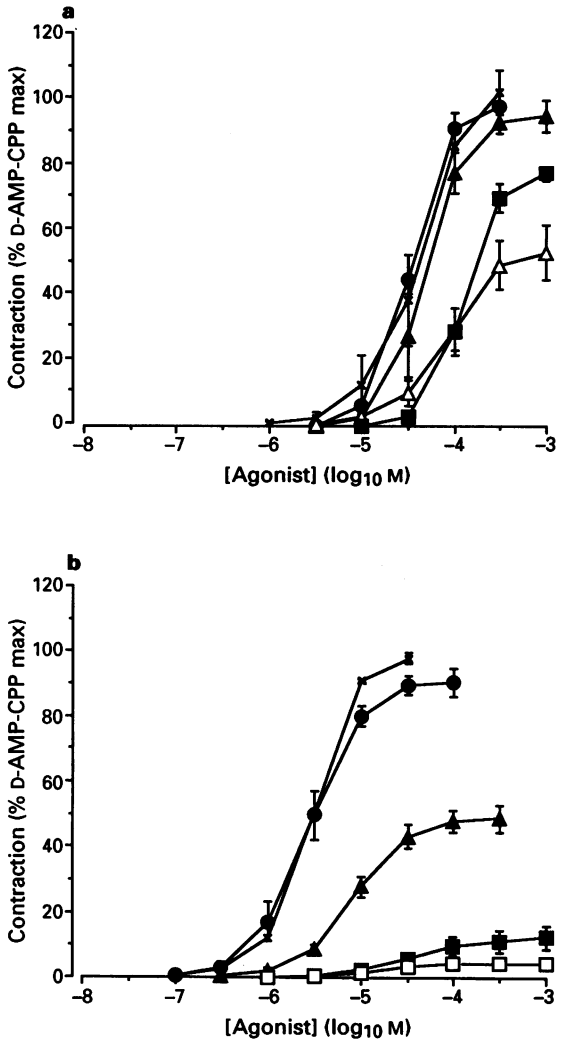

Figure 1 E/[A] curves for a series of D- (a) and L- (b) substituted adenosine 5'-(β,γ-methylene)triphosphonates in isolated rings of rabbit ear artery. The points are the mean effects (± s.e.mean, vertical bars, $n = 3-10$) calculated from the second curves obtained in each piece of tissue using the cumulative protocol: (a) (×) D-AMP-PCP; (●) D-AMP-PCF_2P; (▲) D-AMP-$PCCl_2P$ (■) D-AMP-$PCBr_2P$; (△) 2-methylthio-D-AMP-$PCCl_2P$; (b) (×) L-AMP-PCP; (●) L-AMP-PCF_2P; (▲) L-AMP-$PCCl_2P$; (■) L-AMP-$PCBr_2P$; (□) 2-methylthio-L-AMP-$PCBr_2P$. See text for full names.

Table 1 E/[A] Curve shape parameters for D-AMP-CPP (the reference full agonist) and a series of D- and L-adenosine-5'-(β,γ-methylene) triphosphonates

		Parameter	
Analogue	$p[A_{50}]$ ($-\log_{10}$ M)	α (%)	p
D-AMP-CPP	6.49 ± 0.03	97.1 ± 2.1	1.55 ± 0.04
	(6.57 ± 0.05)	(95.3 ± 3.5)	(1.70 ± 0.06)
D-AMP-PCP	4.36 ± 0.12	105.9 ± 6.4	1.94 ± 0.20
D-AMP-PCF_2P	4.47 ± 0.07	99.7 ± 10.7	2.48 ± 0.23
D-AMP-$PCCl_2P$	4.32 ± 0.12	94.2 ± 3.5	2.62 ± 0.21
D-AMP-$PCBr_2P$	3.89 ± 0.07	78.6 ± 2.0****	2.63 ± 0.14
L-AMP-PCP	5.52 ± 0.04	100.2 ± 4.1	1.87 ± 0.09
L-AMP-PCF_2P	5.57 ± 0.10	90.9 ± 3.3*	1.76 ± 0.04
L-AMP-$PCCl_2P$	5.04 ± 0.05	49.7 ± 3.4****	1.54 ± 0.07
	(4.73 ± 0.09)	(69.8 ± 4.9***)	(1.42 ± 0.22)
L-AMP-$PCBr_2P$	4.35 ± 0.11	15.4 ± 2.7****	1.15 ± 0.17
	(4.46 ± 0.13)	(15.5 ± 1.5****)	(0.96 ± 0.10)
2-Methylthio-D-AMP-$PCCl_2P$	4.05 ± 0.06	55.3 ± 8.74**	1.62 ± 0.11

Values are means (± s.e.mean) of parameter estimates made by fitting the logistic equation (equation (i)) to individual, second E/[A] curves, using the cumulative or sequential (values in parentheses) protocol.

Asterisks indicate significant differences between the maximal effect (α) of the agonist and that of D-AMP-CPP; *$P<0.05$; **$P<0.02$; ***$P<0.01$; ***$P<0.001$ (paired Student's *t* test).

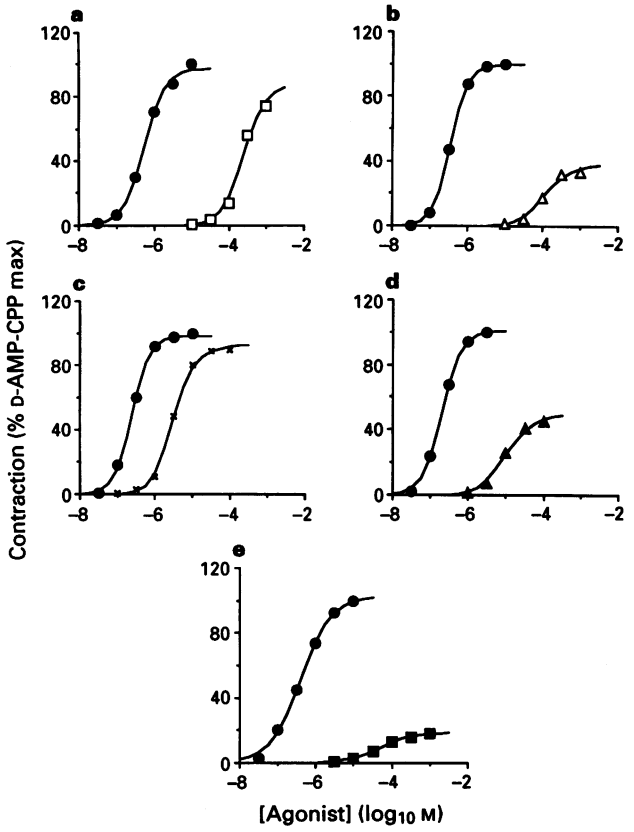

Figure 2 Analysis of partial agonism by the comparative method. Examples of typical experimental data are shown with computer-generated lines obtained by fitting equations (ii) and (iii). Each panel shows an E/[A] curve to the test compound and the corresponding curve to the reference full agonist, D-AMP-CPP (●). The data for each panel were obtained in a single piece of rabbit ear artery using the cumulative protocol. The resulting affinity (pK_A) and efficacy (τ) values from these individual analyses are given below: (a) (□) D-AMP-$PCBr_2P$, $pK_A = 2.94$, $\tau = 4.93$; (b) (△) 2-methylthio-D-AMP-$PCCl_2P$, $pK_A = 4.14$, $\tau = 0.78$; (c) (x) L-AMP-PCF_2P, $pK_A = 4.94$, $\tau = 4.51$; (d) (▲) L-AMP-$PCCl_2P$, $pK_A = 5.04$, $\tau = 1.00$; (e) (■) L-AMP-$PCBr_2P$, $pK_A = 4.27$, $\tau = 0.25$.

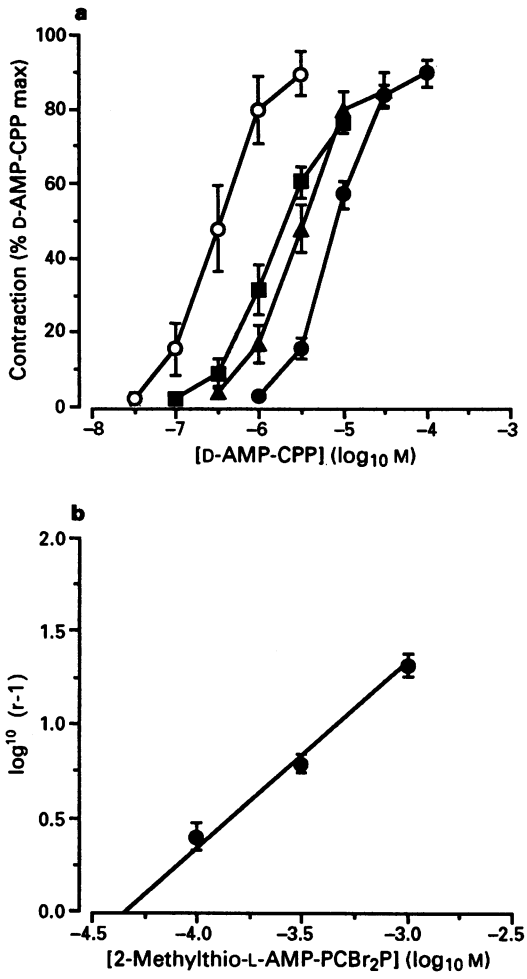

Figure 3 Analysis of antagonism by 2-methylthio-L-AMP-$PCBr_2P$. (a) E/[A] curves for D-AMP-CPP, in the rabbit ear artery, in the presence of zero (○), 0.1 (■), 0.3 (▲) and 1 mM (●) 2-methylthio-L-AMP-$PCBr_2P$. The points are the mean effects (± s.e.mean, vertical bars, $n = 5$) calculated from second curves obtained in each piece of tissue using the cumulative protocol. (b) Corresponding Schild plot of the effect of 2-methylthio-L-AMP-$PCBr_2P$ on E/[A] curves to D-AMP-CPP. Points show mean (dose ratio − 1) values ± s.e.mean.

Table 2 Affinity (pK_A) and efficacy (τ) estimates for partial agonists

Analogue	pK_A ($-\log_{10}$ M)	τ range	*Parameter* E_m (%)	n
D-AMP-$PCBr_2P$	3.49 ± 0.13	2.87 2.40–3.44	96.9 ± 1.49	1.75 ± 0.13
L-AMP-PCF_2P	5.05 ± 0.06	4.18 3.80–4.59	99.2 ± 0.75	1.77 ± 0.06
L-AMP-$PCCl_2P$	4.98 ± 0.02	0.99 0.98–1.00	100.6 ± 0.43	1.52 ± 0.03
	(4.58 ± 0.04*)	(1.26 1.23–1.29)	(99.3 ± 0.72)	(1.63 ± 0.05)
L-AMP-$PCBr_2P$	4.49 ± 0.09	0.26 0.25–0.28	101.2 ± 0.82	1.43 ± 0.04
	(4.63 ± 0.07)	(0.32 0.31–0.34)	(100.3 ± 0.50)	(1.46 ± 0.03)
2-Methylthio-D-AMP-$PCCl_2P$	3.93 ± 0.06	0.95 0.91–0.99	99.7 ± 0.98	1.56 ± 0.07

The values are the weighted averages (± s.e.mean, $n = 4–10$) of the operational model parameters obtained using the comparative analysis. The main table shows estimates obtained from cumulative data. Values in parentheses show estimates obtained by the sequential protocol.

Asterisks indicate significant differences between the pK_A estimates obtained by the cumulative and sequential protocols: $^*P < 0.01$ (Student's t test).

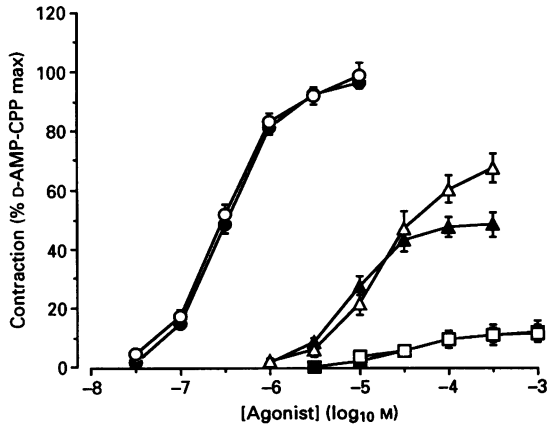

Figure 4 Comparison of E/[A] curves obtained in the rabbit ear artery, using the cumulative (closed symbols) and sequential (open symbols) protocols: (● $n = 29$, ○ $n = 18$) D-AMP-CPP; (▲ $n = 10$, △ $n = 5$) L-AMP-$PCCl_2P$; (■ $n = 5$, □ $n = 5$) L-AMP-$PCBr_2P$. Points show mean ± s.e.mean (vertical bars).

compound, 2-methylthio-L-AMP-$PCBr_2P$, produced effects which were only barely measurable. The remaining three D- and one L- analogues produced maximal effects which were indistinguishable from that of D-AMP-CPP. Table 1 summarizes these findings. In all cases, responses were obliterated in the presence of 3×10^{-5} M D-AMP-CPP.

The five compounds which gave reduced but measurable maximal effects were then analysed by the comparative method (Barlow *et al.*, 1967) in order to estimate affinity and efficacy values. Figure 2 illustrates typical experimental data for the compounds, each obtained in a single piece of tissue. Each example shows the corresponding E/[A] curve for D-AMP-CPP, the reference full agonist and computer-generated lines obtained by fitting equations (ii) and (iii). The resulting affinity (pK_A) and efficacy (τ) values from these individual analyses are given in the legend to Figure 2. Average estimates from replicate analyses are shown in Table 2.

2-Methylthio-L-AMP-$PCBr_2P$, which was virtually inactive as an agonist, was analysed as a potential competitive antagonist. Figure 3a shows the effects of this compound on E/[A] curves to D-AMP-CPP. These data are the averages of the second curves obtained in each tissue and are shown for display purposes only. As explained in the Methods section, tests for parallelism were based on paired comparisons. One way analyses of variance on computed asymptote (α) and slope (p) estimates revealed no significant differences ($P > 0.05$ in both cases). Figure 3b illustrates the corresponding Schild plot. The slope of the linear regression was 0.92 ± 0.08 (s.e.mean, 13 d.f.). Therefore, this analogue fulfilled the criteria for simple competition. The pK_B estimate, with n constrained to unity, was 4.35 ± 0.03 (s.e.mean, 14 d.f.). In addition, this compound, at 10^{-3} M, produced no displacement of E/[A] curves constructed to phenylephrine, histamine and KCl (each $n = 4$) (data not shown).

The influence of experimental design was then examined, comparing analysis of E/[A] curves obtained by the cumulative and sequential curve protocols. Figure 4 illustrates data for two agonists, L-AMP-$PCCl_2P$ and L-AMP-$PCBr_2P$ obtained by the sequential curve design. The cumulative curve for each agonist is included for comparison. Evidently, the two curves for the latter compound are superimposed, whereas for the former agonist the sequential curve is significantly larger and rightward-shifted compared to the corresponding cumulative curve (see Table 1 for parameter estimates). Also shown are data on the reference agonist, D-AMP-CPP, which, like the dibromo- analogue, exhibited no differences. The two halogenated compounds were analysed by the comparative method. Minimal differences in affinity and efficacy estimates were computed for the dibromo- analogue but significant differences were found for the dichloro- compound (see Table 2). It was notable from the chart tracings that the contractile effects of the L-AMP-$PCCl_2P$ were subject to more pronounced fade than those of L-AMP-$PCBr_2P$ and D-AMP-CPP although this was not quantified. Finally, an experiment was performed in which L-AMP-$PCBr_2P$ was interacted with D-AMP-CPP. This involved the generation of D-AMP-CPP E/[A] curves in the presence of different concentrations of the analogue. Preliminary experiments showed that cumulative curves to D-AMP-CPP constructed in the presence of partial agonists suffered a depression of their maxima. Since one of the aims of this experiment was to estimate the affinity of L-AMP-$PCBr_2P$ as an antagonist of D-AMP-CPP and curve depression would compromise this, it was decided to try the sequential design. The results obtained with this protocol are shown in Figure 5. L-AMP-$PCBr_2P$ produced rightward displacements of D-AMP-CPP curves which, from visual inspection, were consistent with the interaction between a partial and a full agonist. Analysis of the $[A_{50}]$s of the curves using equation (iv) gave an affinity (pK_B) estimate of 4.18 ± 0.07 (s.e. mean, 5 d.f.). This value was significantly (although only 2 fold) below the estimate made by the comparative method (see Table 2).

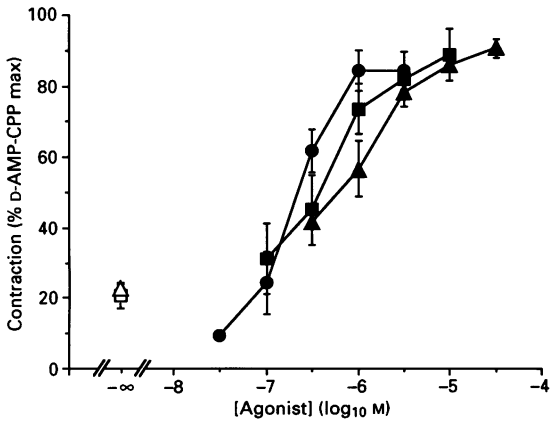

Figure 5 Antagonist effects of L-AMP-$PCBr_2P$. E/[A] curves were obtained in the rabbit ear artery to D-AMP-CPP, alone (●), and in the presence of L-AMP-$PCBr_2P$, (■) 100 μM and (▲) 300 μM. Points are the mean second curve data (± s.e.mean, vertical bars, $n = 3$) obtained using the sequential protocol. The open symbols show the mean contraction induced by (□) 100 μM and (△) 300 μM L-AMP-$PCBr_2P$, before the addition of the full agonist (± s.e.mean, $n = 15$ and 16 respectively).

Discussion

The general objective of this study was to determine whether affinity and efficacy information could be generated for P_{2x}-purinoceptor agonists. To this end we have shown that certain dihalomethylene- and 2-methylthio- substituted ATP analogues demonstrate apparently partial agonist activity in the rabbit ear artery preparation, in principle allowing affinity and efficacy estimates to be obtained by the comparative method (Barlow *et al.*, 1967). However, for the results of such an analysis to be accepted it must be shown that the agonists in question are genuinely partial, that their reduced maximal effects are not the consequence of other pharmacological properties and that their effects are mediated by the same receptor. A number of pieces of evidence support this. Firstly, the effects of all the agonists were obliterated in the presence of a maximum dose of D-AMP-CPP, confirming that they were mediated by the P_{2x}-purinoceptor (this procedure has been shown not to affect non-P_{2x}-contractile agents, O'Connor *et al.*, 1990). Secondly, no relaxation responses were observed in the presence or

absence of this 'occupancy/desensitization' manouevre which could have opposed the constrictor effects of the agonists. Indeed previous reports have shown that the rabbit ear artery lacks relaxant P_{2Y}-purinoceptors (Burnstock & Kennedy, 1985; O'Connor *et al.*, 1990) and the use of 8-SPT in the present study ruled out possible interactions with P_1-purinoceptors. Thirdly, the ability of L-AMP-$PCBr_2P$ (an apparent partial agonist) and its 2-methylthio analogue (a 'silent' compound) to displace E/[A] curves to D-AMP-CPP was consistent with expected actions of a partial agonist and a competitive antagonist respectively, these data confirming mutual interactions of the three compounds with the same receptor and indicating that the two dihalo- analogues had genuinely low efficacy whilst retaining affinity.

On this basis the analogues demonstrating reduced maximal effects were justifiably subjected to affinity and efficacy analysis. A factor which still remained, however, was the possible influence of experimental design. A common feature of all the agonists examined was the propensity of their contractile effects to fade with time. A possible consequence of the cumulative design is accumulation of error due to the progressive superimposition of transient response-time measurements as curves are constructed. This in turn could result in poor E/[A] curve definition and errors in affinity and efficacy estimation. Two partial agonists were studied using the sequential curve protocol which, in principle, eliminates this kind of error. In fact the results were different for the two compounds, the sequential and cumulative curves being superimposed for L-AMP-$PCBr_2P$ but distinct for L-AMP-$PCCl_2P$. For D-AMP-CPP, the full agonist, the curves were superimposed. By inspection of the chart recordings, the discrepancy for the dichloro- analogue appeared to correlate with the more pronounced fade that this agonist exhibited compared to the others. However, a large number of compounds would need to be analysed in order to establish the generality or otherwise of such differences. The resulting affinity and efficacy estimates showed minimal changes in both for the dibromo- analogue but significant changes for the dichloro-compound. For example, the pK_A estimates showed an increase of 0.14 in one case and a decrease of 0.40 in the other. On the basis of these relatively small differences it would seem premature to discount values obtained by the cumulative design, but since the possibility remains that fade may affect response definition it would be cautious to recommend that the sequential protocol, although practically less convenient, is used at least to check results obtained by the cumulative design.

Finally, comparable affinity estimates were obtained for L-AMP-$PCBr_2P$ when analysed as an agonist, by the comparative method, and as an antagonist, by Schild analysis. Although the estimates (4.49 and 4.18 respectively) were significantly different, the separation was only 2 fold. With some caution, therefore, we conclude that the estimates are mutually supportive.

Accepting the validity of the affinity and efficacy estimates shown in Table 2, a number of structure-activity features emerge. Comparing pairs of analogues between the D- and L-series it is evident that D-analogues are generally of higher efficacy than L-analogues. Only D-AMP-$PCBr_2P$ showed overt signs of partial agonist behaviour in the absence of 2-methylthio- substitution, whereas all the L-dihalomethylene- compounds exhibited low efficacy.

Efficacy clearly varied amongst the analogues. For the L-series the order was methylene > difluoromethylene > dichloromethylene > dibromomethylene (the un-halogenated compound being a full agonist). In the D-series, although the difluoro- and dichloro- analogues were full agonists, the dibromo- compound exhibited reduced efficacy. The effect of the 2-methylthio substituent in both series was a further fall in efficacy, converting the D-dichloro- analogue from a full into a partial agonist, and the L-dibromo- analogue from a partial agonist into a 'silent', competitive antagonist. Therefore, in both series of compounds halogenation and 2-methylthio substitution conferred independent reductions in efficacy, in the former case the extent of reduction being proportional to the atomic weight of the halogen.

Regarding affinity, the effects of substituents were not so dramatic. For example, in the L-series, the difluoro- and dichloro- analogues exhibited indistinguishable affinities, and the dibromo- analogue had a 3 fold lower value. The effect of 2-methylthio incorporation was also marginal, causing less than a 2 fold drop in affinity between the unsubstituted agonist dibromo- analogue ($pK_A = 4.49$) and the substituted antagonist compound ($pK_B = 4.35$).

Since most of the compounds in the D- series behaved as full agonists it was not possible to compare affinity estimates amongst different halogenated analogues. However, some general comparisons between the D- and the L- series can still made. It is evident from the computed $p[A_{50}]$ values that compounds in the D- series exhibited lower potency than equivalent members of the L- series. For example, D-AMP-$PCCl_2P$ was a full agonist with a $p[A_{50}]$ of 4.32, whereas L-AMP-$PCCl_2P$ was a partial agonist with a $p[A_{50}]$ of 5.04. Moreover, the pK_A (affinity) estimate for the latter compound was 4.98, a value which is clearly to the more potent side of the $p[A_{50}]$ for the D- analogue. In general, it is theoretically impossible for the E/[A] curve for a full agonist to lie well to the right (on the $\log_{10}$[A] scale) of the pK_A. This can be shown by the operational model equation which defines the relationship between $[A_{50}]$ and K_A (Black & Leff, 1983; Leff, 1987). Conceptually, this situation is unreasonable since it would allow an agonist to produce a threshold effect after saturating the receptors and for two indistinguishable levels of occupancy to produce different pharmacological effects. Therefore, for compounds such as D-AMP-PCP, D-AMP-PCF_2P and D-AMP-$PCCl_2P$, which behaved as full agonists, it can be deduced that their pK_A values are substantially lower than their computed $p[A_{50}]$ values, meaning that they are likely to be less than 4.0 on the $\log_{10}$ scale. These are all an order of magnitude lower than the estimates for the corresponding L-analogues. In fact, in the one case where it was possible to compare pK_A estimates between the two series, D-AMP-$PCBr_2P$ (3.49) with L-AMP-$PCBr_2P$ (4.49), the difference was consistent with this reasoning.

It is important to recognise that the provision of reliable affinity information, as with potency measurements, presumes that the possible influence of ectonucleotidase activity is absent. It has been reported that the dihalomethylene analogues used in the present study (with the exception of the dibromo compounds) are resistant to metabolism, showing no degradation over a period during which ATP was completely dephosphorylated (Cusack *et al.*, 1987). The biochemical experiments which provided those results do not strictly represent the events occuring in the biophase where the receptors and nucleotidase enzymes may be located. Nor were they performed in the tissue which was the subject of the present study. However, with these limitations, the stability data suggest that if errors can occur they are likely to be minimal with these analogues. Definitive experiments must await the availability of a nucleotidase inhibitor for routine pharmacological use.

On the basis of these results it would appear that, predominantly, D-analogues owe their potency to efficacy whereas the L-analogues owe their potency to affinity. For example, D-AMP-PCP and L-AMP-$PCBr_2P$ had virtually identical potencies ($p[A_{50}]$s of 4.36 and 4.35, respectively), the first resulting from a combination of low affinity and high efficacy, the latter from a combination of high affinity and low efficacy. This serves to emphasise one of the important reasons for estimating affinity and efficacy for agonists, rather than relying on potency measurements alone. Using the potency scale, compounds of the same $[pA_{50}]$ would be regarded as having the same activity despite, as the example shows, the possibility that the compounds are different in the relative contributions of the two underlying properties which determine potency. Since, in addition, these two properties

can depend quite differently on chemical structure, the provision of affinity and efficacy information is obviously fundamental to medicinal chemistry on agonists. For example, in the rational design of molecules to probe P_{2x}-purinoceptors, the present data would suggest that D-analogues, having higher efficacies, are better leads to agonists, whereas L-analogues, having lower efficacies, are better leads to antagonists.

In the light of the present analysis it is interesting to re-evaluate the data produced by Cusack *et al.* (1987) which, as mentioned in the Introduction, contained evidence for partial agonist action using the guinea-pig urinary bladder preparation. In particular, L-AMP-$PCCl_2P$ and 2-methylthio-D-AMP-$PCCl_2P$ exhibited lower maximal effects than the other agonists studied (unfortunately, those authors did not report data on dibromo-substituted compounds). The present study shows both these analogues to be partial agonists in the rabbit ear artery. Although from inspection there appear to be some problems of E/[A] curve shape definition in the bladder preparation, meaning that detailed analysis of the data is not possible, the qualitative consistency in the behaviour of the two agonists between the tissues is noteworthy and points to the way in which affinity and efficacy estimation may be of value in the classification of P_{2x}-purinoceptors. Comparison of 'fingerprints' of affinity and efficacy information for series of agonists has been useful in the classification of receptors in other areas, establishing the similarity or difference between receptors in different tissues (Leff & Dougall, 1992). The power of this approach as compared with the use of potency orders is that each agonist provides two pieces of information for the purposes of receptor classification rather than only one. Also, as implied by preceding arguments, the fact that potency depends on both affinity and efficacy means that the same potency order could emerge from different combinations of affinity and efficacy, and receptors which are in fact different could be wrongly classified as the same. Estimation of affinities and efficacies avoids such potential pitfalls. In the present case, for example, the same 'fingerprint' of affinity and efficacy estimates in the ear rabbit ear artery and guinea-pig bladder would provide strong quantitative support to the belief that these preparations contain the same receptor type. While selective antagonists remain scarce in this field of receptor research, quantitative data on agonist action could make an important contribution to receptor classification.

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Evidence against vasoactive intestinal polypeptide as the relaxant neurotransmitter in human cavernosal smooth muscle

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1 The putative role of vasoactive intestinal polypeptide (VIP) as the relaxant neurotransmitter in human cavernosal smooth muscle has been studied in isolated tissue preparations.

2 Consistent neurogenic relaxations were evoked by electrical field stimulation (EFS; 2–64 pulses/train, 0.8 ms pulse duration, 10 Hz). VIP (0.1–3 μM) relaxed cavernosal smooth muscle in a dose-dependent fashion. Relaxant responses to both EFS and VIP were reduced in tissue from impotent men.

3 Neurogenic relaxant responses were not diminished in the presence of the VIP-inactivating peptidase, α-chymotrypsin (α-CT, 2 units ml^{-1}). In contrast VIP-induced relaxations were completely abolished.

4 Inhibition of nitric oxide synthase by N^G-nitro-L-arginine (30 μM), and of guanylate cyclase by methylene blue (50 μM) caused highly significant reductions of neurogenic relaxant responses whereas VIP-evoked relaxations were unaffected.

5 It is concluded that VIP-evoked relaxations are not mediated by the NO-guanosine 3′:5′-cyclic monophosphate (cyclic GMP) pathway and that VIP release is not essential for neurogenic relaxation of human cavernosal smooth muscle. VIP does not therefore act as the major relaxant neurotransmitter in this tissue.

Keywords: Vasoactive intestinal polypeptide; penile erection; nitric oxide

Introduction

Recent studies have provided good evidence that nerve-evoked relaxation of human cavernosal (penile) smooth muscle is mediated by the nitric oxide-guanosine 3′:5′-cyclic monophosphate (cyclic GMP) pathway (Kim *et al.*, 1991; Pickard *et al.*, 1991; Rajfer *et al.*, 1992; Holmquist *et al.*, 1992). However, the site of nitric oxide (NO) synthesis in this tissue remains unknown and hence the status of NO as a true neurotransmitter or as a secondary messenger is undecided.

Previous *in vitro* and *in vivo* findings lend some support for vasoactive intestinal polypeptide (VIP) as a relaxant neurotransmitter in this tissue. Nerves immunoreactive for VIP are found adjacent to the arterioles and vascular spaces of the corpora cavernosa (Polak *et al.*, 1981) and exogenous VIP relaxes isolated cavernosal tissue in a dose-dependent fashion (Willis *et al.*, 1983). In addition one study found increased levels of VIP in penile blood during erection (Ottesen *et al.*, 1984), although this finding was not confirmed by others (Kiely *et al.*, 1987). The putative role of VIP as a relaxant neurotransmitter has been strengthened by the finding that the VIP-inactivating peptidase, α-chymotrypsin attenuates neurogenic smooth muscle relaxation in other tissues (Angel *et al.*, 1983; Ellis & Farmer, 1989). In addition there is evidence that peptidergic (VIP) smooth muscle relaxation is mediated by the nitric oxide-cyclic GMP pathway in sheep cerebral artery and rat gastric fundus (Gaw *et al.*, 1991; Li & Rand, 1990).

In the present study we have investigated the possibility that VIP acts as the primary stimulator of NO release in human cavernosal tissue by examining the effect of α-chymotrypsin (α-CT), N^G-nitro-L-arginine (L-NOARG), an inhibitor of nitric oxide biosynthesis and methylene blue, an inhibitor of cyclic GMP formation, on relaxant responses evoked by VIP and nerve stimulation.

A preliminary account of this work was presented at a meeting of the Physiological Society (Pickard *et al.*, 1992).

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Methods

The use of human tissue in this study was approved by the Newcastle Joint Ethics Committee.

Cavernosal tissue samples were obtained during penile operations from 5 potent and 10 impotent men and were transported to the laboratory in chilled Krebs-Henseleit solution (composition, mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11). Tissue strips measuring approximately 2 × 3 × 5 mm were fashioned from each sample and suspended under 1 g of tension from a force transducer in a 1 ml organ bath. The bath contained Krebs solution at 37°C gassed with 95% O_2/5% CO_2 mixture. Each strip was left to equilibrate for 90 min. The bathing medium contained atropine 1 μM and guanethidine 10 μM in order to block muscarinic receptors and noradrenergic neuronal activity respectively throughout the experiments. To enable the recording of relaxant responses to electrical field stimulation (EFS) and VIP, the strips were precontracted by the addition of phenylephrine (PE) 10 μM.

Once a stable level of increased tone had been reached following the addition of PE, control relaxant responses to EFS were recorded. EFS comprised trains of pulses (0.8 ms pulse duration, 30 V) delivered by an electronic stimulator (Bell & Stein, 1971). EFS was applied both as trains of 2, 4, 8, 16, 32 and 64 pulses at a constant frequency of 10 Hz and as trains of 16 pulses at increasing frequencies of 2, 4, 8 and 16 Hz. The responses to the addition of cumulative doses of VIP from 0.1–3 μM were then recorded.

The strip was then washed repeatedly with fresh Krebs solution and allowed to re-equilibrate for 15 min. Next the strips were incubated with either α-CT (2 units ml^{-1}) for 10 min or L-NOARG (30 μM) for 10 min or methylene blue (50 μM) for 60 min. The strips were then re-contracted with PE and relaxant responses to both EFS and VIP recorded in the continued presence of the respective inhibitor. In experiments using methylene blue only one train length (16 pulses) at each frequency and one concentration of VIP (1 μM) was used.

Results are expressed as mean ± s.e.mean of data from *n* individuals. Statistical significance was tested by Student's *t*

test for paired and unpaired data and accepted if $P < 0.05$ (one-tailed).

Stock solutions of α-chymotrypsin (Sigma), atropine sulphate (Sigma), guanethidine sulphate (CIBA), methylene blue (Sigma), N^G-nitro-L-arginine (Sigma), phenylephrine (Sigma), porcine vasoactive intestinal polypeptide (Sigma) and tetrodotoxin (Sigma) were made up in distilled water and stored at − 20°C.

Results

Response to electrical field stimulation

Under conditions of sub-maximal contraction induced by PE, EFS produced relaxant responses that were rapid in onset with fast recovery to the original tension following cessation of the stimulus. The magnitude of the relaxation was dependent upon train length and, to a lesser extent, upon the pulse frequency (Figure 1a,b). The responses to EFS were fully sensitive to tetrodotoxin, 1 μM. Tissue strips from impotent men showed a reduction in the magnitude of relaxant responses to EFS at all train lengths (Figure 2).

Response to vasoactive intestinal polypeptide

The cumulative addition of VIP to the bath giving concentrations of 0.1, 0.3, 1 and 3 μM produced dose-dependent relaxation of tissue strips that was slow in onset with no recovery towards the original level of tone (Figure 1c). The rapidity of response varied between strips from different individuals. The response to VIP was reduced in strips from impotent men compared to those from potent men although the difference did not reach statistical significance (Figure 3).

Effect of α-chymotrypsin

Incubation with α-CT completely abolished the relaxant response to VIP at all concentrations tested. The presence of α-CT caused a small increase in the magnitude of relaxations evoked by EFS at most train lengths (Figure 4). This effect was observed at all frequencies tested. The increase was greatest and statistically significant when trains of 4 and 8 pulses were used at a frequency of 10 Hz, being 43 ± 14% and 15 ± 5% respectively ($n = 14$, $P < 0.05$).

Effect of N^G-nitro-L-arginine

In the presence of L-NOARG, relaxations evoked by EFS were markedly diminished (Figure 5). This inhibition occurred at all frequencies tested and was highly significant when longer trains of stimulation were used, maximum inhibition being 90 ± 8% with trains of 64 pulses ($n = 11$, $P < 0.01$). In

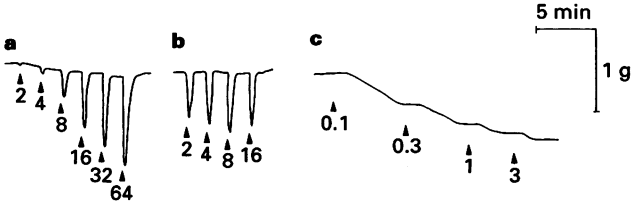

Figure 1 Relaxant responses of isolated preparations of human corpus cavernosum to electrical field stimulation (EFS, a,b) and vasoactive intestinal polypeptide (VIP, c). Atropine (1 μM), guanethidine (10 μM) and phenylephrine (10 μM) were present throughout. EFS given as a sequence of 2, 4, 8, 16, 32 and 64 pulse trains at a constant frequency of 10 Hz produced rapid relaxant responses of increasing magnitude (a). The magnitude of responses also increased when 16 pulse trains were delivered using increasing pulse frequencies of 2, 4, 8 and 16 Hz (b). The relaxant responses to cumulatively increasing VIP concentrations of 0.1, 0.3, 1 and 3 μM were slow in onset and concentration-dependent (c).

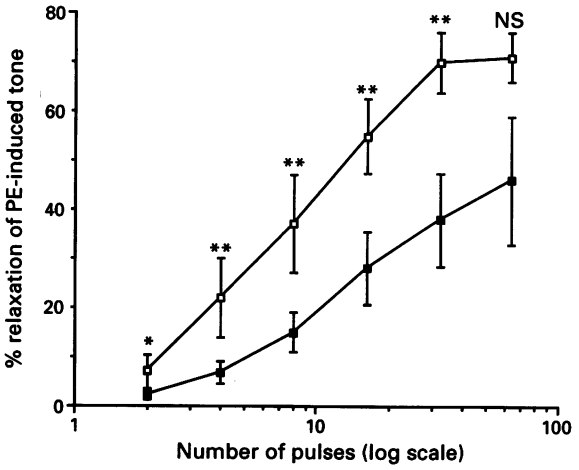

Figure 2 Relaxant responses to electrical field stimulation (2–64 pulse trains, 10 Hz) in isolated preparations of corpus cavernosum from 5 potent men (□) compared to those demonstrated in tissue from 10 impotent men (■). Each point is the mean relaxation of phenylphrine (PE, 10 μM)-induced tone with bars representing s.e.mean. NS = not significant; $*P < 0.05$; $**P < 0.01$.

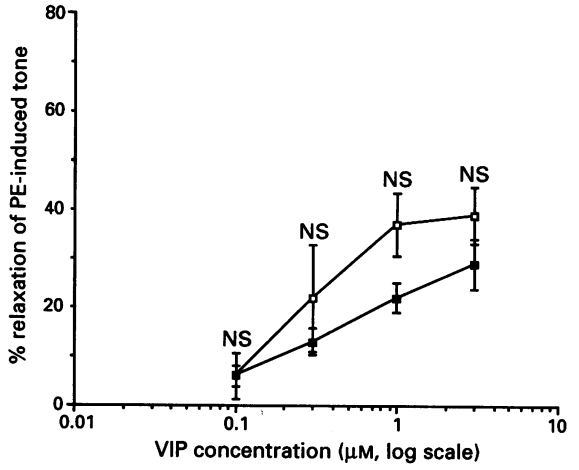

Figure 3 Comparison of relaxant responses to the cumulative addition of vasoactive intestinal polypeptide (VIP) giving concentrations of 0.1, 0.3, 1 and 3 μM in isolated preparations of corpus cavernosum from 5 potent (□) and 10 impotent men (■). Each point represents the mean relaxation of phenylephrine (PE, 10 μM)-induced tone with bars depicting s.e.mean. NS = not significant.

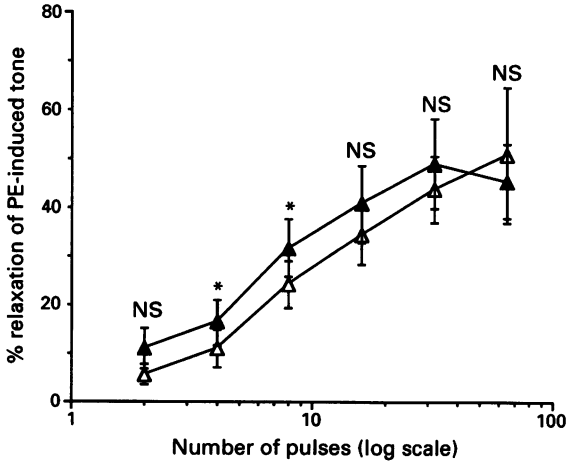

Figure 4 The effect of incubation of human isolated cavernosal strips in α-chymotrypsin (α-CT, 2 units ml^{-1}) on relaxations evoked by electrical field stimulation (2–64 pulse trains, 10 Hz). Each point shows the mean relaxation of phenylephrine (PE, 10 μM)-induced tone before (△) and following (▲) the addition of α-CT with bars representing s.e.mean ($n = 14$). NS = not significant; $*P < 0.05$.

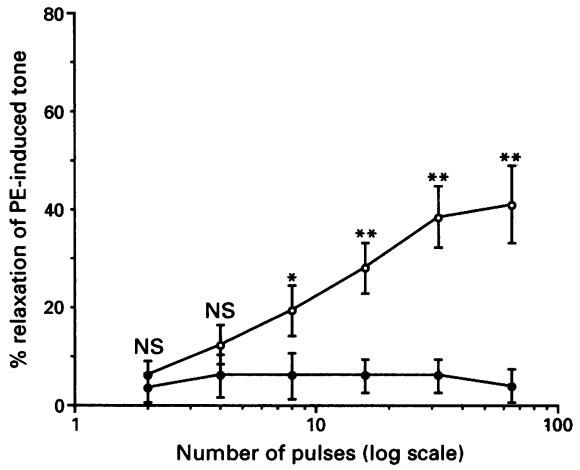

Figure 5 The effect of incubation with N^G-nitro-L-arginine (L-NOARG, 30 μM) on relaxations of human cavernosal strips evoked by electrical field stimulation (2–64 pulses/train, 10 Hz). Each point indicates mean relaxation of phenylephrine (PE, 10 μM)-induced tone before (○) and following (●) the addition of L-NOARG with vertical bars showing s.e.mean ($n = 11$). NS = not significant; $*P < 0.05$; $**P < 0.01$.

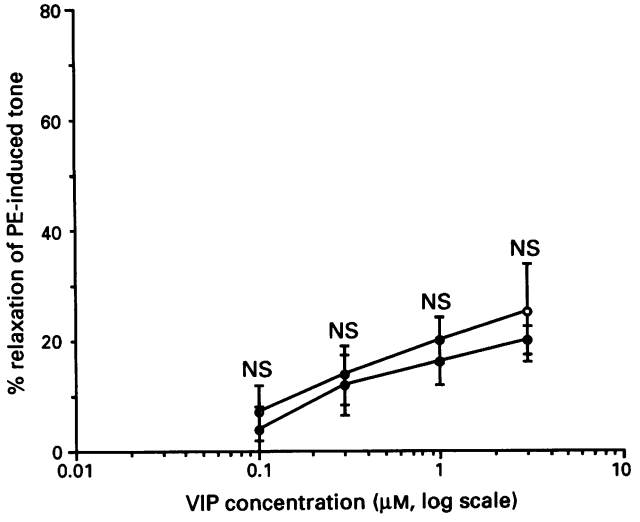

Figure 6 The effect of N^G-nitro-L-arginine (L-NOARG, 30 μM) on relaxations of human isolated cavernosal strips evoked by the cumulative addition of vasoactive intestinal polypeptide (VIP, 0.1, 0.3, 1 and 3 μM). Points show the mean relaxation of phenylephrine (PE, 10 μM)-induced tone before (○) and following (●) the addition of L-NOARG with bars representing s.e.mean ($n = 11$). NS = not significant.

contrast, only small, statistically insignificant reductions in the relaxant responses to VIP were observed at all concentrations tested (Figure 6), the maximum inhibition being 20 ± 20% at a VIP concentration of 1 μM ($n = 11$, NS).

Effect of methylene blue

Treatment with methylene blue significantly attenuated the relaxant response to EFS by 38 ± 12% at a frequency of 10 Hz ($n = 5$, $P < 0.01$, Figure 7). This inhibitory effect was similar at all frequencies used, reaching a maximum of 54 ± 19% at 8 Hz ($n = 4$, $P < 0.01$). In contrast, the magnitude of VIP-evoked relaxations was slightly increased by 4 ± 19% ($n = 5$, NS, Figure 7). Following treatment with methylene blue the contractile response to PE was reduced by 11 ± 25% ($n = 5$, NS).

The effects of α-CT, L-NOARG and methylene blue on relaxant responses to EFS and VIP were similar in tissues from potent and impotent men.

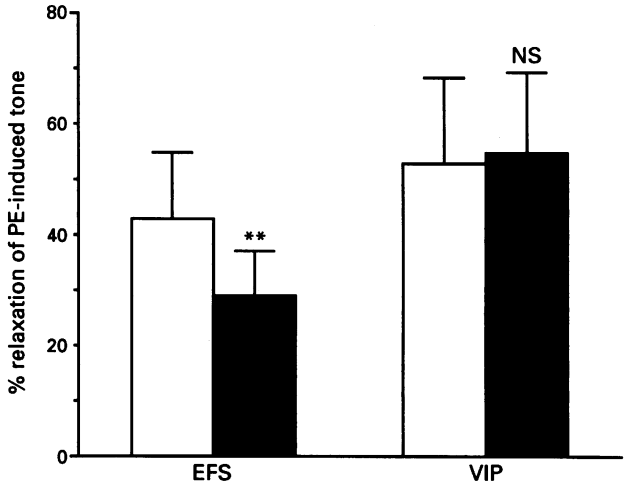

Figure 7 The effect of incubation with methylene blue (50 μM) on relaxant responses of isolated strips of human corpus cavernosum evoked by electrical field stimulation (EFS, 16 pulse trains, 10 Hz) and vasoactive intestinal polypeptide (VIP, 1 μM). Each pair of columns represents the mean relaxant responses before (open columns) and after (solid columns) the addition of methylene blue expressed as a percentage of phenylphrine (PE 10 μM)-induced tone. Vertical bars show s.e.mean ($n = 5$). NS = not significant; $**P < 0.01$.

Discussion

The neurogenic relaxation of cavernosal smooth muscle is essential for the initiation and maintenance of physiological penile erection in man (Krane *et al.*, 1989). The nerves responsible for cavernosal relaxation are overwhelmingly non-adrenergic, non-cholinergic (NANC) in nature (Saenz de Tejada *et al.*, 1988) and although the identity of the NANC neurotransmitter has yet to be unequivocally determined, VIP has been proposed as a strong candidate for this role (Willis *et al.*, 1983; Ottesen *et al.*, 1984; Adaikan *et al.*, 1986). The present investigation has tested the putative role of VIP in the neurogenic relaxation of human cavernosal smooth muscle using two different approaches and the results are incompatible with VIP being a major relaxant neurotransmitter.

In the first series of experiments incubation of corpus cavernosum strips with the peptidase, α-chymotrypsin (α-CT), which has been shown to abolish the smooth muscle relaxant effects of both endogenous and exogenous VIP in canine stomach and guinea-pig trachea (Angel *et al.*, 1983; Ellis & Farmer, 1989), caused no inhibition of neurogenic relaxant responses; on the contrary they were significantly increased by this treatment when short trains of stimuli were used. The failure of α-CT to reduce or abolish neurogenic relaxant responses was not attributable to weak peptidase activity since in these experiments under identical conditions, treatment with α-CT led to complete abolition of relaxant responses to exogenous VIP. Immunohistochemical studies have shown that the density of peptidergic (VIP) nerves is reduced in cavernosal tissue from impotent men, particularly those with diabetes (Gu *et al.*, 1984; Lincoln *et al.*, 1987). In our study although the magnitude of both neurogenic and VIP-evoked relaxations was somewhat reduced in tissue from impotent men the effects of α-CT were similar in strips from potent and impotent men. It is therefore unlikely that the lack of effect of α-CT was due to the pathological absence of peptidergic (VIP) nerves. These findings therefore indicate that neurogenic relaxation of human cavernosal smooth muscle is not dependent upon the release of VIP.

There is incontrovertible evidence that NANC nerve-evoked relaxation of human cavernosal smooth muscle is mediated by nitric oxide (NO) or a NO-like substance which acts through the activation of soluble guanylate cyclase in the smooth muscle cell (Kim *et al.*, 1991; Pickard *et al.*, 1991;

Rajfer et al., 1992; Holmquist et al., 1992). The implication of this knowledge is that for VIP to serve as the main NANC neurotransmitter in human cavernosal smooth muscle it must act predominately through the release of NO which in its turn activates guanylate cyclase leading to formation of cyclic GMP and consequent smooth muscle relaxation. In the second set of experiments we have tested this hypothesis by comparing the effect of inhibitors of NO-synthase and guanylate cyclase on neurogenic and VIP-evoked relaxations of human corpus cavernosum and our findings indicate that it should be rejected. The NO-synthase inhibitor, L-NOARG virtually abolished the neurogenic relaxant responses of cavernosal smooth muscle but did not affect the relaxant response to VIP, thus implying that functional integrity of NO-synthase is an essential prerequisite for evoking relaxant responses to nerve stimulation alone. In this respect human cavernosal smooth muscle activity is similar to that of the rabbit (Holmquist et al., 1992). These results are in conformity with the view that NO is the mediator of nerve-evoked relaxation whilst VIP does not require NO to exert its relaxant effect. Additional evidence for this contention comes from our finding that methylene blue, an inhibitor of guanylate cyclase activation also failed to reduce significantly VIP-evoked relaxation in this tissue, thereby indicating that VIP-evoked relaxation, unlike neurogenic relaxation is independent of the NO-cyclic GMP pathway.

Our exclusion of VIP as a major inhibitory neurotransmitter in this tissue is in keeping with the recent immunocytochemical localization of NO-synthase in nerves supplying erectile tissue of the bull and rat indicating that NO acts as a true neurotransmitter in these tissues (Sheng et al., 1992; Burnett et al., 1992). Although the site of NO-synthesis has yet to be determined in human corpus cavernosum it seems likely that NO is also neuronal in origin in man.

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In vitro characterization of prostanoid receptors on human myometrium at term pregnancy

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1 Prostanoid receptors present on the pregnant human myometrium *in vitro* have been characterized according to the receptor classification proposed by Coleman *et al.* (1984) using natural prostanoids and synthetic, selective analogues and antagonists where available.

2 Prostaglandin E_2 (PGE_2) produced a biphasic effect consisting of an initial excitation followed by a dose-related inhibition. The EP_2/EP_3-receptor agonists, rioprostil and misoprostol, produced similar effects to PGE_2, however, the excitatory event of the misoprostol response was related to dose. The EP_1/EP_3-receptor agonist, sulprostone, evoked a purely excitatory response which was unaffected by AH6809. The selective EP_2-receptor agonist butaprost produced a long-lasting dose-dependent inhibition of activity. The results from these prostanoids indicated that inhibitory EP_2- and excitatory EP_3-receptors are present on myometrium from pregnant donors at term.

3 $PGF_{2\alpha}$ and the synthetic FP-receptor agonist, fluprostenol, caused equipotent excitatory effects, indicating the presence of contractile FP-receptors.

4 PGD_2 produced a biphasic effect of which the inhibition appeared dose-related and was antagonized by the selective DP-receptor antagonist BW A868C. The selective DP-receptor agonist, BW245C, produced a potent inhibitory effect that was competitively antagonized by BW A868C ($pA_2 = 8.6$).

5 PGI_2 produced a biphasic response qualitatively similar to PGE_2. The EP_1/IP-receptor agonist, iloprost, produced an occasional unquantifiable excitation and dose-related inhibition. The selective IP-receptor prostanoid, cicaprost, evoked only an inhibitory response.

6 The stable thromboxane A_2 (TXA_2)-mimetic, U46619, produced potent excitation which was competitively antagonized by the TP-receptor antagonist, GR32191 ($pA_2 = 7.2$).

7 The prostanoids tested indicate that a heterogeneous population of prostanoid receptors are present on human myometrium from pregnant donors. It may be concluded that excitation is EP_3-, FP- and TP-receptor-mediated and inhibition is EP_2-, DP- and IP-receptor-mediated. Comparison of data obtained from non-pregnant specimens indicates that the lower segment tissue from pregnant donors demonstrated more pronounced responses to EP_2 and IP-receptor activation.

Keywords: Human myometrium; pregnant; prostanoids; synthetic analogues; antagonists; prostanoid receptors

Introduction

Present prostanoid receptor classification states that each of the natural prostanoids has its own receptor termed the P receptor where that particular prostanoid is at least ten times more potent than any of the other prostanoids. Thus the prostaglandin E_2 (PGE_2)- sensitive receptor is termed the EP-receptor and likewise $PGF_{2\alpha}$, the FP-receptor; PGD_2, the DP-receptor; PGI_2, the IP-receptor and thromboxane A_2 (TXA_2), the TP-receptor (Kennedy *et al.*, 1982; Coleman *et al.*, 1984). The EP-receptor is further subdivided into the EP_1-, EP_2- and EP_3-receptor subtypes (Coleman *et al.*, 1987a,b,c).

Numerous workers have identified high and low affinity PGE_2 and $PGF_{2\alpha}$ binding sites on the human myometrium (Wakeling & Wyngarden, 1974; Crankshaw *et al.*, 1979; Bauknecht *et al.*, 1981; Giannopoulos *et al.*, 1985; Chegini *et al.*, 1986; Adelantado *et al.*, 1988). Hofmann *et al.* (1983) and Giannopoulos *et al.* (1985) began to elucidate PGE and $PGF_{2\alpha}$ receptor topography in the human uterus. The findings of the former group would appear to have a functional correlation (PGE and $PGF_{2\alpha}$ receptor concentration was highest in the fundus and decreased towards the cervix in parallel with the degree of smooth muscle content present), as the upper uterine segment has to generate the expulsive force required during labour, whilst the lower segment remains passive and dilates to allow passage of the foetus (Wiqvist *et al.*, 1983). However, the findings of Giannopoulos *et al.* (1985) did not fully concur with those of Hofmann *et al.* (1983) probably due to the high levels of endogenous prostaglandin in these tissues. Adelantado *et al.* (1988) using radiolabelled PGE_2, proposed the existence of two PGE receptors in the uterus namely high affinity sites in the myometrium and low affinity sites in the cervix. However, the lack of receptor specificity of the natural prostanoids renders the interpretation of these binding studies difficult. Conclusions about the distribution of prostanoid receptors in the myometrium have come mainly from preliminary functional studies: use of U46619 and fluprostenol has demonstrated TP- and FP-receptor sites respectively (Clayton *et al.*, 1986a,b); BW245C has been shown to relax human uterus (Sanger *et al.*, 1982); and cicaprost evokes relaxant responses in human myometrium (Dyal & Crankshaw, 1988).

The aim of this functional study was to investigate comprehensively the effect of natural and synthetic prostanoids and their antagonists on the human myometrium, taken at term, in order to characterize the prostanoid receptors present.

Methods

Human myometrial samples were obtained during elective Caesarean section, between 38–40 weeks of pregnancy from consenting donors. The specimens were taken from the lower

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segment of the uterus and then immediately placed in Krebs solution. Myometrial strips were set up within a 60 min post-operative period and superfused with oxygenated Krebs solution (95% O_2/5% CO_2) containing 2.79 μM indomethacin at 37°C as previously described (Senior *et al.*, 1991). In order to investigate the possible action of compounds through TP-receptors, the TP-receptor antagonist, GR32191, was included in the Krebs solution at a concentration of 1 μM (see Senior *et al.*, 1991). Only one dose-effect curve was obtained from each myometrial strip; group size (*n*) indicates the number of tissues used with each tissue coming from a different donor.

Measurement of response and analysis of results were undertaken as detailed by Senior *et al.* (1991). Excitatory responses were expressed as T/B ratios i.e. ratio of the area of the excitatory response produced by a dose of agonist to the area of the previous spontaneous background contraction. Potency for excitatory agonists was expressed as an ED_1 value i.e. the dose of agonist required to produce a T/B ratio equal to 1. The inhibitory actions of the agonists were expressed in terms of how they modified the periodicity of spontaneous activity, measured in minutes. A measure of the potency of an agonist producing inhibition was obtained by calculating an inhibitory dose ID_4 value which was defined as the dose of agonist required to extend the normal interval between two myogenic contractions by a period of 4 min. When an agonist produced a biphasic response each component of the response was analysed separately; it has to be accepted that these two events are inter-related.

Sample traces showing responses to $PGF_{2\alpha}$ (excitatory) and PGE_2 (excitation followed by inhibition) are shown in Figure 1.

ED_1 and ID_4 values were obtained from individual dose-effect curves and expressed as geometric means with 95% confidence limits. These measures of potency are the same as those used previously for myometrium from non-pregnant donors. Where antagonists were used potency was expressed as the pA_2 value as determined by the method of Arunlakshana & Schild (1959). Statistical comparisons were made by use of an unpaired Student's *t* test (Snedecor & Cochran, 1979).

Compounds

With the exception of the natural prostaglandins (including 11α, 9α -epoxymethano-PGH_2, U46619) and indomethacin, which were obtained from Sigma the following compounds were gifts which we gratefully acknowledge: BW245C (5-(6-carbohexyl)-1 (3-cyclohexyl-2-hydroxylpropyl)-hydantion) and BW A868C (3-benzoxyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxy-ethylamino) hydantoin) (Wellcome); butaprost (Bayer); misoprostol (Searle); rioprostil (Ortho); ICI 181008 (*m*-trifluoromethyl-16-phenoxy 17,18,19,20-tetrano-$PGF_{2\alpha}$, fluprostenol) (ICI); cicaprost and iloprost (Schering); AH6809 (6-isoproxy-9-oxoxanthene-2-carboxylic acid) and GR32191 {[1R-[1α(Z)2β,3β,5α]-(+)-7-5-([1,1-biphenyl)-4-yl) methyloxyl}-3-hydroxy-2-(1-piperidinyl) cyclopentyl]-4-heptanoic acid hydrochloride) (Glaxo). All stock solutions and vehicles were the same as have been previously published (Senior *et al.*, 1991; 1992).

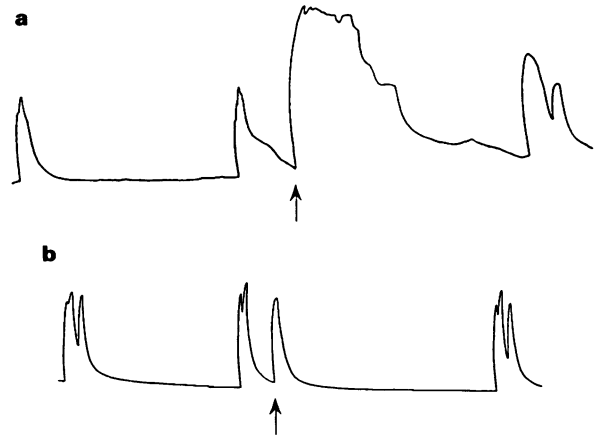


Figure 1 Sample traces showing the effect of a dose of (a) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10 nmol) and (b) PGE_2 (3 nmol) on the myogenic activity of human myometrium from pregnant donors.

Results

The effect of EP-receptor agonists

PGE_2 always produced a biphasic response, namely a stimulation at lower doses (0.14 nmol) and a predominantly inhibitory response at higher doses (0.28–140 nmol). The stimulatory component of the PGE_2 response was not dose-related, whereas the inhibitory response did increase with dose (Figure 2a and Table 1). The EP_2/EP_3 receptor agonists, rioprostil and misoprostol, both evoked responses qualita-

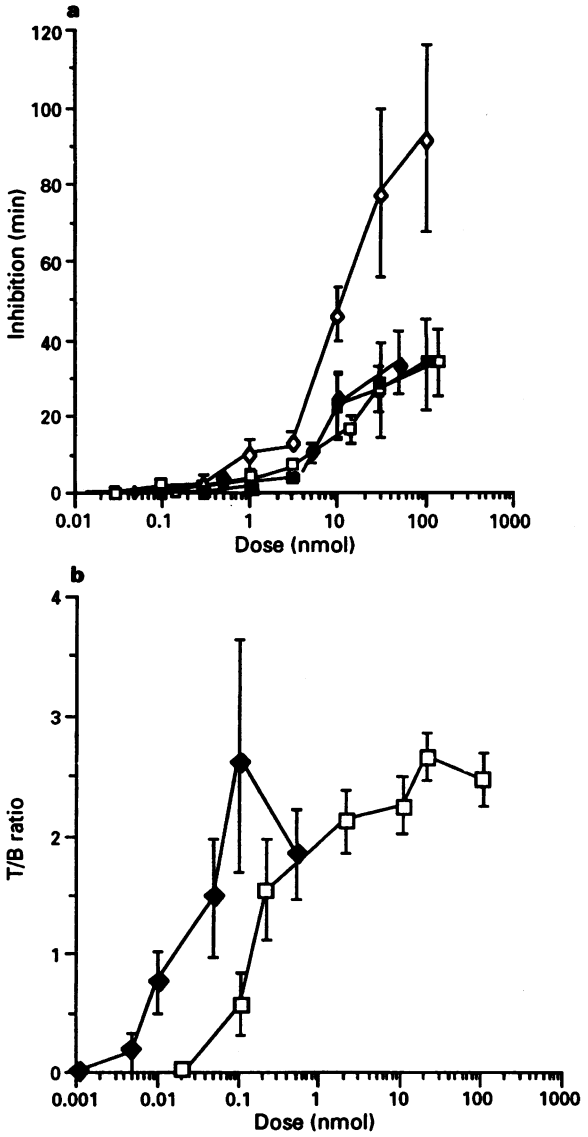


Figure 2 (a) Mean inhibitory dose-effect curves to prostaglandin E_2 (PGE_2, □) $n = 6$, rioprostil (■) $n = 7$, misoprostol (◆) $n = 6$ and butaprost (◇) $n = 6$. Data are expressed as arithmetic means and vertical bars represent s.e.mean. (b) Mean stimulatory dose-effect curves to sulprostone (□) $n = 6$ and misoprostol (◆) $n = 6$. Data are expressed as arithmetic means and vertical bars represent s.e.mean.

Table 1 Mean ED_1, ID_4 and maximum responses for excitation and inhibition by natural and synthetic prostanoids

	Excitation			*Inhibition*		
Prostanoid	ED_1 (95% c.l.)	max (s.e.mean)	*n*	ID_4 (95% c.l.)	max (s.e.mean)	*n*
PGD_2	?		6	*	4.75 (0.95)	6
PGE_2	?		6	1.14 (0.57–2.84)	33.8 (7.83)	6
$PGF_{2\alpha}$	0.5 (0.28–0.66)	5.07 (0.75)	6	>14	NR	6
PGI_2	?		9	4.78 (0.08–11.16)	21.9 (5.64)	9
U46619	0.1 (0.05–0.26)	3.82 (1.39)	6	>140	NR	6
BW245C	>100	NR	7	0.36 (0.07–0.65)	31.5 (5.44)	7
Butaprost	>100	NR	6	0.41 (0.06–3.0)	92.5 (21.98)	6
Misoprostol	0.02 (0.0035–0.3)	2.66 (0.97)	6	0.86 (0.65–1.4)	34.0 (9.11)	6
Rioprostil	?		7	1.71 (0.7–3.0)	33.7 (11.63)	7
Sulprostone	0.14 (0.065–0.4)	2.67 (0.24)	6	>110	NR	6
Fluprostenol	0.61 (0.5–0.75)	3.08 (0.3)	5	>110	NR	5
Cicaprost	>12.6	NR	9	0.2 (0.13–0.50)	34.1 (8.47)	9
Iloprost	?		9	0.56 (0.18–6.39)	24.7 (3.20)	9

NR = no response
? = not quantifiable
ED_1 and ID_4 values are expressed as geometric means (nmol) with 95% c.l. in parentheses
Maximum responses for excitation are expressed as multiples of background activity (i.e. maximum T/B ratios) and maximum responses for inhibition are expressed in minutes
*Maximum response for PGD_2 inhibition is shown. However, a reliable ID_4 value could not be obtained because an inhibition of 4 min was not achieved by PGD_2 in all experiments

tively similar to that of PGE_2. However, the initial stimulatory effect of misoprostol differed from PGE_2 and rioprostil in that it was dose-related and sulprostone (EP_1/EP_3) and butaprost (EP_2) produced only monophasic reactions of contraction and inhibition respectively (Figures 2a and b): butaprost was the most potent of the inhibitory mediators in terms of maximum inhibition of myogenic activity achieved.

The EP_1-receptor antagonist AH6809 (10^{-5} M) (Coleman *et al.*, 1985) was tested against sulprostone but it failed to elicit any rightward displacement of the dose-effect curve.

The effect of FP-receptor agonists

The natural FP-receptor agonist, $PGF_{2\alpha}$, produced a purely contractile response, similar to the synthetic analogue fluprostenol, both compounds being approximately equipotent (Figure 3 and Table 1).

The effect of DP-receptor agonists and antagonists

PGD_2, like PGE_2, produced a biphasic response which consisted of a dose-independent contraction followed by a dose-dependent inhibition (Figure 4a). Since PGD_2 was a much weaker inhibitory agent than PGE_2 a reliable ID_4 value could not be obtained because individual ID_4 values for PGD_2 were not within the 95% c.l. from the mean, nor could an inhibition of 4 min always be obtained. In contrast the selective DP-receptor agonist, BW245C, elicited a potent inhibition of myometrial activity (Figure 4b, Table 1).

The effect of the selective DP-receptor antagonist, BW A868C, was tested against both PGD_2 and BW245C. At a concentration of 10^{-7} M, BW A686C appeared to abolish the weak inhibition previously seen with PGD_2 (Figure 4a). It was also noted that the PGD_2-mediated excitatory event was potentiated (not significantly) in the presence of the antagonist. At increasing concentrations of the antagonist (10^{-8}, 10^{-7} and 10^{-6} M) a parallel rightward displacement of the dose-effect curve to BW245C was seen (Figure 4b). The mean data from Schild analysis for each experiment is shown below:

Schild regression parameter	*Value*
pA_2 (range)	8.6 (8.3–8.9)
Slope (range)	0.86 (0.68–1.07)
Regression coefficient (range)	0.99 (0.97–1.0)

All values are the geometric mean of $n = 5$ determinations.

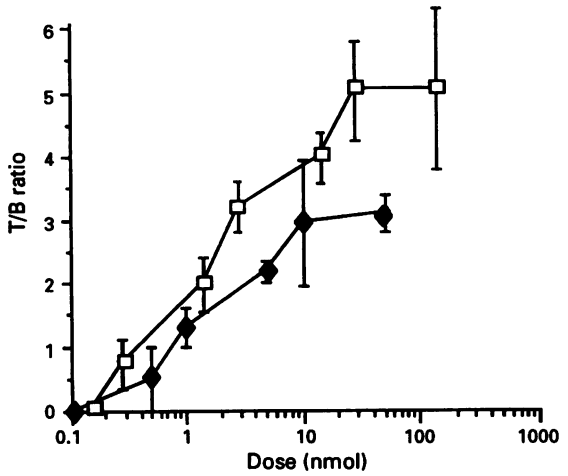
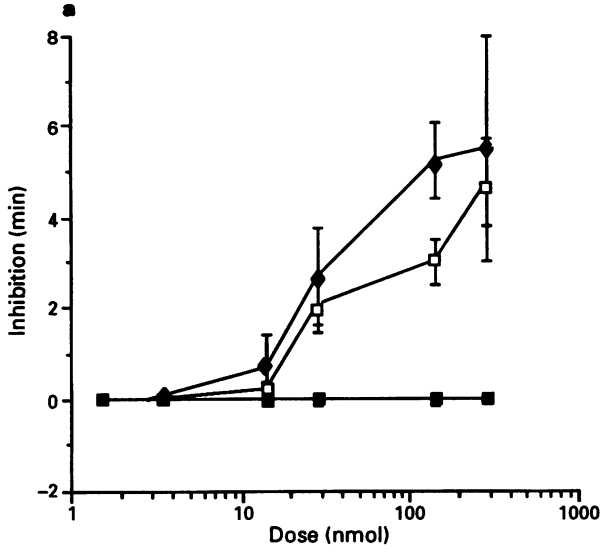


Figure 3 Mean stimulatory dose-effect curves to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, □) $n = 6$ and fluprostenol (◆) $n = 5$. Data are expressed as arithmetic means and vertical bars represent s.e.mean.



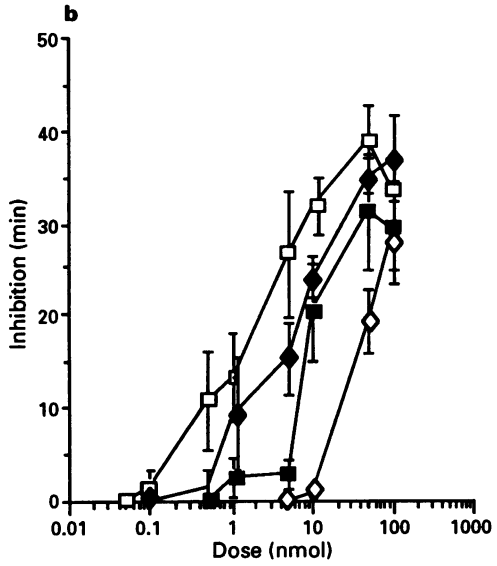


Figure 4 (a) Mean inhibitory dose-effect curves to prostaglandin D_2 (PGD_2) alone (□) and in the presence of BW A868C 10^{-8} M (◆) and 10^{-7} M (■). Data are expressed as arithmetic means of 6 determinations and vertical bars represent s.e.mean. (b) Mean inhibitory dose-effect curves to BW245C alone (□) and in the presence of BW A868C 10^{-8} M (◆), 10^{-7} M (■) and 10^{-6} M (◇). Data are expressed as arithmetic means of 5 determinations and vertical bars represent s.e.mean.

The effect of IP-receptor agonists

The natural IP-receptor agonist, PGI_2, produced a biphasic response which consisted of a none dose-related stimulation followed by a dose-related inhibition of activity (Figure 5, Table 1). The EP_1/IP-receptor agonist, iloprost, evoked an inhibitory response (Figure 5) but at the two highest doses (13.9 and 70 nmol) a biphasic response was observed. The selective IP-receptor agonist, cicaprost, was purely inhibitory in nature at all doses tested (Figure 5).

The effect of the TP-receptor agonist U46619

Responses to the synthetic TP-receptor agonist, U46619, were dose-related and like $PGF_{2\alpha}$ were only excitatory (Figure 6, Table 1). The U46619 responses were tested against the TP-receptor antagonist, GR32191: increasing the

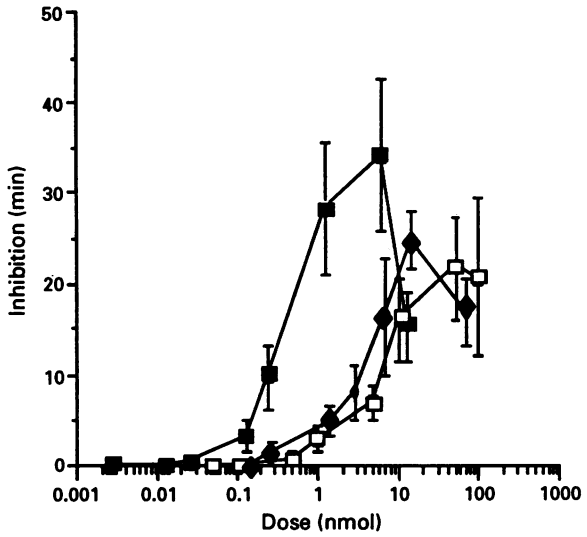


Figure 5 Mean inhibitory dose-effect curves to prostacyclin (PGI_2, □) $n = 9$, iloprost (◆) $n = 9$ and cicaprost (■) $n = 9$. Data are expressed as arithmetic means and vertical bars represent s.e.mean.

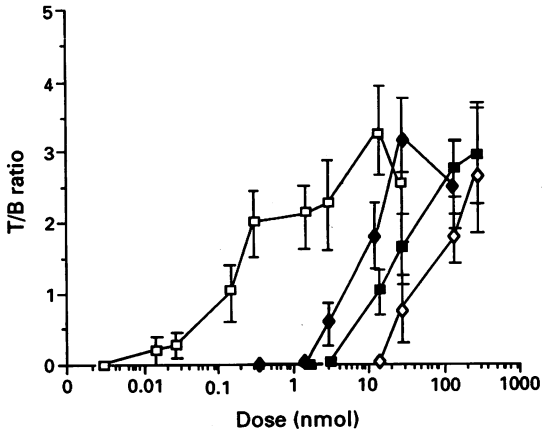


Figure 6 Mean stimulatory dose-effect curves to U46619 alone (□) and in the presence of GR32191 10^{-7} M (◆), 3×10^{-7} M (■) and 10^{-6} M (◇). Data are expressed as arithmetic means of 7 determinations and vertical bars represent s.e.mean.

concentration of the antagonist (10^{-7}, 3×10^{-7} and 10^{-6} M) caused a parallel rightward displacement of the dose-effect curve. The mean data from Schild analysis for each experiment is shown below:

Schild regression parameter	*Value*
pA_2 (range)	7.1 (7.0–7.3)
Slope (range)	0.93 (0.6–1.02)
Regression coefficient (range)	0.91 (0.97–1.0)

All values are the geometric mean of $n = 7$ determinations.

Discussion

All the prostanoids tested, both natural and synthetic, showed some activity on myometrial strips from pregnant donors at term (Table 1). The biphasic response evoked by PGE_2 indicated that both contractile and inhibitory EP-receptors could be present, hence the need to employ more specific synthetic agonists in this investigation. To date there are many EP-receptor agonists but few of them show absolute selectivity for the individual EP_1-, EP_2- and EP_3-receptor subtypes.

Sulprostone, which acts via EP_1- and EP_3-receptors, produced a purely contractile effect and is a potent agonist, measured in terms of the ED_1 value and the T/B ratio it elicited. In order to investigate further the receptor subtype involved in mediating this contractile response, the competitive EP_1-receptor antagonist, AH6809 (Coleman *et al.*, 1985) was used but this EP_1-receptor antagonist had no effect on the dose-response curve to sulprostone, thereby suggesting a paucity of EP_1-receptors on human myometrium at term. The present evidence suggests that the contractions evoked by sulprostone are mediated via the EP_3-receptor.

Misoprostol, an EP_1/EP_3-receptor agonist, evoked a biphasic response consisting of a contraction at lower doses and an inhibition at higher doses. This prostanoid was the most potent contractile agonist tested in terms of ED_1 values, thus providing further evidence for the presence of EP_3-receptors. Misoprostol was produced by chemical modification of PGE_1 (Collins *et al.*, 1985) and in the rat has been shown to be approximately four times more potent than PGE_2 in terms of inhibition of gastric acid secretion (EP_3-receptor-mediated, Reeves *et al.*, 1988).

The spasmolytic effects produced by the prostanoids, PGE_2, rioprostil and misoprostol, provide evidence for the presence of inhibitory EP_2-receptors. More conclusive support for this, however, is obtained by the use of butaprost. This is the most selective EP_2-receptor agonist used to date, having no apparent activity at EP_1-, EP_3-, DP-, FP-, IP- or TP-receptors (Gardiner, 1986) although Lawrence & Jones (1992) noted a contractile response on chick ileum but were unable to identify the receptor. In the present study, butaprost produced a maximum inhibition of myometrial activity of 92.5 min, although in terms of the ID_4 value, butaprost was of similar potency to the inhibitory prostanoids, BW 245C and cicaprost (Table 1). However, the different maxima obtained for these agonists may reflect differences in bioavailability.

In the light of current knowledge we postulate that myometrium taken from pregnant subjects at term contains two subtypes of EP-receptors. Firstly, the EP_3 subtype which initiates contraction and is activated at the lower end of the dose-range to PGE_2 and secondly the EP_2 subtype which promotes relaxation and is activated by higher doses of PGE_2. When these results are compared with those obtained on the myometrium from non-pregnant donors (Senior *et al.*, 1991) it can be seen that the excitatory EP-mediated events are less pronounced on myometrium from pregnant than non-pregnant donors. Sulprostone is ten times less potent on myometrium from pregnant donors and neither PGE_2 nor rioprostil show any quantifiable excitatory responses (Table 1). These differences may be attributed to the different regions of the uterus used in the two studies; for ethical reasons it is not possible to obtain tissue from the upper segment of the uterus in the gravid patient. Alternatively these observations may represent a true decrease in EP-receptor-mediated excitatory events in the myometrium before parturition. The ED_1 value obtained from $PGF_{2\alpha}$, which always produces a dose-related increase in uterine activity, showed that $PGF_{2\alpha}$ is a potent excitatory agonist while the synthetic analogue, fluprostenol, which has a similar profile of action indicated that specific FP-receptor sites are present.

PGD_2, although less potent than PGE_2, produced a qualitatively similar response. The low potency of PGD_2 raises the question, however, whether this prostanoid actually acts through a DP-receptor to produce a biphasic effect. To investigate this biphasic response the selective DP-receptor antagonist, BW A868C (10^{-7} M), (Giles *et al.*, 1989) was used. At this dose, BW A868C (lower doses were ineffective) antagonized only the weak inhibitory PGD_2 response. Although, PGD_2 was the least potent of all the inhibitory prostanoids, this antagonism of PGD_2 suggests that there are DP-receptors in the at term myometrium. Because of the low potency of PGD_2, a more selective synthetic analogue, BW245C (Town *et al.*, 1983) was used to confirm this finding. On at term myometrium, BW245C did not, however, produce any stimulation but was a potent inhibitory agonist, this effect being competitively antagonized by BWA868C. Therefore, the inhibitory effect seen with PGD_2 and BW245C on human myometrium at term suggests that this is the true DP-receptor response.

The weak excitatory event exhibited by PGD_2 may be via the EP_3- or FP-stimulatory receptors since use of a thromboxane receptor antagonist, GR32191, failed to modify the PGD_2 response. As the EP_3-receptor stimulants are less active on myometrium from pregnant subjects than from non-pregnant donors this could explain the weak excitatory response to PGD_2 but it should also be noted that the ED_1 for $PGF_{2\alpha}$ was ten times greater on the myometrium from pregnant subjects than from non-pregnant donors (Senior *et al.*, 1992).

PGI_2 displayed a response which consisted of a dose-related inhibition of activity preceded by a contraction apparently unrelated to dose. PGI_2, as an inhibitory agonist, was approximately 4–5 times less potent than PGE_2 on the pregnant myometrium. The EP_1-/IP-receptor agonist, iloprost, also produced a dose-related inhibition of myogenic activity with an occasional contraction observed at the highest doses. The inhibitory dose-effect curve from iloprost had a bell shaped appearance (Figure 5), which may be due to the inhibitory response becoming biphasic as the dose was increased. Iloprost has been shown to be as potent as PGI_2 at the IP-receptor but it also has high potency at the EP_1-receptor, for example, in guinea-pig fundus (an EP_1-receptor containing preparation) it is equipotent with PGE_2 and twenty times more potent than PGI_2 (Coleman, 1987); this effect is antagonized by SC19220 and AH6809 (Dong *et al.*, 1986; Sheldrick *et al.*, 1988). The paucity of EP_1-receptors on the myometrium from pregnant donors renders the excitatory effect of iloprost unpredictable on this tissue. This is unlike the result from non-pregnant tissue where iloprost gave a consistent, dose-related contractile response which was antagonized by AH6809. Because of the unpredictability of the contractile iloprost response on tissue from pregnant donors the effect could not be investigated in the presence of AH6809.

Cicaprost was the most potent inhibitory agonist, in terms of ID_4 value, on the pregnant human myometrium *in vitro*. The dose-effect response curve to cicaprost also had a bell-shaped appearance similar to iloprost. Early studies showed cicaprost to be only weakly active at EP_1-receptors (Dong *et al.*, 1986), thus the bell-shaped curve obtained from cicaprost in this study may represent an activity at other contractile prostanoid receptors as suggested for PGD_2.

The contraction seen with PGI_2, as discussed previously with PGD_2, is unlikely to be mediated via EP_1- or TP-receptors. Therefore, this effect may occur via EP_3- or FP-receptors. PGI_2 is 300 times less potent than $PGF_{2\alpha}$ at the FP-receptor but only 50 times less potent than PGE_2 at EP-contractile receptors (Kennedy *et al.*, 1982). Until more antagonists become available no firm conclusions can be drawn regarding the contractile effect observed with PGI_2. Sangha (1991), showed that the PGI_2-induced stimulation was not abolished by morphone (10^{-6} M) which eliminates the possibility of an indirect action on neuronal mechanisms (similarly, the contractions observed with PGE_2 and PGD_2 were unaffected by morphine).

The observations made with PGI_2, iloprost and cicaprost suggest that IP-receptors mediating relaxation exist in the pregnant human myometrium *in vitro*. The inhibitory effects of PGI_2, iloprost and cicaprost were similar to, but of longer duration than, those obtained on myometrial tissue taken from non-pregnant donors (Senior *et al.*, 1991; 1992), despite the fact that the myometrium was obtained from different sites on the uterus.

The stable thromboxane-mimetic, U46619, caused excitation and was of similar potency to sulprostone. U46619 is at least ten times more potent at TP-receptors than at the four

other prostanoid receptors (Coleman, 1987); thus its high potency suggests that TP-receptors are present on human myometrial tissue from pregnant donors. The action of U46619 was antagonized by the selective TP-receptor antagonist, GR32191, which further supports this proposal. Again the potency of U46619 and the pA_2 value for GR32191 in this study are similar to the results found on non-pregnant myometrium, suggesting that the site of myometrium excision may not be so important in terms of TP-receptors for these *in vitro* functional studies.

In conclusion, the data presented in this study provide evidence for the existence of a heterogeneous population of prostanoid receptors in human myometrium from term pregnancy. The excitation may be EP_3-, FP- and TP-receptor-mediated and inhibition through EP_2-, DP- and IP-receptors. These results are qualitatively similar to those found on non-pregnant myometrium; quantitatively the excitation mediated via EP_3-receptors appears to be less pronounced in the lower segment myometrial strips at term and there is a paucity of EP_1 receptors. This could be related to the functional stability of the lower segment prior to parturition, an effect enhanced by the pronounced inhibitory action seen using the compounds affecting the relaxant EP_2, DP and IP receptors. The potent inhibitory action of some synthetic prostaglandin analogues at these receptors could be useful in clinical hyperactivity states such as pre-term labour.

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Investigations into factors determining the duration of action of the β_2-adrenoceptor agonist, salmeterol

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1 This study has explored the mechanism underlying the long duration of action of the β_2-adrenoceptor agonist, salmeterol.

2 Salmeterol, salbutamol and isoprenaline caused a concentration-related inhibition of electrically-induced contractile responses of the guinea-pig superfused trachea preparation. The effects of both isoprenaline and salbutamol were rapid in onset and rapidly reversed upon removal of the agonist. In contrast, the effects of salmeterol were slower in onset and could not be reversed by superfusion of the tissue with agonist-free Krebs solution even for periods of up to 10 h.

3 The effects of salmeterol were, however, readily reversed by a number of β-adrenoceptor blocking drugs, as was the effect of a continuous infusion of isoprenaline. Upon removal of the antagonist, however, the effects of salmeterol and of the isoprenaline infusion were reasserted at a rate which was inversely related to the lipophilicity of a β-adrenoceptor blocking drugs.

4 Salmeterol inhibited the binding of [^{125}I]-(−)-iodopindolol (100 pM) to rat lung membranes (pIC_{50} 7.1), with isoprenaline (pIC_{50} 6.2) and salbutamol (pIC_{50} 5.1) having lower potencies. The inhibition of binding by salmeterol was apparently non-competitive, whereas that produced by salbutamol and isoprenaline was competitive in nature.

5 Isoprenaline and salbutamol rapidly dissociated from their binding sites, whereas in marked contrast, the binding of salmeterol showed no dissociation for periods of up to 1 h.

6 These data are consistent with a mechanism in which salmeterol binds adjacent to the active site of the β_2-adrenoceptor, such that the drug cannot be washed out of the tissue, yet can interact with and activate the receptor. This latter property is susceptible to antagonism by β-adrenoceptor blocking drugs but is reasserted when the antagonists are removed.

Keywords: β-Adrenoceptor agonists; β-adrenoceptor antagonists; salmeterol; guinea-pig trachea; rat lung membrane; duration of action; exosite

Introduction

Salmeterol is a potent and selective, long-acting β_2-adrenoceptor agonist both *in vitro* and *in vivo* (Ball *et al.*, 1987a, b; 1991; Bradshaw *et al.*, 1987; Johnson, 1990). Moreover, clinical studies have shown that, following aerosol administration, salmeterol is also a long-acting bronchodilator in man (Ullman & Svedmyr, 1988; Maconochie *et al.*, 1987) and is effective in the treatment of bronchial asthma (Britton, 1990; Palmer, 1990).

We have previously suggested that the remarkably sustained β_2-adrenoceptor agonist activity of salmeterol is due to binding to an 'exosite' rather than to an irreversible interaction with the β_2-adrenoceptor itself (Bradshaw *et al.*, 1987; Nials & Coleman, 1988; Jack, 1991). This mechanism involves an association of the lipophilic N-substituent phenylalkyloxyalkyl 'tail' of salmeterol with an exosite which holds the molecule in such a position that the phenylethanolamine 'head' can repeatedly interact with, and activate, the β_2-adrenoceptor. This proposal was based mainly upon two observations. Firstly, the long-lasting effects of salmeterol can be competitively antagonized by propranolol (Ball *et al.*, 1987a). Secondly, the smooth muscle relaxant activity of salmeterol, which can be reversed by the β-adrenoceptor antagonist, sotalol, reasserts itself when the sotalol is removed (Ball *et al.*, 1991), implying some form of specific retention of salmeterol adjacent to the active site of the β_2-adrenoceptor.

In this present study, the interaction of salmeterol with β_2-adrenoceptors has been explored further. Specifically, we have investigated the effects of a number of β-adrenoceptor antagonists on the rates of reversal and reassertion of salmeterol-induced smooth muscle relaxation in a superfused, electrically-stimulated guinea-pig trachea preparation. In addition, we have also investigated the interaction of salmeterol with rat lung β_2-adrenoceptors using a radioligand binding assay.

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Methods

Preparation of isolated tracheal strip

Tracheal strips were prepared as described by Coleman & Nials (1989). Briefly, adult, male guinea-pigs (400–550 g) were killed by a blow to the head with subsequent exsanguination. The tracheae were excised and placed in Krebs solution in a Petri dish. The adherent connective tissue was dissected away and the lumen gently flushed with Krebs solution. Tracheae were dissected into rings containing 3–4 cartilage bands (Castillo & DeBeer, 1947; Akcasu, 1959) and the rings opened to form strips by cutting through the cartilage on the side opposite to the smooth muscle band (Coburn & Tomita, 1973). A long cotton thread was attached to the cartilage at one end of the strip for attachment to a strain gauge, and a cotton loop to the other end for anchoring the tissue in the superfusion chamber.

Tissue superfusion

The superfusion apparatus employed in these experiments has been described previously (Coleman *et al.*, 1986; Coleman & Nials, 1989). Preparations were mounted under a

resting tension of 1 g, and superfused at a rate of 2 ml min^{-1} with oxygenated (5% CO_2 in O_2) modified Krebs solution (Apperley *et al.*, 1976) maintained at 37°C. Bipolar platinum electrodes were positioned parallel with and in close proximity to, the superfused tissue.

Determination of agonist activity on electrically-stimulated guinea-pig trachea

For experiments involving electrical stimulation, preparations were taken from guinea-pigs pretreated with 6-hydroxydopamine (200 mg kg^{-1}, i.p.), in order to eliminate any adrenergic component in the electrically-induced response (Coleman *et al.*, 1986; Coleman & Nials, 1989). To measure relaxant activity in the guinea-pig trachea, phasic contractile responses were induced by electrical field stimulation with 10 s trains of square wave pulses of 5 Hz frequency, 0.1 ms duration and just maximal voltage (8–16 V) every 2 min. These electrically-induced responses were highly reproducible for periods of at least 8–10 h of stimulation.

For each preparation, constant responses to the standard agonist, isoprenaline, were first obtained by infusing increasing concentrations in a sequential manner, until sensitivity was constant. A 30 min equilibration period was allowed before a concentration-effect curve to a test agonist was started. The magnitude of each response was measured and calculated as a percentage inhibition of the electrically-induced contractile response.

Potency values for the β-adrenoceptor agonists were expressed in both absolute terms (concentration required to induce 50% inhibition, EC_{50}) and relative to isoprenaline, as an equipotent concentration (EPC, i.e. EC_{50} for the test agonist/EC_{50} for isoprenaline).

Determination of rate of onset of action

The time for onset of action (Ot_{50}) is defined as the time from starting the administration of the test agonist to attainment of 50% of the response maximum for an EC_{50} concentration. Ot_{50} values were determined by interpolation from a plot of % response against time to attainment of 50% of each response maximum.

Determination of rate of offset of action

The time for offset of action (Rt_{50}) is defined as the time from stopping administration of the test agonist to attainment of 50% recovery from an EC_{50} concentration. Rt_{50} values were determined by interpolation from a plot of % response against time to 50% recovery of each response (Rt_{50}).

Interaction of β-adrenoceptor antagonists with salmeterol and isoprenaline

Electrically-induced contractile responses were inhibited by infusion of salmeterol (10–100 nM), and when the inhibitory responses had equilibrated, the infusion was stopped. In control preparations, an infusion of isoprenaline (30–100 nM) was continued throughout the experiment. Some of the preparations were left with no further treatment for the duration of the experiment in order to determine whether any spontaneous recovery from the inhibitory responses of salmeterol and isoprenaline occurred. On other preparations, a β-adrenoceptor blocking drug was infused. When the antagonism of the β-adrenoceptor agonist effect had equilibrated, the antagonist infusion was halted, and the tissue superfused with an antagonist-free Krebs solution. A range of seven β-adrenoceptor blocking drugs of varying potency, lipophilicity and β_1/β_2 adrenoceptor selectivity were used: sotalol, propranolol, atenolol, ICI 118551, labetalol, pindolol and timolol. Each β-adrenoceptor blocking drug was tested at a single concentration which approximated to its pA_{100} (i.e. $\sim 100 \times pA_2$) at β_2-adrenoceptors. For the β_1-adrenoceptor selective antagonist, atenolol, this was not possible due to its low β_2-adrenoceptor antagonist potency, and therefore an approximate pA_{10} concentration was used. We attempted to identify on each preparation a concentration of salmeterol or isoprenaline which caused 70–90% inhibition of electrically-induced contractions. On each preparation, the maximum degree of reversal of the agonist-induced inhibition, the rate of reversal, and the rate at which the agonist effect reasserted itself once the antagonist infusion had been halted (Ra_{50} the time taken for the agonist to reassert to 50% of its pre-antagonist level) were measured.

Radioligand binding assays

The binding of the radioligand [^{125}I]-(−)-iodopindolol ([^{125}I]-PIN) to rat lung membrane preparations was determined by an assay based upon that described by Barovsky & Brooker (1980). [^{125}I]-PIN was either synthesized from (-)-pindolol and Na^{125}I using the chloramine-T oxidation method (Barovsky & Brooker, 1980) or was purchased.

Membrane preparation

All procedures were performed on ice or at 4°C. Lungs were removed from female Wistar rats (200 g, killed by cervical dislocation), scissor-minced and homogenized (3 × 5 s bursts, Polytron homogenizer) in 10 volumes of ice-cold buffer (50 mM Tris-HCl, pH 7.5). The homogenate was centrifuged at 800 *g* for 10 min and the pellet discarded. The supernatant fraction was recentrifuged at 40,000 *g* for 20 min to produce a crude membrane pellet. This was washed by resuspension in ice-cold assay buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mg ml^{-1} L-ascorbic acid, pH 7.8) using a loose-fitting glass Dounce homogenizer (Jencons), and recentrifuged at 70,000 *g* for 20 min. The washed membrane pellet was resuspended in ice-cold assay buffer and the protein content, determined by the method of Lowry *et al.* (1951), was adjusted to 1 mg ml^{-1} with assay buffer. This suspension was stored as aliquots at − 60°C for periods of up to 1 month, and was thawed for use as required.

Binding assay

Binding assays were performed in triplicate. Assay mixtures (final volume 0.2 ml), comprising [^{125}I]-PIN (0–400 pM), guanosine-5'-triphosphate (GTP, 100 μM), membrane suspension (50 μg protein) and drugs where appropriate, all prepared in assay buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mg ml^{-1} L-ascorbic acid, pH 7.8), were incubated for 30 min at 37°C. Non-specific [^{125}I]-PIN binding was defined as that occurring in the presence of 200 μM (-)-isoprenaline, and constituted ~5% of total binding at a radioligand concentration of 100 pM. Assays were terminated by dilution with ice-cold assay buffer followed by rapid vacuum filtration through glass fibre filters (Whatman GF/B) mounted in a 12-place manifold (Millipore). Assay tubes and filters were washed with ice-cold assay buffer and, when dry, filters were counted in an LKB gamma counter to determine the amount of membrane-bound radioactivity trapped in the filter.

For experiments examining the reversibility of β_2-adrenoceptor agonist binding, aliquots of rat lung membranes (2 ml of a 1 mg ml^{-1} suspension) were pretreated for 30 min at 37°C with assay buffer (controls), salmeterol (10 μM), salbutamol (300 μM) or isoprenaline (100 μM); all solutions were prepared with assay buffer containing 100 μM GTP. The membrane suspensions were then centrifuged for 2 min at 12,000 *g* and the supernatant fractions discarded. The pellets were surface rinsed and resuspended in assay buffer (containing 100 μM GTP) to a membrane protein concentration of 1 mg ml^{-1}. Each pretreated membrane suspension was then divided into two samples which were further diluted (1:4) with assay buffer containing [^{125}I]-PIN (final concentration

Table 1 Superfused, electrically-stimulated guinea-pig trachea: potencies and durations of action of isoprenaline, salbutamol and salmeterol

Agonist	EC_{50} (nM)	*EPC* (Isopren = 1)	*Onset* (Ot_{50}, min)	*Duration* (Rt_{50}, min)	n
Isoprenaline	15.4 (9.3–25.5)	1	<2	>2 <4	8
Salbutamol	40.2 (8.0–208)	3.9 (1.9–8.1)	2.8 (1.9–5.3)	6.4 (3.1–10.7)	5
Salmeterol	4.8 (2.5–9.1)	0.3 (0.26–0.47)	27.6 (21.8–33.7)	>470	6

Data are geometric (EC_{50}, EPC) or arithmetic (onset, duration) means with 95% confidence limits shown in parentheses. EC_{50} is defined as the concentration of agonist required to induce 50% inhibition of the electrically-induced contractile response. Ot_{50} is defined as the time from starting the administration of the agonist to attainment of 50% of the response maximum for an EC_{50} concentration. Rt_{50} is defined as the time from stopping administration of the test agonist to attainment of 50% recovery from an EC_{50} concentration (see Methods). Isopren = isoprenaline.

100 pM) and GTP (100 μM final concentration) with or without isoprenaline (200 μM final concentration) to define non-specific binding.

This procedure results in a 250 to 300 fold dilution of the original drug solutions as estimated from experiments applying the same procedure to solutions of [^{125}I]-PIN. These mixtures were subsequently incubated at 37°C, and at timed intervals, samples (200 μl, 50 μg protein) were removed, diluted with ice-cold buffer and filtered to terminate binding. The time course for specific (i.e. total minus non-specific) [^{125}I]-PIN binding was compared for drug-treated or buffer-treated (control) membranes, and the binding to drug-treated membranes expressed as a percentage of that obtained for binding to control membranes.

Data (counts per minute) were analysed with the computer programmes LIGAND (Munson & Rodbard, 1980) and ALLFIT (DeLean *et al.*, 1978) or by the procedures detailed by Weiland & Molinoff (1981).

Drugs

The drugs used in this study were adrenaline bitartrate (Sigma, U.K.), atenolol (ICI, U.K.), erythro-DL-1 (7-methylindan-4-yloxy)-3-isopropylamino-butan-2-ol (ICI 118551, ICI, U.K.), 6-hydroxydopamine hydrobromide (Sigma, U.K.) indomethacin (Sigma, U.K.), isoprenaline sulphate (Sigma, U.K.), labetalol (Glaxo, U.K.), noradrenaline bitartrate (Sigma, U.K.), pindolol (Sandoz, Switzerland), propranolol hydrochloride (Sigma, U.K.), salbutamol (Glaxo, U.K.), salmeterol 1-hydroxy-2-naphthoate, (Glaxo, U.K.), sotalol hydrochloride (Mead Johnson, U.S.A.), timolol maleate (Merck, U.S.A.).

Sodium [^{125}I]-iodide and [^{125}I]-(−)-iodopindolol were obtained from Amersham International and New England Nuclear respectively.

For the functional studies, isoprenaline, salbutamol and salmeterol were dissolved in a small volume (40 μl) of glacial acetic acid and made up to final volume with phosphate buffer (pH 7.0). Indomethacin was dissolved in 10% (w/v) $NaHCO_3$ and made up to volume with 0.9% (w/v) saline. All other compounds were dissolved in distilled water. Drug dilutions were made up to volume in 0.9% saline. Solutions of the β-adrenoceptor agonists contained ascorbic acid (11 mM). For the binding studies, salmeterol was dissolved in 10% final volume of 1 M acetic acid and diluted in assay buffer to volume. All other drugs were dissolved and diluted in assay buffer.

Results

Potency and duration of action of isoprenaline, salbutamol and salmeterol

All three β-adrenoceptor agonists caused a concentration-related inhibition of electrically-induced contractile responses of the guinea-pig superfused trachea. The results are summarized in Table 1. Salmeterol was approximately 3 fold more potent than isoprenaline, and approximately 12 fold more potent than salbutamol. Marked differences were observed in the rates of onset and offset of action of the agonists (Table 1). Thus, while the onset of action of both

Table 2 Superfused, electrically-stimulated guinea-pig trachea: interaction of a range of β-adrenoceptor blocking drugs with the inhibitory effects of salmeterol (persisting after stopping salmeterol administration) and isoprenaline (continuous infusion)

		vs *Salmeterol* (10–30 nM)				vs *Isoprenaline* (10–100 nM)			
Antagonist	*Conc* (μM)	*Agonist response* (% inhibition of electrically-induced contractile response)	*Antagonist-induced reversal* (% max)	*Onset of antagonist activity* (Ot_{50}, min)	*Agonist reassertion time* (Ra_{50}, min)	*Agonist response* (% inhibition of electrically-induced contractile response)	*Antagonist-induced reversal* (% max)	*Onset of antagonist activity* (Ot_{50}, min)	*Agonist reassertion time* (Ra_{50}, min)
Atenolol	10	85.2 (± 8.7)	47.3 (± 8.0)	2.9 (± 0.4)	4.7 (± 0.9)	93.1 (± 3.1)	77.8 (± 6.9)	5.0 (± 1.3)	10.0 (± 2.2)
Sotalol	10	75.7 (± 9.5)	95.8 (± 2.7)	6.6 (± 0.7)	21.8 (± 3.1)	85.9 (± 2.2)	88.5 (± 3.2)	6.7 (± 1.2)	18.4 (± 3.2)
Pindolol	0.1	69.2 (± 5.2)	86.1 (± 3.8)	10.3 (± 0.6)	53.6 (± 10.2)	89.1 (± 4.2)	93.8 (± 4.1)	3.8 (± 0.4)	47.0 (± 7.9)
Labetalol	0.1	83.7 (± 9.3)	71.2 (± 12.5)	4.6 (± 0.8)	81.7 (± 18.5)	71.9 (± 5.8)	67.4 (± 6.9)	4.4 (± 0.8)	81.5 (± 23.0)
Timolol	0.1	81.6 (± 4.6)	87.9 (± 4.4)	5.2 (± 0.6)	136.4 (± 22.8)	85.8 (± 4.0)	94.3 (± 2.9)	5.0 (± 0.4)	113.5 (± 30.7)
Propranolol	0.1	83.1 (± 4.9)	83.5 (± 4.1)	4.2 (± 1.1)	220.1 (± 39.9)	92.9 (± 2.1)	93.2 (± 2.2)	11.0 (± 2.5)	180.6 (± 18.5)
ICI 118551	0.1	87.2 (± 3.7)	87.3 (± 3.7)	3.4 (± 0.7)	155 ->886	85.7 (± 3.2)	68.8 (± 5.4	2.9 (± 0.3)	179.6 (± 21.9)

Results are arithmetic means ± s.e.mean in parentheses of at least 4 experiments.

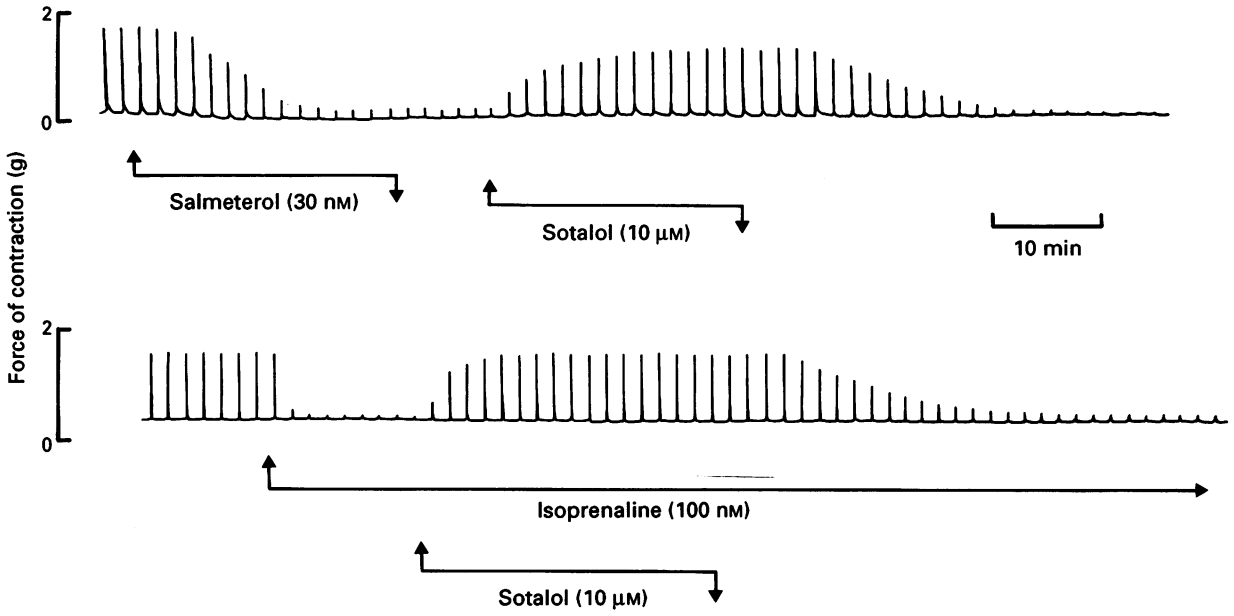


Figure 1 Superfused, electrically-stimulated guinea-pig trachea: typical experiments illustrating the effects of administration and subsequent withdrawal of sotalol (10 μM) on the inhibitory responses to salmeterol (persisting after salmeterol withdrawal) and isoprenaline (continuous infusion).

isoprenaline and salbutamol was rapid ($Ot_{50} < 3$ min), that of salmeterol was slower ($Ot_{50} = 27.6$ min). Isoprenaline and salbutamol were also both short-acting, ($Rt_{50} < 7$ min); however, no recovery was observed from responses to salmeterol even after periods in excess of 8 h, despite continuous superfusion of the tissue with agonist-free Krebs solution.

Interaction of a range of β-adrenoceptor antagonists with salmeterol and isoprenaline

On electrically-stimulated guinea-pig trachea preparations, infusions of sotalol and atenolol (10 μM), and propranolol, ICI 118551, labetalol, pindolol and timolol (0.1 μM) reversed

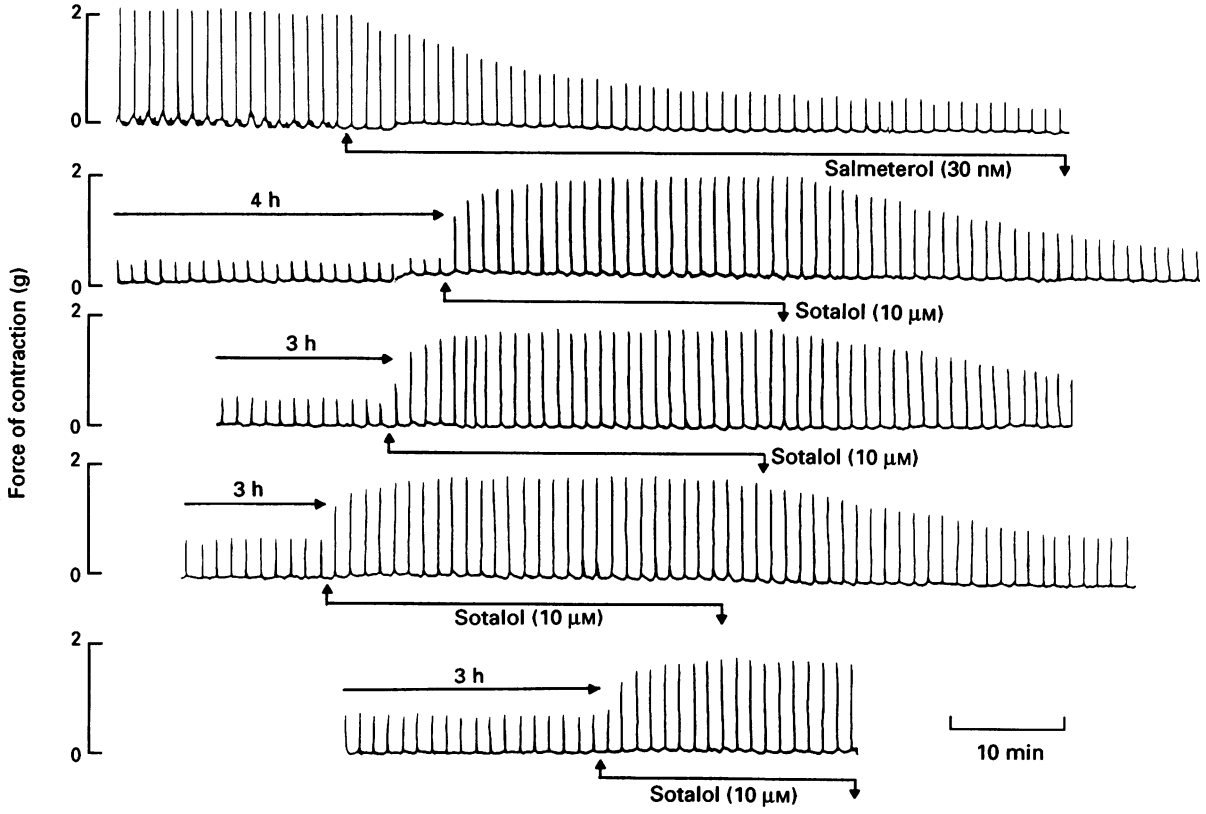
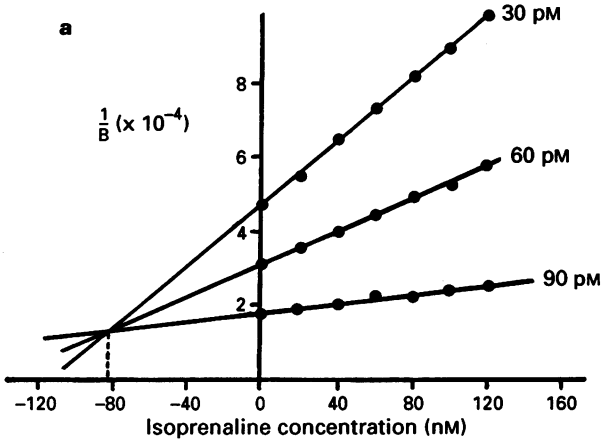
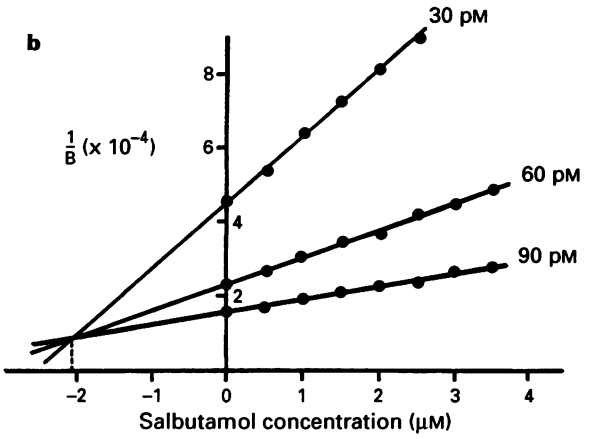


Figure 2 Superfused, electrically-stimulated guinea-pig trachea: repeated administration and subsequent withdrawal of sotalol (10 μM) on the inhibitory responses to salmeterol (persisting after salmeterol withdrawal).





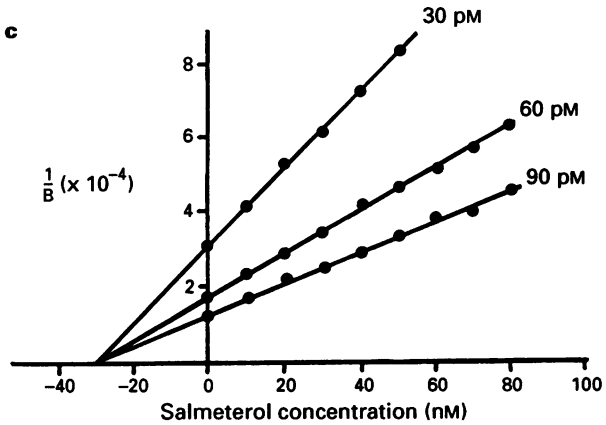


Figure 3 Rat lung membranes: Dixon plots for the inhibition of specific [^{125}I]-(-)-pindolol ([^{125}I]-PIN) binding (B) to rat lung β_2-adrenoceptors by (a) isoprenaline, (b) salbutamol and (c) salmeterol. Inhibition curves were constructed at three radioligand concentrations, 30 pM, 60 pM and 90 pM, and the data plotted according to the method of Dixon (Boeynaems & Dumont, 1980). Note that the profiles of isoprenaline and salbutamol are that of a competitive inhibitor, whereas the inhibition produced by salmeterol is apparently non-competitive. Results are from a single representative experiment which was repeated on at least 3 occasions.

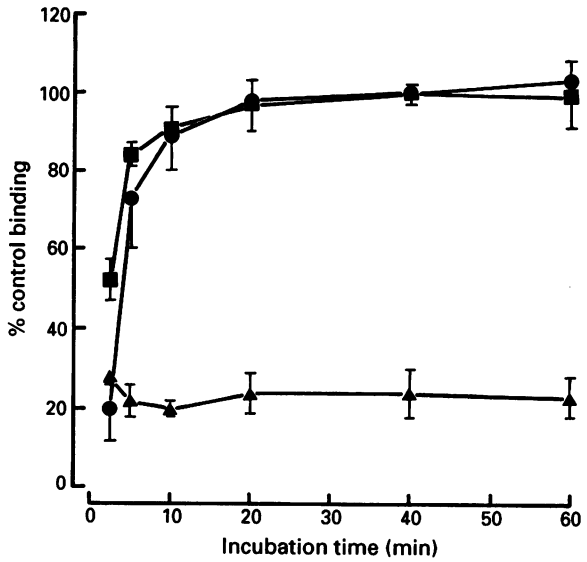


Figure 4 Rat lung membranes: dissociation of β_2-adrenoceptor agonists from rat lung membrane β_2-adrenoceptors. Rat lung membranes were pretreated for 30 min at 37°C with assay buffer (controls), isoprenaline (100 μM, ●), salbutamol (300 μM, ■) or salmeterol (10 μM, ▲) and then diluted 250 to 300 fold with buffer containing [^{125}I]-(-)-pindolol (100 pM) to induce drug dissociation. The time course of radioligand binding was followed and compared in drug-treated and buffer-treated (control) membranes. Results are means ± s.e.mean (vertical bars) of at least 3 separate determinations.

both the persistent salmeterol-induced inhibition and the inhibition resulting from a continuous isoprenaline infusion. The rate of reversal of both salmeterol and isoprenaline-responses by each antagonist was rapid, the time to 50% reversal of agonist activity being 11 min or less (Table 2). With the exception of atenolol and ICI 118551 against salmeterol, and isoprenaline respectively, at least 80% reversal was obtained (Table 2). When the response to the β-adrenoceptor blocking drug had equilibrated, the infusion of the antagonist was halted. In every case, the salmeterol-induced inhibition, or the inhibition resulting from the continuous isoprenaline infusion was reasserted (e.g. Figure 1). However, the rates at which the reassertion of agonist

Table 3 Rat lung membranes: inhibition of [^{125}I]-(-)-pindolol binding to β_2-adrenoceptors

Inhibitor	*pIC_{50}*	*Slope*	n
Agonists			
Salmeterol	7.1 ± 0.1	0.93 ± 0.05	9
Isoprenaline	6.2 ± 0.1	0.78 ± 0.02*	8
Adrenaline	5.8 ± 0.1	0.71 ± 0.15*	3
Salbutamol	5.1 ± 0.2	0.95 ± 0.05	4
Noradrenaline	4.8 ± 0.1	0.70 ± 0.12*	3
Antagonists			
Propranolol	8.7 ± 0.1	0.92 ± 0.02	3
(-)-Pindolol	8.6 ± 0.1	0.96 ± 0.12	3
ICI 118551	8.4 ± 0.2	0.91 ± 0.06	3
Atenolol	4.9 ± 0.1	0.93 ± 0.10	3

Rat lung membranes (50 μg protein) were incubated for 30 min at 37°C with [^{125}I]-PIN (100 pM) and drugs (0.1 nM–0.1 mM) and the IC_{50} and slope of the concentration-effect curve for inhibition of specific [^{125}I]-PIN binding determined. The pIC_{50} value is the negative $\log_{10}$ of the IC_{50} value. Results are means ± s.e.mean of *n* determinations. Significantly different from unity: *$P < 0.05$.

activity occurred differed markedly with the various antagonists (Table 2). Thus, following atenolol, Ra_{50} values of ~5 and 10 min against salmeterol and isoprenaline respectively were obtained. In contrast, with ICI 118551, the corresponding values were at least 2.5 and 4 h. Despite the differences in the rates of agonist reassertion following exposure to the different antagonists, in each case, the rates were of a similar order for both salmeterol and isoprenaline. Thus, the rank order of reassertion rate for salmeterol (persistent) and isoprenaline (continued infusion) responses was: atenolol > sotalol > pindolol > labetalol > timolol > propranolol > ICI 118551.

Repeated blockade and reassertion with sotalol

In one series of experiments, after full reassertion of the response to salmeterol following sotalol (10 μM)-treatment, sotalol was re-administered and the salmeterol response inhibited once again. When this antagonism had equilibrated, the sotalol infusion was stopped a second time and the salmeterol response was again fully reasserted. This process was repeated four times over a period of approximately 15 h, after which the final salmeterol response was little changed from the original response before the first sotalol infusion (see Figure 2).

Binding studies

The radioligand, [^{125}I]-PIN, bound with a high affinity (equilibrium dissociation constant, $K_d = 67 \pm 2$ pM) to a non-interacting (Hill coefficient 1.03 ± 0.05) population of sites (density 111 ± 13 fmol mg^{-1} protein, $n = 3$). This binding was rapid, equilibrating within 30 min at 37°C (association rate constant, $k_{+1} = 2.23 \pm 0.45 \times 10^7$ M^{-1} s^{-1}) and was readily displaced by 200 μM isoprenaline (dissociation rate constant, $k_{-1} = 8.57 \pm 0.76 \times 10^{-4}$ s^{-1}). The kinetically-derived radioligand equilibrium dissociation constant (from $K_d = k_{-1}/k_{+1}$) was 38 pM, compared to the value of 67 pM obtained from saturation studies performed over the radioligand concentration range 0–400 pM. A radioligand concentration of 100 pM was employed in all subsequent experiments.

The binding of [^{125}I]-PIN (100 pM) to rat lung membranes was inhibited, with a similar maximum in each case, by a range of β-adrenoceptor agonists and antagonists (Table 3), but not by agents selective for other receptor types, including 5-hydroxytryptamine, ketanserin, mepyramine, ranitidine, phentolamine or atropine (all at 1 μM). Inhibition curves for isoprenaline, adrenaline and noradrenaline had slopes significantly less than unity (Table 3). All other compounds tested produced inhibition curves with slopes of unity. The rank order of potency of the β-adrenoceptor agonists for inhibiting [^{125}I]-PIN binding (Table 3) was salmeterol > isoprenaline > adrenaline > salbutamol ≥ noradrenaline, a profile in keeping with binding to adrenoceptors of the β_2-subtype. This was confirmed by the greater affinity for the selective β_2-adrenoceptor antagonist, ICI 118551 (Lemoine *et al.*, 1985) than for the selective β_1-antagonist, atenolol (Lemoine *et al.*, 1988) (Table 3). Since the binding of salmeterol was apparently irreversible (see below), estimated IC_{50} values were not converted to equilibrium dissociation constants, since the Cheng & Prusoff transformation (1973) applies only to competitive inhibitors. Accordingly, only IC_{50} values are quoted in Table 3. The non-competitive nature of the interaction of salmeterol with rat lung β_2-adrenoceptors is illustrated by Dixon plots (Boeynaems & Dumont, 1980) of the inhibition data for salmeterol compared with those for salbutamol and isoprenaline (Figure 3). Thus, the Dixon plots of the inhibition of [^{125}I]-PIN binding, measured at three radioligand concentrations, by isoprenaline (Figure 3a) and salbutamol (Figure 3b) clearly show intercepts above the abscissa scale, indicating competitive inhibition. In contrast, the Dixon plot for salmeterol (Figure 3c) shows an intercept on the abscissa, a profile expected for a non-competitive inhibitor (Boeynaems & Dumont, 1980).

In an attempt to investigate the reversibility of the binding of β_2-adrenoceptor agonists, rat lung membranes were pretreated with a concentration of agonist which was estimated to produce at least 80% occupation of binding sites as judged from inhibition curves with these agonists, and the dissociation of the agonist measured indirectly by measuring the association of [^{125}I]-PIN following dilution to induce drug dissociation. Using this approach, it was found that both isoprenaline and salbutamol rapidly dissociated from the membrane binding sites (Figure 4), with estimated $t_{\frac{1}{2}}$ values of 4 min and 2.5 min respectively, whereas salmeterol showed no signs of dissociation, at least over the 60 min time period examined (Figure 4).

Discussion

The results of this study confirm those previously reported on guinea-pig trachea (Ball *et al.*, 1987a,b; 1991), in that salmeterol is slightly more potent as a bronchorelaxant than isoprenaline, and approximately ten fold more potent than the 'prototype' β_2-adrenoceptor agonist, salbutamol. Furthermore, salmeterol proved to be long-acting, despite continuous washing of tissues with agonist-free medium, whereas under similar conditions, the effects of isoprenaline and salbutamol were rapidly lost.

We have previously shown that the β-adrenoceptor blocking drug, sotalol, is capable of inhibiting persistent relaxant responses to salmeterol, but that these responses are fully reasserted when the sotalol is washed from the tissue. Indeed, it proved possible to repeatedly reverse, and on each occasion to fully reassert, the effect of salmeterol on individual preparations over a 15 h period. It was results such as these which led us to propose that salmeterol is held in the vicinity of the β-adrenoceptors in such a way that it is free to interact with the receptors, but is not free to diffuse away. We further proposed the existence of 'exosite' binding to account for these observations (Bradshaw *et al.*, 1987). However, from these original studies, it was not clear whether the salmeterol reassertion phenomenon would be observed with other β-adrenoceptor blocking drugs. It was also not clear whether the reassertion rate after sotalol removal reflected the rate at which sotalol dissociated from the β-adrenoceptors, or the rate at which salmeterol re-engaged with them. In order to address such questions, we undertook the present study, in which we have extended the range of β-adrenoceptor blocking drugs to include compounds of differing potency, β-adrenoceptor subtype selectivity and physico-chemical characteristics. We have also studied isoprenaline, for comparative purposes. Because responses to isoprenaline are rapidly lost on washing, it was necessary to use continuous infusion to mimic, as far as possible, the persistent effects following exposure to salmeterol.

In these experiments, we attempted to standardize both the β-adrenoceptor agonist response and the degree of antagonist-induced reversal. With all of the antagonists except atenolol, we achieved 70–90% inhibition of salmeterol-induced relaxant responses, whereas that obtained with atenolol was only 47%. The rather modest effect of atenolol against salmeterol reflects its low β_2-adrenoceptor blocking potency (pA_2 at β_2-adrenoceptors in guinea-pig trachea of 5.6; O'Donnell & Wanstall, 1979), and thus a concentration of 10 μM would only be expected to cause a rightward shift of the order of 5 fold. This is supported by the observation that it was more effective against isoprenaline (78% inhibition) which interacts with both β_1- and β_2-adrenoceptors in guinea-pig trachea (O'Donnell & Wanstall, 1979). The results with the selective β_2-adrenoceptor blocking drug, ICI 118551, also support this finding, in that it was more effective against the selective β_2-adrenoceptor agonist, salmeterol, (87% inhibition) than against isoprenaline (69% inhibition). The other five antagonists show little or no β_1/β_2-adrenoceptor selectivity (Main, 1990), and produced similar degrees of inhibi-

tion against both salmeterol and isoprenaline.

A number of interesting observations were made in these studies. Most obviously, it was clear that the ability to reverse salmeterol-induced relaxant responses, and to permit salmeterol reassertion on washout is not restricted to sotalol. However, the rate of reassertion of salmeterol activity differs for the different antagonists, with that after atenolol being particularly rapid, occurring within 5 min, whilst that after ICI 118551 was the slowest, with 50% reassertion taking about 2.5 h. It therefore seems unlikely that the rate of reassertion following antagonist washout is a function of re-equilibration of salmeterol with the β-adrenoceptors, but rather the rate at which the antagonist dissociates from the tissue. This being the case, it is perhaps not surprising that the rank order of rate of reassertion after the seven antagonists corresponds to their rank order of lipophilicity (Cruickshank, 1980). However, these data alone do not rule out some influence of salmeterol/receptor re-equilibration in the reassertion process, although bearing in mind the relatively rapid rate of the reassertion following atenolol, it would appear to be a minor contribution. Significantly, there were no differences in the rates of reassertion for salmeterol and isoprenaline after washout of any of the antagonists.

It is interesting to consider what information these results provide towards understanding the mechanism of action underlying the persistent agonist activity of salmeterol. The rates of reversal of β-agonist activity for all of the antagonists were of the same order (3–11 min), and there were no consistent differences in these rates for the various antagonists against salmeterol when compared with those against isoprenaline. These experiments do not prove that salmeterol and isoprenaline dissociate from the active site of the β-adrenoceptors at the same rate, but they do indicate that dissociation is not rate-limiting as far as onset of action of antagonist activity is concerned. Thus, bearing in mind the rapid onset of action of atenolol in particular, both agonists must obviously dissociate within this time period, and slow dissociation from β-adrenoceptors cannot therefore be the mechanism of the long duration of action of salmeterol.

Further insight into the nature of the interaction of salmeterol with β_2-adrenoceptors is provided by studies on rat lung membrane β_2-adrenoceptors labelled with the radioligand,[^{125}I]-PIN (Barovsky & Brooker, 1980; Neve *et al.*, 1986). These binding sites were confirmed as β_2-adrenoceptors by the characteristic catecholamine agonist rank-order of potency (isoprenaline > adrenaline > noradrenaline; Lands *et al.*, 1967) and by the 1000 fold greater affinity for the β_2-adrenoceptor antagonist, ICI 118551 than for the β_1-adrenoceptor antagonist, atenolol (O'Donnell & Wanstall, 1980; Lemoine *et al.*, 1988). Of the agonists examined, salmeterol showed the highest apparent binding affinity. The non-competitive nature of the interaction between salmeterol and the β-adrenoceptor blocking drug, [^{125}I]-PIN reported in rat lung membranes contrasts with that previously reported between salmeterol and propranolol in functional studies in guinea-pig trachea, where a competitive interaction was observed (Ball *et al.*, 1991). However, it is important to appreciate that in the functional studies, we allowed the antagonist to equilibrate with the β-adrenoceptors before adding salmeterol, whereas in the binding studies, salmeterol was allowed to equilibrate before addition of antagonist. When one considers the particular characteristics of the interaction of salmeterol with the β_2-adrenoceptor, it is quite clear that different patterns of competition could result.

Surprisingly, despite the continued presence of GTP to minimize complications such as interconvertible receptor states (DeLean *et al.*, 1980; Abramson & Molinoff, 1984), isoprenaline, adrenaline and noradrenaline consistently produced inhibition curves with shallow slopes (Hill coefficients 0.7–0.8). In contrast, the slopes of the inhibition curves produced by salbutamol, salmeterol and the β-adrenoceptor antagonists were not significantly different from unity. These differences in slope parameters might reflect binding site heterogeneity, since [^{125}I]-PIN recognises β_2-adrenoceptors with only a 3 fold greater affinity than β_1-adrenoceptors (Neve *et al.*, 1986). However, this seems unlikely in view of the monophasic inhibition curves obtained with all other compounds, including β_2-adrenoceptor-selective agonists and β_1 and β_2-selective antagonists. A more plausible explanation is that the concentration of GTP was insufficient to ensure all of the receptor population was in its ground state. If this were so, it is interesting to speculate that the differences seen between slope parameters for the β-adrenoceptor agonists might reflect differences in agonist efficacies. By this token, it would be predicted that salbutamol and salmeterol might be partial (low efficacy) agonists compared with isoprenaline, an observation in keeping with several reports of functional studies (Ball *et al.*, 1991; Dougall *et al.*, 1991; Waldeck & Kallstrom, 1991).

The major finding of this part of the study was the apparent irreversibility of the binding of salmeterol. Unfortunately, the interaction of salmeterol with the β_2-adrenoceptor could not be examined directly because suitably radiolabelled salmeterol with sufficiently high specific activity was not available. Accordingly, the binding of salmeterol was examined indirectly by its ability to inhibit the binding of the radioligand, [^{125}I]-PIN. Using this approach, there was a clear distinction between the apparent dissociation profile (measured as the association of [^{125}I]-PIN to the vacated binding sites) for salmeterol compared with that for isoprenaline or salbutamol. Essentially, both salbutamol and isoprenaline dissociated from the binding sites so rapidly that there was little difference in the rate of [^{125}I]-PIN binding to membranes pretreated with these agonists compared with that to membranes pretreated with vehicle. This contrasted markedly with the apparent lack of dissociation of salmeterol. These differences in dissociation of the β-adrenoceptor agonists therefore produced different inhibition profiles, with salmeterol behaving as an apparently irreversibly bound, non-competitive inhibitor.

The apparent inconsistency between the irreversibility of binding in the rat lung membranes and the functional studies in guinea-pig trachea may result from the nature of the two types of experiment. In the functional studies, high concentrations of antagonist were employed which compete with the persisting local levels of salmeterol for occupancy of the β_2-adrenoceptors. In contrast, in the binding studies, low concentrations of radiolabelled antagonist were employed, which were insufficient to compete with the relatively high local levels of β_2-agonist, but which will bind increasingly to the receptors as agonist is removed from the membranes by dilution. While the concentration of isoprenaline and salbutamol at the β_2-adrenoceptors will be reduced by dilution, allowing progressively more [^{125}I]-PIN to associate with the β_2-adrenoceptors, dilution does not appear to affect the amount of salmeterol in the vicinity of the β_2-adrenoceptors, and thus there is no such increase in [^{125}I]-PIN binding. Indeed, the results of the functional and binding studies lead to exactly the same conclusion, that washing the tissues even for extended periods of time fails to reduce the effective tissue concentration of salmeterol accessible to the β_2-adrenoceptors. In contrast, both techniques demonstrate rapid and complete loss of both isoprenaline and salbutamol. If higher concentrations of [^{125}I]-PIN were used, comparable to those of the antagonists used in the functional studies, then increasing β-antagonist binding would presumably be observed even in the face of maintained tissue levels of salmeterol. Unfortunately, at [^{125}I]-PIN concentrations above nanomolar levels, the degree of non-specific binding would render the assay useless.

These observations are consistent with the 'exosite' hypothesis, originally described by Bradshaw *et al.* (1987) and subsequently developed by Johnson (1990) and Jack (1991). This envisages the 'anchoring' of the lipophilic N-substituent phenylalkyloxyalkyl 'tail' of salmeterol to a site adjacent to or within the β-adrenoceptor, allowing the phenylethanol-

amine head group to interact with the active site of the receptor. This mechanism, described as the 'charnière' (hinge) effect, was first proposed by Rocha e Silva (1969) to explain the slow offset of action of the histamine H_1-receptor blocking drug, diphenhydramine.

Although lipophilic compounds generally tend to be long-acting *in vitro*, they do not normally exhibit the persistence of action associated with salmeterol. In the present study, the duration of action of the β-adrenoceptor blocking drugs tested, correlated with their rank order of lipophilicity (Cruikshank, 1980; Conway *et al.*, 1987). The most lipophilic of the antagonists, ICI 118551, has a calculated log P (octanol: water) of 3.85 (Medchem Data Base), which is similar to that of salmeterol (4.05, Johnson *et al.*, 1992). However, while the agonist activity of salmeterol persists apparently unchanged for periods of at least 10 h, there was obvious, albeit slow, recovery from the effects of ICI 118551, resulting in 50% recovery usually within 3 h.

Although, lipophilicity might be expected to hinder the molecule leaving the lipid environment of the cell membrane and re-entering the aqueous phase, it would not be expected to prevent it. Under the conditions of these superfusion experiments, there would effectively be an infinite concentration gradient from the hydrophobic membrane lipid phase, to the extracellular aqueous phase, and any molecule within the membrane would slowly re-enter the aqueous phase. This clearly does not happen to salmeterol, which *in vitro* at least, shows persistence of action for periods of 10 h or more, far greater than the time over which substantial loss of activity was observed for the isolipophilic β-adrenoceptor antagonist, ICI 118551. These results argue strongly for 'anchoring' of the salmeterol molecule in the vicinity of the active site of the receptor and this we believe is affected by the postulated exosite. If this is so, then what and where is the exosite? It is now well established that the β-adrenoceptor protein is arranged in the cell membrane in a series of 7 transmembrane domains (Dohlman *et al.*, 1987; Lefkowitz & Caron, 1988; O'Dowd *et al.*, 1989). There is now considerable knowledge, based on deletion experiments, of the amino acids essential for β_2-agonist binding, these being Asp113, Ser204 and Ser207, located in the IIIrd and Vth domains (Strader *et al.*, 1988; 1989). To interact with the appropriate residues, the agonist molecule must sit within the central pore where it can span the appropriate binding sites on the receptor protein (Dixon *et al.*, 1987). The binding of β-adrenoceptor agonists within the β-adrenoceptor has been modelled (Jack, 1991), and it has been suggested that the phenylethanolamine head of salmeterol binds in similar fashion to the natural agonist, adrenaline, to the appropriate Asp and Ser residues (Strader *et al.*, 1989), and that the N-substituent is orientated 'downwards' in an intracellular direction. However, such modelling cannot be regarded as definitive, and the N-substituent may equally be orientated 'upwards', in an extracellular direction, or even laterally. It is not clear how the phenylalkyloxyalkyl chain would interact with the receptor protein if it assumed these different orientations, or if it did, with which amino acid residues the flexible N-substituent of salmeterol could interact (Anderson, 1991). In the case of the lateral orientation, it is unlikely that such a long chain could be accommodated in the receptor pore at all, and perhaps the most likely arrangement would involve a protrusion between the transmembrane helices, into the membrane itself. It is important to appreciate that whereas hydrophilic agonists such as isoprenaline and salbutamol almost certainly access the active site of the receptor directly from the extracellular aqueous phase, more lipophilic compounds rapidly accumulate in the membrane lipid (Herbette *et al.*, 1988), and would almost certainly access the receptor from the cell membrane. To achieve this, it would be necessary for the phenylethanolamine end of the molecule to enter the receptor pore between the transmembrane helices. If this is so, the postulated exosite may not be a part of the receptor protein itself, but rather the 'peri-receptor' lipid, defined as that lipid around and between the receptor transmembrane domains. Salmeterol has a fully extended length of 25 angstroms, which is sufficient in theory to allow the phenylethanolamine head to interact with the active site, yet for the lipophilic phenylalkyloxyalkyl side-chain to protrude from the receptor into the 'peri-receptor' lipid. The possibility that the 'peri-receptor' lipid or a hydrophobic domain of the receptor protein itself is the postulated exosite is currently being investigated.

In conclusion, although the theory of 'exosite' binding to explain the persistent action of salmeterol is not proven, the data presented in the present report are all consistent with it. Indeed, in view of the lack of evidence from functional studies for avid binding of salmeterol to the β_2-adrenoceptors themselves, it is difficult to envisage another explanation for the pharmacological profile of this compound. In the light of this, we have speculated that the postulated exosite may be the 'peri-receptor' lipid or a hydrophobic domain of the receptor itself. However, there is scope for further experimental work aimed at investigating the validity of the 'exosite' hypothesis. Increased understanding of the mechanism may enable it it be used in extending the duration of action of other classes of therapeutic agent, where this cannot be achieved by more conventional approaches.

We would like to acknowledge the key roles played by Dr D. Jack and Dr R.T. Brittain in suggesting many of the experiments presented here, and in their invaluable contributions to the interpretation of the data obtained. We would also like to thank them for their continued support and interest in this work.

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The pharmacology of RS-15385-197, a potent and selective α_2-adrenoceptor antagonist

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1 RS-15385-197 ((8a**R**, 12a**S**, 13a**S**)-5,8,8a,9,10,11,12,12a,13,13a-decahydro-3-methoxy-12-(methylsulphonyl)-6H-isoquino [2,1-g][1,6]-naphthyridine) was evaluated in a series of *in vitro* and *in vivo* tests as an antagonist at α_2-adrenoceptors.

2 RS-15385-197 had a pK_i of 9.45 for α_2-adrenoceptors in the rat cortex (pA_2 in the guinea-pig ileum of 9.72), whereas the 8aS, 12aR, 13aR enantiomer, RS-15385-198, had a pK_i of only 6.32 (pA_2 6.47) indicating a high degree of stereoselectivity. The racemate RS-15385-196 had a pK_i of 9.18.

3 RS-15385-197 showed unprecedented α_2 vs. α_1 adrenoceptor selectivity *in vitro*. In the rat cortex, RS-15385-197 had a pK_i of 9.45 in displacing [^{3}H]-yohimbine and 5.29 in displacing [^{3}H]-prazosin (α_2/α_1 selectivity ratio in binding experiments >14000). The compound had a pA_2 of 9.72 as a competitive antagonist of the inhibitory effects of UK-14,304 in transmurally-stimulated guinea-pig ileum and 10.0 against BHT-920-induced contractions in dog saphenous vein (DSV); this latter value was unaltered by phenoxybenzamine. An apparent pK_B of 5.9 was obtained against cirazoline-induced contractions in DSV, whilst a pA_2 of 6.05 was obtained against phenylephrine-induced contractions in the rabbit aorta (α_2/α_1 selectivity ratio in functional experiments >4000).

4 RS-15385-197 was highly selective for α_2-adrenoceptors over other receptors: the compound showed low affinity for 5-HT_{1A} (pK_i 6.50) and 5-HT_{1D} (pK_i 7.00) receptor subtypes, and even lower affinity ($pK_i \leqslant 5$) for other 5-HT receptor subtypes, dopamine receptors, muscarinic cholinoceptors, β-adrenoceptors and dihydropyridine binding sites. RS-15385-197 was devoid of affinity for the non-adrenoceptor imidazoline binding site, labelled by [^{3}H]-idazoxan, which provides further evidence that these sites are not related to α_2-adrenoceptors. In the DSV, contractile responses to 5-hydroxytryptamine (5-HT) were unaffected by a concentration of 1 μM RS-15385-197.

5 RS-15385-197 was non-selective for the α_{2A}- and α_{2B}-adrenoceptor subtypes in that the pK_i for the α_{2A}-adrenoceptor in human platelets was 9.90 and the pK_i for the α_{2B}-adrenoceptor in rat neonate lung was 9.70. However, RS-15385-197 showed lower affinity for the α_2-adrenoceptor subtype in hamster adipocytes (pK_i 8.38).

6 In anaesthetized rats, RS-15385-197 was a potent antagonist of the mydriasis response induced by UK-14,304 or clonidine (AD_{50} 5 and 7 μg kg^{-1}, i.v., respectively; 96 μg kg^{-1}, p.o.) and of UK-14,304-induced pressor responses in pithed rats (AD_{50} 7 μg kg^{-1}, i.v.); the compound therefore is both centrally and orally active. Even at a high dose (10 mg kg^{-1}, i.v.), RS-15385-197 did not antagonize pressor responses to cirazoline in pithed rats, indicating that the selectivity for α_2 vs. α_1-adrenoceptors was maintained *in vivo*.

8 RS-15385-197 is therefore a very potent, selective, competitive α_2-adrenoceptor antagonist, both *in vitro* and *in vivo*, is orally active and readily penetrates the brain. It will thus be a powerful pharmacological tool for exploring the various physiological roles of α_2-adrenoceptors.

Keywords: RS-15385-197; α_2-adrenoceptors; yohimbine; idazoxan

Introduction

The role of α_2-adrenoceptors in mediating or modulating pre- and postjunctional events has previously been explored using poorly selective antagonists such as yohimbine, its diastereoisomer rauwolscine, and idazoxan (Langer, 1974; Ruffolo *et al.*, 1991). Using these relatively imprecise pharmacological tools, the distribution and function of α_2-adrenoceptors in a variety of species has been inferred (Goldberg & Robertson, 1983) and therapeutic applications of antagonists at these receptors proposed (Clark *et al.*, 1986; MacDonald *et al.*, 1988). However, yohimbine and rauwolscine have high affinity for subtypes of 5-hydroxytryptamine (5-HT) receptors (Convents *et al.*, 1988; Brown *et al.*, 1990c) and idazoxan has equivalent affinity for an imidazoline-preferring site compared with α_2-adrenoceptors (Hamilton *et al.*, 1988; Yablonsky *et al.*, 1988; MacKinnon *et al.*, 1989; Michel *et al.*, 1989; Brown *et al.*, 1990a). Yohimbine has been shown to cause a variety of effects in man, such as anxiety and increases in blood pressure and heart rate (Goldberg *et al.*, 1983), but it is not clear whether all of these effects arise from antagonism of ongoing activation of α_2-adrenoceptors. In order to investigate more definitively the role of α_2-adrenoceptor activation in different physiological systems we have synthesized a novel α_2-adrenoceptor antagonist, RS-15385-197 ((8a**R**, 12a**S**, 13a**S**)-5-8,8a,9,10,11,12,12a,13,13a-decahydro-3-methoxy-12-(methylsulphonyl)-6H-isoquino [2,1-g][1,6]-naphthyridine; Figure 1), and the present paper describes its affinity and selectivity for α_2-adrenoceptors *in vitro* and *in vivo*. RS-15385-197 is the active enantiomer and the affinity of the racemate (RS-15385-196) and the inactive enantiomer (RS-15385-198) are listed. From our data it would appear that RS-15385-197 is the most potent and selective α_2-adrenoceptor antagonist thus far described. Preliminary reports of its pharmacology

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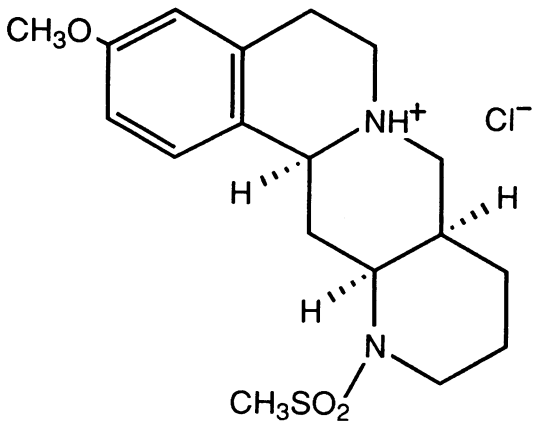

Figure 1 Structure of RS-15385-197.

have been published (Clark *et al.*, 1989a; 1990; Brown *et al.*, 1990b).

Methods

In vitro *binding studies*

Membrane preparation A washed, total membrane fraction was used in the majority of binding studies. Species and tissues are shown in Table 1. Tissues were rapidly removed and carefully dissected on ice. Tissue was homogenized in 25 vol ice cold Tris HCl buffer (50 mM, pH 7.4) in a polytron PT10 tissue disrupter (at setting 10; 2 × 10 s). The homogenate was centrifuged at 48,000 *g* in a refrigerated centrifuge (Sorvall RC-RB) at 4°C for 15 min. The resultant pellet was washed a further three times by resuspension and centrifugation and the final pellet was resuspended in 3 ml buffer and stored under liquid nitrogen until required.

Hamster adipocyte membranes were prepared as previously described (MacKinnon *et al.*, 1989). White adipose tissue taken from male Syrian hamsters (100–150 g) was finely chopped and suspended in Krebs-Ringer bicarbonate buffer (3 ml g^{-1} tissue) containing 1 mg ml^{-1} collagenase (Sigma type 1) and 3.5% w/v fatty acid free bovine serum albumin. The suspension was incubated at 37°C for 20 min and shaken vigorously every 5 min. The suspension was filtered through muslin and the cells washed three times by suspension in collagenase-free buffer. Isolated adipocytes were lysed by suspension in 10 vol lysing buffer (5 mM Tris HCl pH 7.4, 0.5 mM EDTA) at room temperature and centrifuged at 400 *g* for 1 min. The membrane fraction was collected and the unbroken cells lysed by a further two washes in lysing buffer. The membrane fractions were pooled and centrifuged at 30,000 *g* for 15 min at 4°C. The final pellet was suspended in 50 mM Tris HCl pH 7.4 containing 10 mM $MgCl_2$ and stored under liquid nitrogen until required.

Binding assays The membrane fraction (0.05–0.5 mg protein) was incubated to equilibrium with radiolabelled drug in the presence of 8–15 concentrations of RS-15385-197 or a saturating concentration of a specific drug to define non-specific binding. Conditions for the various assays are summarized in Table 1. Separation of bound from free ligand was achieved with a Brandel M24 cell harvester at a constant vacuum pressure of 22 mmHg. Bound radioactivity was determined by liquid scintillation spectrometry. α_2-Adrenoceptor saturation assays were performed with [^{3}H]-yohimbine and [^{3}H]-idazoxan (0.25–12 nM). Non-specific binding was determined with 10 μM phentolamine. Membrane protein was determined by use of Pierce BCA protein assay reagent (Sorensen & Brodbeck, 1986).

Saturation analysis Fitting of data to the appropriate models was achieved by use of the Scafit non-linear regression analysis programme LIGAND (Munson & Rodbard, 1980). Comparisons between different binding models were made using the extra sum of the squares principle as outlined by Munson & Rodbard (1980), and shown in equation 1.

$$F = \frac{(SS_1 - SS_2)/(d.f._1 - d.f._2)}{(SS_2/d.f._2)} \quad (1)$$

SS and d.f. refer to the residual sum of the squares and degrees of freedom associated with the two fits being compared. The subscript 1 is designated to the fit with the highest number of degrees of freedom (i.e. the least complicated model of analysis). Statistical significance of the F value was determined from a table of the percentage of the F distribution using $d.f._1 - d.f._2$ and $d.f._1$ degrees of freedom as the numerator and denominator respectively.

Competition analysis The inhibition of the radioligands by competing ligands was analysed graphically to estimate the IC_{50} (concentration of competitor displacing 50% of specifi-

Table 1 Binding assays

Receptor	*Tissue*	*Ligand*	*Incubation*	*Non-specific drug* (M)	*Standard*
α_1	Rat cerebral cortex	0.5 nM [^{3}H]-prazosin	30 min 25°C	10^{-5} phentolamine	prazosin
α_2	Rat cerebral cortex	2.0 nM [^{3}H]-yohimbine	30 min 25°C	10^{-5} phentolamine	yohimbine
α_2	Rat cerebral cortex	1.0 nM [^{3}H]-idazoxan	15 min 25°C	3×10^{-6} phentolamine	yohimbine
α_2	Baboon cortex	2.0 nM [^{3}H]-yohimbine	30 min 25°C	10^{-6} phentolamine	yohimbine
α_2	Baboon cortex	1.0 nM [^{3}H]-idazoxan	30 min 25°C	10^{-6} phentolamine	yohimbine
α_2	Hamster adipocytes	1.0 nM [^{3}H]-idazoxan	30 min 25°C	10^{-6} phentolamine	yohimbine
α_{2A}	Human platelets	1.0 nM [^{3}H]-yohimbine	30 min 25°C	10^{-6} phentolamine	yohimbine
α_{2B}	Rat neonate lung	1.0 nM [^{3}H]-yohimbine	45 min 25°C	10^{-5} phentolamine	yohimbine
β_1	Guinea-pig left ventricle	1.0 nM [^{3}H]-dihydroalprenolol	30 min 25°C	10^{-4} isoprenaline	propranolol
β_2	Rat lung	0.9 pM [^{125}I]-cyanopindolol	30 min 25°C	10^{-4} isoprenaline	propranaol
D_1	Rat striatum	0.3 nM [^{3}H]-SCH23390	30 min 25°C	10^{-7} SCH23390	SCH23390
D_2	Rat striatum	0.1 nM [^{3}H]-spiperone	30 min 37°C	10^{-6} (+)-butaclamol	haloperidol
5-HT_{1A}	Rat cerebral cortex	1.0 nM [^{3}H]-8-OH-DPAT	15 min 37°C	3×10^{-6} buspirone	buspirone
5-HT_{1B}	Rat striatum	30 pM [^{125}I]-cyanopindolol	40 min 37°C	10^{-5} 5-HT	RU 24969
5-HT_{1C}	Pig choroidal plexus	1.0 nM [^{3}H]-mesulergine	15 min 37°C	10^{-5} 5-HT	mesulergine
5-HT_{1D}	Baboon cortex	2.0 nM [^{3}H]-5-HT	15 min 37°C	10^{-5} 5-HT	rauwolscine
5-HT_2	Rat frontal cortex	1.0 nM [^{3}H]-ketanserin	10 min 37°C	2×10^{-6} methysergide	ritanserin
M_1	Rat cerebral cortex	0.2 nM [^{3}H]-methylscopolamine	3 h 32°C	10^{-6} atropine	atropine
M_2	Rat heart	0.2 nM [^{3}H]-methylscopolamine	3 h 32°C	10^{-6} atropine	atropine
DHP	Rat striatum	0.2 nM [^{3}H]-PN200-110	3 h 32°C	10^{-6} nifedipine	nifedipine

cally bound radioligand), by a non-linear least squares programme specially designed for the interpretation of sigmoidal concentration curves in terms of total and non-specific binding as well as inhibition constants and curve steepness. When Hill coefficients were not significantly different from unity the concentration of competitor displaying 50% of specific binding (IC_{50}) was converted to an affinity constant (K_i) using the expression derived by Cheng & Prusoff (1973), as shown in equation 2.

$$K_i = \frac{IC_{50}}{1 + ([L]/K_d)} \quad (2)$$

In this expression [L] and K_d represent the radioligand concentration and dissociation constant respectively. All data were initially analysed assuming a one site model of radioligand binding. The data with Hill coefficients less than unity were then analysed assuming a two site model, and the results of the curve fitting were statistically compared with those of the one site fit by an F test. The two site model was accepted if the observed fit was significantly better ($P < 0.05$) than the one site fit.

In vitro *functional studies*

Composition of physiological salt solution (PSS) Apart from the guinea-pig ileum preparation, the composition of the PSS in each of the *in vitro* assays was Krebs bicarbonate solution, as follows (mM): NaCl 118.93, KCl 4.69, $MgSO_4.7H_2O$ 1.01, KH_2PO_4 1.18, glucose 11.1, $NaHCO_3$ 25.0 and $CaCl_2.6H_2O$ 2.5, gassed with 95% O_2/5% CO_2 and maintained at 37°C. Inhibition of neuronal or extraneuronal uptake were not included.

Guinea-pig ileum preparation Ileum preparations taken from female Dunkin Hartley guinea-pigs (200–400 g), were set up in 30 ml isolated organ baths containing physiological Tyrode solution of the following composition (mM): NaCl 136.89, KCl 2.68, $MgCl_2.6H_2O$ 1.05, $NaH_2PO_4.2H_2O$ 0.42, glucose 5.55, $NaHCO_3$ 11.9 and $CaCl_2.6H_2O$ 1.8, gassed with 100% O_2 and maintained at 37°C. An initial tension of 1 g was applied. The preparations were stimulated co-axially at 0.1 Hz (1 ms pulse durations, supramaximal voltage) via parallel stainless steel electrodes with the anode passing through the lumen. The resulting contractions were recorded isometrically on a LectroMed oscillograph. After a period of 1 h an initial cumulative concentration-response curve (CCRC) to the agonist (UK-14,304 or clonidine) was obtained. The preparations were then washed three times and left to equilibrate for a further 40 min, after which a second agonist CCRC was obtained and measured as the control. The antagonist was added to the bath for a period of 40 min before repeating the agonist CCRC. In washout experiments, following the agonist CCRC in the presence of the antagonist, the preparations were washed by emptying and filling the bath 5 times and left to equilibrate for 40 min before repeating the agonist CCRC.

Vas deferens preparation Prostatic portions of vas deferens were taken from Sprague-Dawley rats (250–350 g) and set up in 30 ml organ baths. An initial tension of 0.5 g was applied. The preparations were field stimulated at 0.01 Hz (1 ms pulse durations, supramaximal voltage) via stainless steel electrodes running parallel to the tissue. The protocol was similar to that used for the ileum.

Dog saphenous vein Saphenous veins (DSV) were obtained from mongrel dogs of either sex under pentobarbitone (35 mg kg^{-1}, i.v.) anaesthesia. Tissues were studied after 24 h storage at 4°C in PSS. Veins were cleared of connective tissue, cut into rings of approximately 5 mm length and denuded of endothelium by carefully rubbing with forceps. The success of this technique was shown in certain tissues by demonstrating the failure of acetylcholine to relax a phenylephrine-mediated contraction. Preparations were mounted in 10 ml organ baths on steel hooks, under 2 g resting tension in PSS containing propranolol (1 μM). Isometric contractile concentration-response curves were obtained in separate preparations with BHT-920, cirazoline or 5-HT and responses displayed on a Gould BS-274 chart recorder.

Two consecutive CCRCs were obtained to BHT-920 before incubating tissues with RS-15385-197 for 30 min at varying concentrations, using only one concentration in each preparation ($n = 4-7$ tissues/group); the CCRC to BHT-920 was then repeated. In a further series of experiments the antagonist effects of RS-15385-197 were evaluated in DSVs treated with phenoxybenzamine (10 nM; 30 min followed by 30 min washout) in order to eliminate any α_1-adrenoceptor stimulation by BHT-920 at high concentrations. The effects of cirazoline washed out slowly, so only one concentration-response curve per preparation was obtained to this agonist in vehicle or antagonist-treated DSV rings after an initial contraction to KCl (80 mM) which served as a 100% reference response. Response curves to 5-HT, mediated by stimulation of 5-HT_1-'like' receptors, were studied in the presence of ketanserin (0.1 μM) in order to antagonize any 5-HT_2 receptor effects of the agonist (Humphrey *et al.*, 1988).

Rabbit aorta Aortic ring preparations were taken from male New Zealand white rabbits (2 kg). The endothelium was removed by rubbing and the rings set up in 30 ml organ baths. An initial tension of 1.5 g was applied to the tissue. After 30 min equilibration time the tissues were contracted by phenylephrine (10 nM–10 μM) in the presence or absence of one concentration of RS-15385-197.

Calculation of results from in vitro *studies* CCRCs to agonists were obtained in the presence of various concentrations of antagonists. Antagonist concentration ratios (CR) were then calculated at the EC_{50} level of the agonist in the presence of each concentration of antagonist [B]. Where possible, pA_2 values were calculated by the method of Arunlakshana & Schild (1959) by plotting $\log_{10}(CR - 1)$ vs. $-\log_{10}[B]$. The slope of this plot should theoretically be equal to 1 for a competitive antagonist and the pA_2 should then equal the antagonist dissociation constant pK_B. For the experiments on the DSV with cirazoline and 5-HT, an apparent pK_B was calculated from a single concentration of antagonist by the method of Furchgott (1972): EC_{50} values calculated for control tissues were used for the calculation of K_B, where

$$K_B = \frac{[B]}{CR - 1} \quad (3)$$

and thus pK_B is $-\log_{10}K_B$.

Pithed rat studies

Male Sprague-Dawley rats (250–350 g) were anaesthetized with halothane (5% in oxygen) or diethyl ether, pithed through the orbit, and then artificially ventilated with room air (1 ml kg^{-1}) at a rate of 50–60 strokes min^{-1} using a Harvard small animal ventilator. Body core temperature was maintained at 37°C with a heating blanket. A carotid artery and jugular (or femoral) vein were cannulated for arterial blood pressure measurement and injection of drugs respectively. Blood pressure was registered and displayed by use of either an Elcomatic 524 pressure transducer connected to a Lectromed Multitrace 4 chart recorder, or a Gould pressure transducer connected to a Gould 8000 S chart recorder.

Reversal of agonist response UK-14,304 (30 μg kg^{-1}, i.v.), was administered at 10 min intervals, following administration of prazosin (100 μg kg^{-1}, i.v.) to exclude any involvement of α_1-adrenoceptors. Increases in diastolic pressure were measured; the pressor responses to the second and third

UK-14,304 challenge doses were averaged and taken as the control response. Beginning 5 min after the third injection of UK-14,304, the antagonist was injected at 10 min intervals in ascending doses, preceding each agonist challenge dose by 5 min. %Original control response was plotted against dose of antagonist; from this, the dose of antagonist which reduced the response to UK-14,304 by 50% (AD_{50}; Berridge *et al.*, 1983) was calculated. In early studies clonidine was used as the agonist, but without prior administration of prazosin.

Displacement of agonist dose-response curves Following injection of prazosin (100 $\mu g\,kg^{-1}$, i.v.), RS-15385-197 (9, 45, 225 or 1000 $\mu g\,kg^{-1}$, i.v.) or saline was injected; separate groups of rats were used for each dose of antagonist. UK-14,304 was administered in ascending doses (1–1000 $\mu g\,kg^{-1}$, i.v.) at 5 min intervals, beginning 5 min after injection of the antagonist. Increase in diastolic blood pressure was plotted against cumulative dose of UK-14,304. To assess α_2- vs. α_1-adrenoceptor selectivity, pressor responses were evoked either by UK-14,304 or by cirazoline, respectively. Cumulative dose-response curves to these agonists were determined in groups of rats given 10 min i.v. infusions of each antagonist at varying doses and in a control group given an i.v. vehicle infusion of 0.5 $ml\,kg^{-1}$ for 10 min. Increases in diastolic blood pressure were plotted vs. dose of agonist. Separate groups of rats were used for each dose of antagonist. From these curves, '*in vivo* Schild plots' (i.e. plots of log (dose ratio-1) vs. log dose of antagonist) were constructed to estimate the i.v. dose of antagonist giving a dose-ratio of 2 (DR_2; Clineschmidt *et al.*, 1988).

Prejunctional α_2-adrenoceptor effects All rats were bilaterally vagotomized and given (+)-tubocurarine (1 $mg\,kg^{-1}$, i.v.) and atropine (0.5 $mg\,kg^{-1}$, i.v.). Heart rate was raised by about 100 beats min^{-1} by continuous electrical stimulation of the thoracic sympathetic outflow (50 V; 0.3–0.5 ms; 0.3–0.5 Hz) via an uninsulated portion of the pithing rod. After a period of 20 min stabilization under electrical stimulation, cardioinhibitory response curves to UK-14,304 were constructed in vehicle- or antagonist-treated groups of rats. Separate groups of rats were used for each dose of antagonist.

Mydriasis studies

The methods used were based on those described by Berridge *et al.* (1983). Male Sprague-Dawley rats (200–400 g) were anaesthetized with sodium pentobarbitone (60 $mg\,kg^{-1}$, i.p.). Body core temperature was maintained at 37°C with a heating blanket. A jugular vein was cannulated for drug injections. Pupil diameter was measured with an illuminated inspection glass with × 7 magnification (RS Components Ltd).

Reversal of agonist response In early studies, mydriasis was produced by injection of clonidine (300 $\mu g\,kg^{-1}$, s.c.), and the antagonist was administered intravenously at 10 min intervals, the pupil diameter being measured 5 min after each dose. Alternatively, the antagonist was administered orally in increasing doses every 20 min, the pupil diameter being measured 15 min after each dose. In subsequent studies, mydriasis was produced by injection of UK-14,304 (100 $\mu g\,kg^{-1}$, i.v.), and the antagonist was administered at 5 min intervals, beginning 5 min after injection of the agonist, and pupil diameter was measured 4.5 min after each dose of antagonist. A graph of % original increase in pupil diameter vs. dose of antagonist was plotted, from which the AD_{50} (see above) was calculated.

Displacement of agonist dose-response curves Rats received a single dose of antagonist 5 min before performing a cumulative dose-response curve to UK-14,304 (1–1000 $\mu g\,kg^{-1}$, i.v.), administered at 5 min intervals. Increase in pupil diameter was plotted vs. dose of UK-14,304. In order to assess the time course of antagonism following oral administration, conscious rats were treated orally with antagonist or vehicle. They were prepared as described above at appropriate intervals following gavage, then a dose-response curve to clonidine (injected i.v. at 5 min intervals) was constructed 1, 2, 4 or 6 h after the oral dose. Pupil diameter was measured 4.5 min after each dose of agonist.

Drugs

Reagents used were of the highest analytical grade. Compounds were kindly donated by their manufacturers, synthesized at the Institute of Organic Chemistry, Syntex (Palo Alto) or purchased. The following compounds were used: [^{3}H]-idazoxan (40 Ci $mmol^{-1}$), [^{3}H]-5-HT (40 Ci $mmol^{-1}$), [^{3}H]-mesulergine (80 Ci $mmol^{-1}$), [^{3}H]-8-OH-DPAT (220 Ci $mmol^{-1}$) and [^{3}H]-prazosin (86 Ci $mmol^{-1}$) from Amersham, U.K., and [^{3}H]-dihydroalprenolol (90 Ci $mmol^{-1}$), [^{125}I]-cyanopindolol (2200 Ci $mmol^{-1}$), [^{3}H]-yohimbine (89 Ci $mmol^{-1}$), [^{3}H]-ketanserin (76 Ci $mmol^{-1}$), PN200110 (80 Ci $mmol^{-1}$), [^{3}H]-spiperone (80 Ci $mmol^{-1}$), [^{3}H]-SCH23390 (80.4 Ci $mmol^{-1}$) and [^{3}H]-n-methylscopolamine (71.3 Ci $mmol^{-1}$) from DuPont, U.K.; atropine sulphate (BDH); BHT-920 HCl (6-allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazolo-[4,5-ol]-azepine; Boehringer Ingleheim); phentolamine mesylate (Ciba); L-659,066 (MSD); prazosin HCl (Pfizer); (+)-butaclamol, ketanserin tartrate and phenoxybenzamine HCl (RBI); methysergide maleate (Sandoz); SCH 23390 maleate (Schering); (−)-adrenaline bitartrate, (−)-noradrenaline bitartrate; clonidine HCl, oxymetazoline HCl, yohimbine HCl, L-phenylephrine HCl, 5-HT creatinine sulphate and (±)-propranolol HCl (Sigma); cirazoline HCl (Synthelabo); buspirone HCl, idazoxan HCl, piperoxan HCl, ritanserin, UK-14,304 HCl (5-bromo-6-[2-imidazolin-2-ylamino] quinoxaline), RS-15385-197, RS-15385-198 and RS-15385-196 (synthesized by Dr R. Clark, Syntex, Palo Alto, CA, U.S.A.).

Results

Binding studies

The affinities of RS-15385-197 for a wide range of receptor binding sites are listed in Table 2. The highest affinity of RS-15385-197 was for α_2-adrenoceptors in a range of different tissues from different species (pK_i 9.18–10.15).

Saturation analysis of [^{3}H]-yohimbine and [^{3}H]-idazoxan binding indicated that it was specific and both ligands bound to a single population of high affinity sites in the baboon cortex (K_d 3 and 2.1 nM; B_{max} 80 and 93 fmol mg^{-1} protein, respectively). The affinities of a number of compounds were determined and showed the site to be characteristic of an α_2-adrenoceptor with high affinity for yohimbine, idazoxan, phentolamine and noradrenaline. Both radioligands labelled a site characteristic of the α_{2A}-like subtype with high affinity for oxymetazoline (pK_i 7.87 ± 0.20 and 8.04 ± 0.21 respectively) and low affinity for prazosin (pK_i 6.23 ± 0.26 and 6.28 ± 0.11 respectively), which has been reported to have higher affinity for both α_{2B}- and α_{2C}-adrenoceptor subtypes (Bylund, 1988).

RS-15385-197 revealed markedly lower affinity for various other receptor binding sites (Table 2). The selectivity ratio for α_2-adrenoceptors was > 14,000 over α_1-adrenoceptors in the rat cortex. The compound showed low affinity for 5-HT_{1A} (pK_i 6.50) and 5-HT_{1D} (pK_i 7.00) and even lower affinity for other 5-HT subtypes, dopamine receptors, muscarinic cholinoceptors, β-adrenoceptors and dihydropyridine binding sites (pK_i ≤ 5). Inhibition of [^{3}H]-yohimbine binding to α_2-adrenoceptors by RS-15385-197 was monophasic (Figure 2), consistent either with an interaction at one site or with equal affinity for subtypes labelled by 2 nM [^{3}H]-yohimbine in the rat cortex. RS-15385-196 was slightly less potent than the

Table 2 Affinity values of RS-15385-197 (pK_i)

Receptor	*Ligand*	*Tissue*	*Affinity*
α_2	[^{3}H]-yohimbine	Rat cortex	9.45 ± 0.12
α_2	[^{3}H]-idazoxan	Rat cortex	9.18 ± 0.13
α_{2A}	[^{3}H]-yohimbine	Human platelets	9.90 ± 0.10
α_{2B}	[^{3}H]-yohimbine	Rat neonate lung	9.70 ± 0.10
α_2	[^{3}H]-yohimbine	Baboon cortex	10.12 ± 0.3
α_2	[^{3}H]-idazoxan	Baboon cortex	10.15 ± 0.31
α_2	[^{3}H]-idazoxan	Hamster adipocyte	8.38 ± 0.01
α_1	[^{3}H]-prazosin	Rat cortex	5.29
I_2	[^{3}H]-idazoxan	Hamster adipocyte	<4
5-HT$_{1A}$	[^{3}H]-8-OH-DPAT	Rat cortex	6.50
5-HT$_{1B}$	[^{125}I]-CYP	Rat striatum	<5
5-HT$_{1C}$	[^{3}H]-mesulergine	Pig choroidal plexus	<5
5-HT$_{1D}$	[^{3}H]-5-HT	Baboon cortex	7.0 ± 0.2
5-HT$_2$	[^{3}H]-ketanserin	Rat frontal cortex	5.10
β_1	[^{3}H]-dihydroalprenolol	Guinea-pig left ventricle	5.27
β_2	[^{125}I]-CYP	Rat lung	<5
D_1	[^{3}H]-SCH23390	Rat striatum	<5
D_2	[^{3}H]-spiperone	Rat striatum	<5
M_1	[^{3}H]-N-methylscopolamine	Rat cortex	<5
M_2	[^{3}H]-N-methylscopolamine	Rat heart	<5
DHP	[^{3}H]-PN 200-110	Rat striatum	<5

Assays were performed as described in methods and Table 1. The results are expressed as the pK_i mean ± s.e.mean of 3 experiments performed in duplicate. DHP = dihydropyridine Ca^{2+} binding sites.

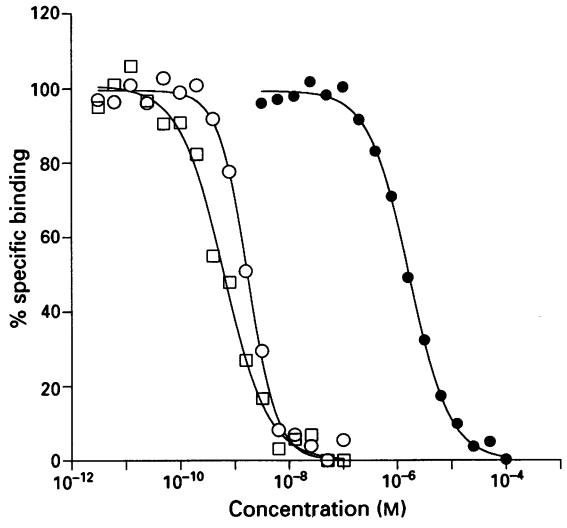

Figure 2 Displacement of [^{3}H]-yohimbine binding from rat cerebral cortex membranes by RS-15385-197 (□, pK_i 9.4), RS-15385-196 (○, pK_i 8.9) or RS-15385-198 (●, pK_i 5.9).

Table 3 Displacement of [^{3}H]-idazoxan, 1.5 nM, from hamster adipocytes

	pK_i		
	Site 1 20–30%	*Site 2* 70–80%	*Selectivity*
Idazoxan	8.34 ± 0.06	–	
Yohimbine	7.28 ± 0.02	4.48 ± 0.16	631
Phentolamine	7.92 ± 0.14	5.21 ± 0.02	513
(−)-Noradrenaline	6.21 ± 0.10	< 4.00	> 162
(−)-Adrenaline	6.92 ± 0.12	< 4.00	> 832
RS-15385-197	8.38 ± 0.01	< 4.00	> 23988

Adipocyte membranes were incubated with 1.5 nM [^{3}H]-idazoxan and 13 concentrations of competing ligand to equilibrium in a final volume of 0.5 ml 50 mM Tris HCl pH 7.4, 10 mM $MgCl_2$ buffer. Non-specific binding was defined using 1 mM phentolamine. The data represent 3–5 experiments performed in duplicate. The fit for a 1 or 2 site model were compared using the differential F value. A 2 site fit was assumed to be better than a 1 site fit if the F value achieved significance of $P<0.05$.

−197 isomer, and the −198 isomer was more than a thousand fold less potent (Figure 2), thus indicating stereoselectivity.

RS-15385-197 was non-selective for the α_{2A} (human platelet pK_i 9.90) and α_{2B} (rat neonate lung pK_i 9.70) subtype of the α_2-adrenoceptor. However, RS-15385-197 showed lower affinity for the α_2 subtype in hamster adipocytes (pK_i 8.38); this subtype has previously been described as α_{2A}-like (MacKinnon *et al.*, 1989) but shows lower affinity for tetracyclic yohimbine-like structures. Yohimbine, phentolamine and noradrenaline inhibited 1.5 nM [^{3}H]-idazoxan binding to hamster adipocytes with Hill slopes less than unity, which were better fitted to a 2 site model (Table 3). RS-15385-197 inhibited only 20–30% specific binding with an affinity constant, pK_i = 8.38, but did not displace the imidazoline specific component at concentrations up to 1 mM. Idazoxan exhibited a monophasic displacement curve on [^{3}H]-idazoxan binding, consistent with this compound having equal affinity for both sites, *viz.* the α_2-adrenoceptor and the non-adrenoceptor imidazoline binding site.

Guinea-pig ileum and rat vas deferens

UK-14,304 concentration-response curves for inhibition of twitch height in the transmurally-stimulated guinea-pig ileum were shifted to the right by RS-15385-197 (0.1–3 nM), RS-15385-196 (0.1–3 nM) and RS-15385-198 (300–3000 nM; Figure 3a–c). The Schild plots derived from these data are shown in Figure 3d; the slopes for all three compounds were not significantly different from unity, which is compatible with a competitive interaction at the α_2-adrenoceptor. The pA_2 for RS-15385-197 was 9.72 ± 0.05 (slope 1.10 ± 0.02, n = 11), for RS-15385-196 was 9.69 ± 0.06 (slope 1.10 ± 0.05, n = 5) and for RS-15385-198 was 6.47 ± 0.06 (slope 0.96 ± 0.04, n = 4) indicating a highly stereoselective interaction of RS-15385-197 with the α_2-adrenoceptor. The effects of RS-

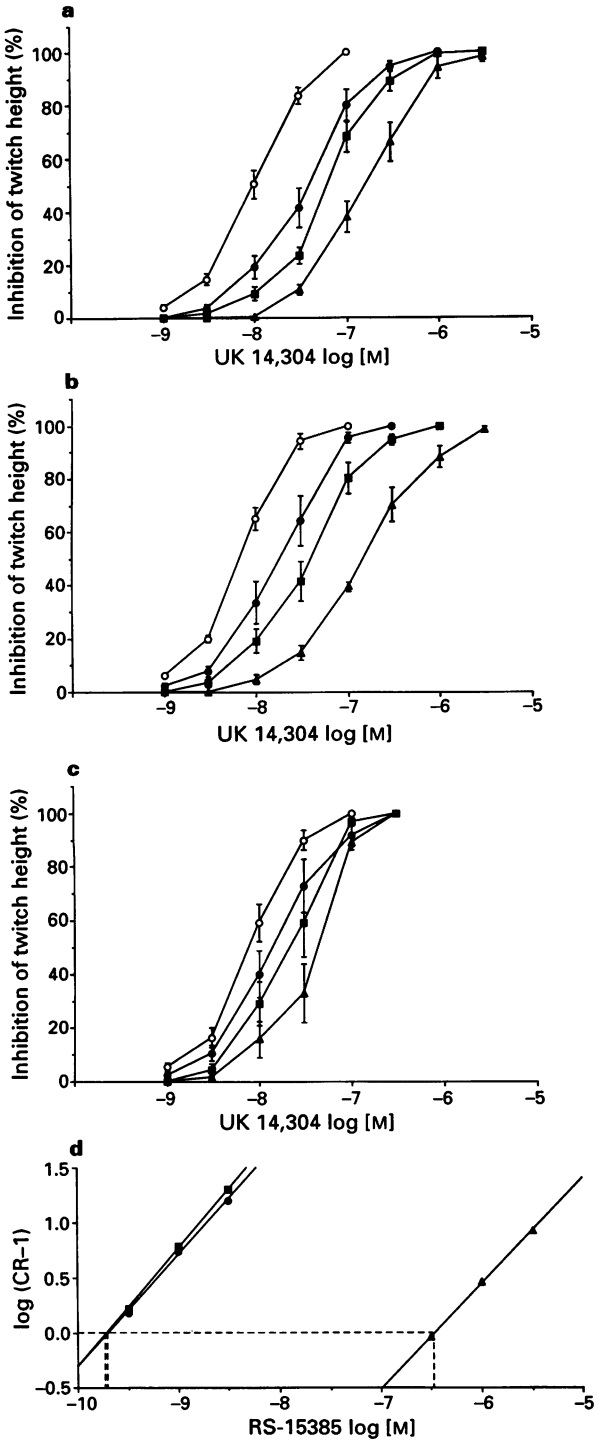

Figure 3 Antagonism of the inhibitory responses to UK-14,304 (controls O) on the contractile responses of guinea-pig ileum prepaations to field stimulation by RS-15385-197 (a: ●, 0.1 nM; ■, 1 nM; ▲, 3 nM); RS-15385-196 (b: ●, 0.1 nM; ■, 1 nM; ▲, 3 nM) or RS-15385-198 (c: ●, 0.3 μM; ■, 1 μM; ▲, 3 μM). The antagonistic effects of RS-15385-197 (■), RS-15385-196 (●) and RS-15385-198 (▲) are quantified by the method of Arunlakshana & Schild (1959) in (d). Vertical bars represent s.e.mean, $n = 5-6$.

15385-197 (3 nM) were reversible as the concentration-ratio for UK-14,304 was <2 after 30 min washout. RS-15385-197 (0.1 to 3 nM) did not modify the responses to co-axial stimulation directly and showed no partial agonist effects. With clonidine as the agonist, RS-15385-197 had a pA_2 of 9.46 ± 0.26 (slope 0.75 ± 0.10, $n = 4$). RS-15385-197 was selective for α_2-adrenoceptors in that high concentrations (1 and 10 μM) did not modify the inhibitory responses to morphine in the ileum (concentration-ratio <2; data not shown).

RS-15385-197 was also a potent antagonist of the response to UK-14,304 in the rat vas deferens preparation (pA_2 9.28 ± 0.10, slope 0.70 ± 0.04, $n = 6$). RS-15385-198 was slightly more potent in the rat vas deferens as an antagonist of UK-14,304 (pA_2 7.42 ± 0.07, slope 1.70 ± 0.09, $n = 4$) than in the guinea-pig ileum.

Dog saphenous vein

Concentration-dependent contractile response curves obtained to BHT-920 were progressively displaced to the right of controls by RS-15385-197 (1.0–100 nM) with little change in the maximum responses to the agonist (Figure 4). In control tissues three subsequent response curves to BHT-920 were superimposable. Schild analysis of these data gave a pA_2 of 10.00 with a slope of 0.85. Pretreatment of the tissues with phenoxybenzamine at a concentration (10 nM) which irreversibly inactivates the α_1-adrenoceptors in this preparation (Ruffolo & Zeid, 1985) did not modify the antagonist effects of RS-15385-197 (pA_2 9.95, slope 0.80; Figure 5).

A high concentration of RS-15385-197 (10 μM) was required to displace the contractile response curves to cirazoline to the right of controls (apparent pK_B 5.9 ± 0.2) and RS-15385-197 (1.0 μM) failed to antagonize the contractile responses to 5-HT in tissues treated with ketanserin (Figure 4).

Rabbit aorta

RS-15385-197 was a weak antagonist of phenylephrine-induced contractions in the rabbit aorta (pA_2 6.05 ± 0.16, slope 0.90 ± 0.06, $n = 4$). This estimate of α_1-adrenoceptor antagonist affinity was similar to that determined on cirazoline-induced contractions in dog saphenous vein. These data indicate a very high α_2 vs. α_1-adrenoceptor selectivity ratio in *in vitro* functional assays of >4000.

Pithed rat studies

UK-14,304 (1–1000 μg kg^{-1}, i.v.) produced a dose-related increase in diastolic blood pressure. The challenge dose used in the agonist reversal study (30 μg kg^{-1}, i.v.) was at approximately the ED_{80} level.

Reversal of agonist response In the presence of prazosin (100 μg kg^{-1}, i.v.), UK-14,304 (30 μg kg^{-1}, i.v.) evoked reproducible increases in diastolic blood pressure of around 30 mmHg. RS-15385-197, idazoxan and L-659,066 reduced this response in a dose-related manner, although in each case there was a small residual response of around 10% of the original pressure response which was resistant to the α_2-adrenoceptor antagonists (Figure 6). When this residual response was corrected for, RS-15385-197 gave an AD_{50} of 7 μg kg^{-1}, i.v. ($n = 6$), which was the same as the value obtained (7 μg kg^{-1}) for reversal of the pressor response to clonidine, and far more potent than the other four antagonists tested (Table 4).

Displacement of agonist dose-response curves The dose-response curve to UK-14,304 was displaced to the right in parallel by RS-15385-197 (45, 225 and 1000 μg kg^{-1}, i.v.) in a dose-related manner, without reduction in the maximum response (Figure 7a). Construction of '*in vivo*' Schild plots' from these data indicated that RS-15385-197 gave a dose-ratio of 2 (DR_2) at a dose of 45 μg kg^{-1}, i.v. (slope = 1.0). In comparison, yohimbine (DR_2 = 180 μg kg^{-1}, i.v.; slope = 0.9) and idazoxan (DR_2 = 200 μg kg^{-1}, i.v.; slope = 0.9) were approximately 4 fold less potent.

α_2- vs. α_1-Adrenoceptor selectivity RS-15385-197 (10 mg kg^{-1}, i.v.) caused only a small rightward shift (dose-ratio =

1.6) in the pressor response curve to cirazoline. The maximal vasoconstrictor to cirazoline was, however, slightly decreased after this dose of RS-15385-197 (Figure 7b). Prazosin (100 μg kg^{-1}, i.v.) caused a rightward displacement of the vasoconstrictor responses to cirazoline with a dose-ratio of 7 (data not shown).

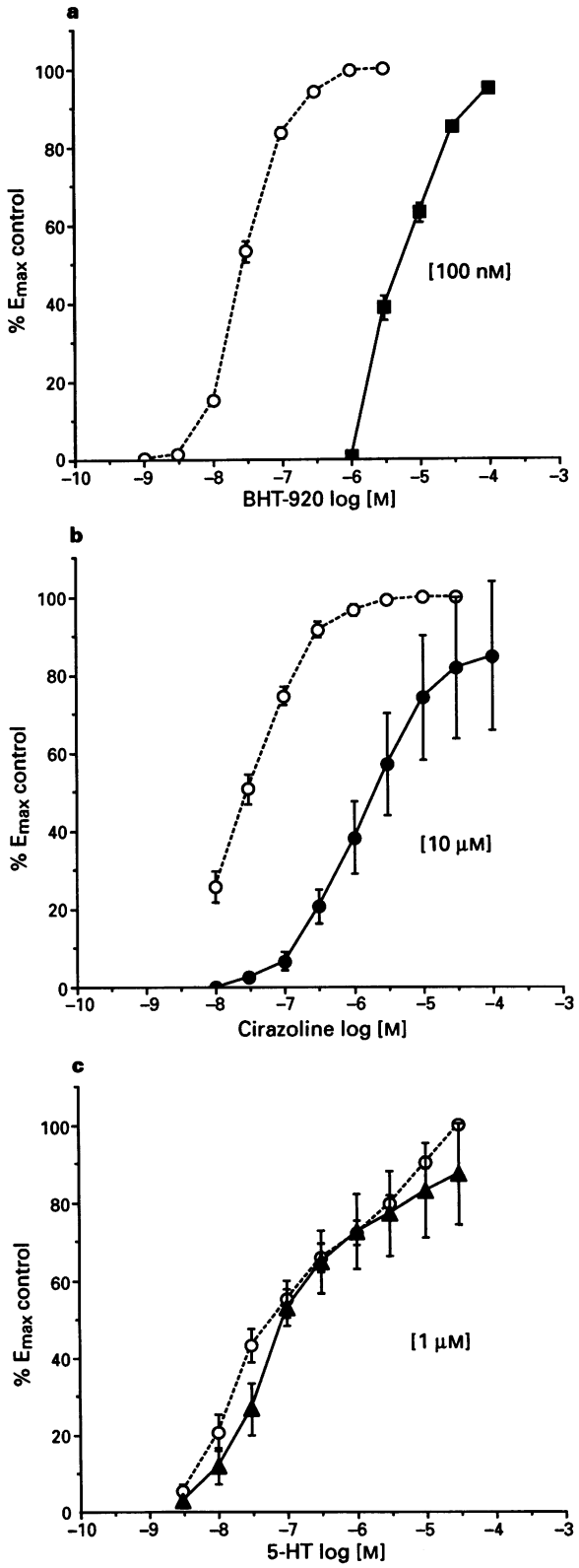

Figure 4 Contractile concentration-response curves for (a) BHT-920, (b) cirazoline and (c) 5-HT in dog saphenous vein rings in the absence (○) or presence of different concentrations of RS-15385-197 (■ 100 nM; ● 10 μM; ▲ 1 μM). Results are expressed as % maximum response to each agonist ± s.e. mean ($n = 4-7$ preparations per curve).

Prejunctional α_2-adrenoceptor effects UK-14,304 produced a dose-related decrease in the tachycardia evoked by continuous electrical stimulation of the thoracic spinal sympathetic outflow. RS-15385-197 progressively displaced the UK-14,304 dose-response curve to the right in a dose-related manner, without reduction in the maximum response (Figure 7c). The displacements were apparently greater than comparable doses tested against the pressor responses to UK-14,304 (cf. Figure 7a and 7c).

Mydriasis studies

Before drug administration, pupil diameter in the anaesthetized rat was around 0.2–0.3 mm. UK-14,304 (1–1000 μg kg^{-1}, i.v.) produced a dose-related increase in pupil diameter, reaching a maximum of about 4 mm. The dose used for the reversal studies (100 μg kg^{-1}, i.v.) was at approximately the

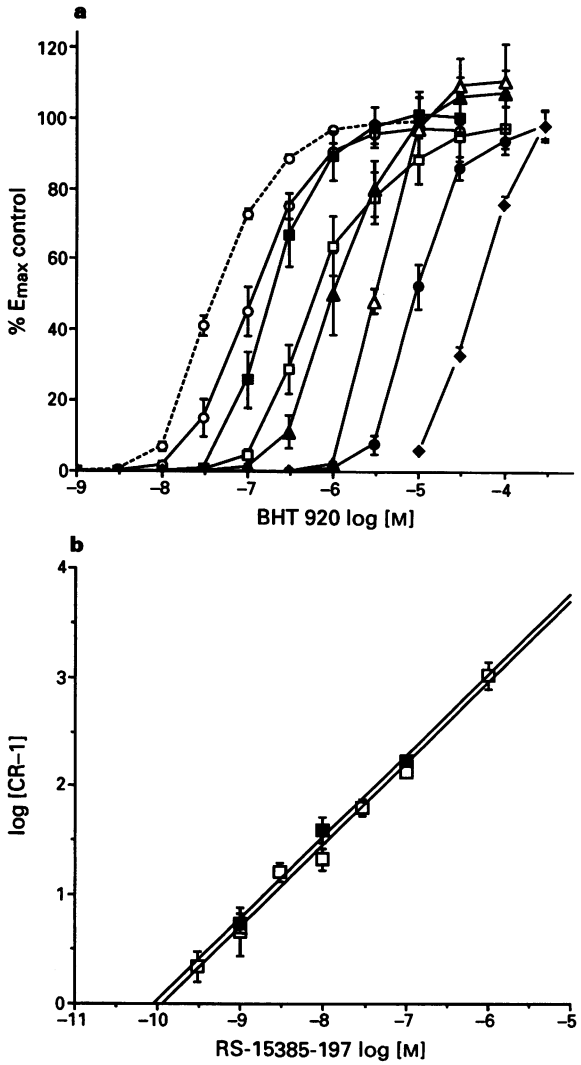

Figure 5 Contractile concentration-response curves for BHT-920 in dog isolated saphenous vein rings treated with phenoxybenzoamine (10 nM) in the absence (○, dashed line), or presence of different concentrations of RS-15385-197: (○) 0.3 nM; (■) 1 nM; (□) 3 nM; (▲) 10 nM; (△) 30 nM; (●) 100 nM; and (◆) 1000 nM. Results are expressed as % max response to BHT ± s.e.mean; n = 4–7 preparations per curve. (b) Schild analysis of the log [CR-1] versus −log [M] concentration of RS-15385-197 obtained in phenoxybenzamine-treated veins (■) and the superimposed Schild plot obtained in non-treated preparations (□).

ED_{90} level, and produced a sustained mydriasis for the duration of the antagonist dose-response curve.

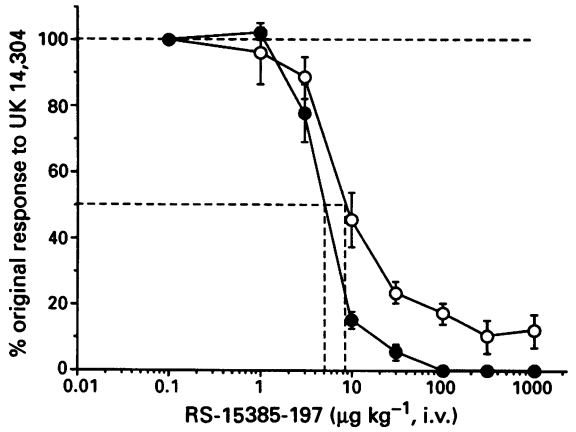

Figure 6 Reversal of response to UK-14,304 by cumulative doses of RS-15385-197: (○) reversal of diastolic pressor response to a standard challenge dos (30 μg kg^{-1}, i.v.) of UK-14,304 in rats pretreated with prazosin (100 μg kg^{-1}, i.v.; $n = 6$). Note the residual pressor response of about 10% of the original control response. (●) Reversal of mydriasis induced by UK-14,304 (100 μg kg^{-1}, i.v.) in anaesthetized rats ($n = 6$).

Reversal of agonist response/oral activity RS-15385-197 reversed the mydriatic response to UK-14,304 (100 μg kg^{-1}, i.v.) with an AD_{50} of 5 μg kg^{-1}, i.v. ($n = 6$), achieving complete reversal at 100 μg kg^{-1}, i.v. (Figure 6). This was far more potent than the other antagonists tested (Table 4). RS-15385-197 reversed the response to clonidine (300 μg kg^{-1}, s.c.) with an i.v. AD_{50} of 7 μg kg^{-1} ($n = 6$) and an oral AD_{50} of 95 μg kg^{-1} ($n = 6$). Idazoxan was less potent by either route (AD_{50} value of 17 and 1200 μg kg^{-1} respectively). Yohimbine and piperoxan were also weaker antagonists than RS-15385-197 when tested intravenously against clonidine (Table 4).

Table 4 Antagonism of responses to UK-14,304 and clonidine *in vivo*

	Peripheral AD_{50} (μg kg^{-1}, i.v.)		Central AD_{50} (μg kg^{-1}, i.v.)	
	UK-14,304	*Clonidine*	*UK-14,304*	*Clonidine*
RS-15385-197	7	7	5	7
L-649,066	650	–	>10000	–
Yohimbine	900	130	80	185
Idazoxan	>1000	79	20	17
Piperoxan	–	2000	–	2400

The AD_{50} value is the antagonist dose which reduced the agonist response by 50%. Data are the mean from at least 5 rats.

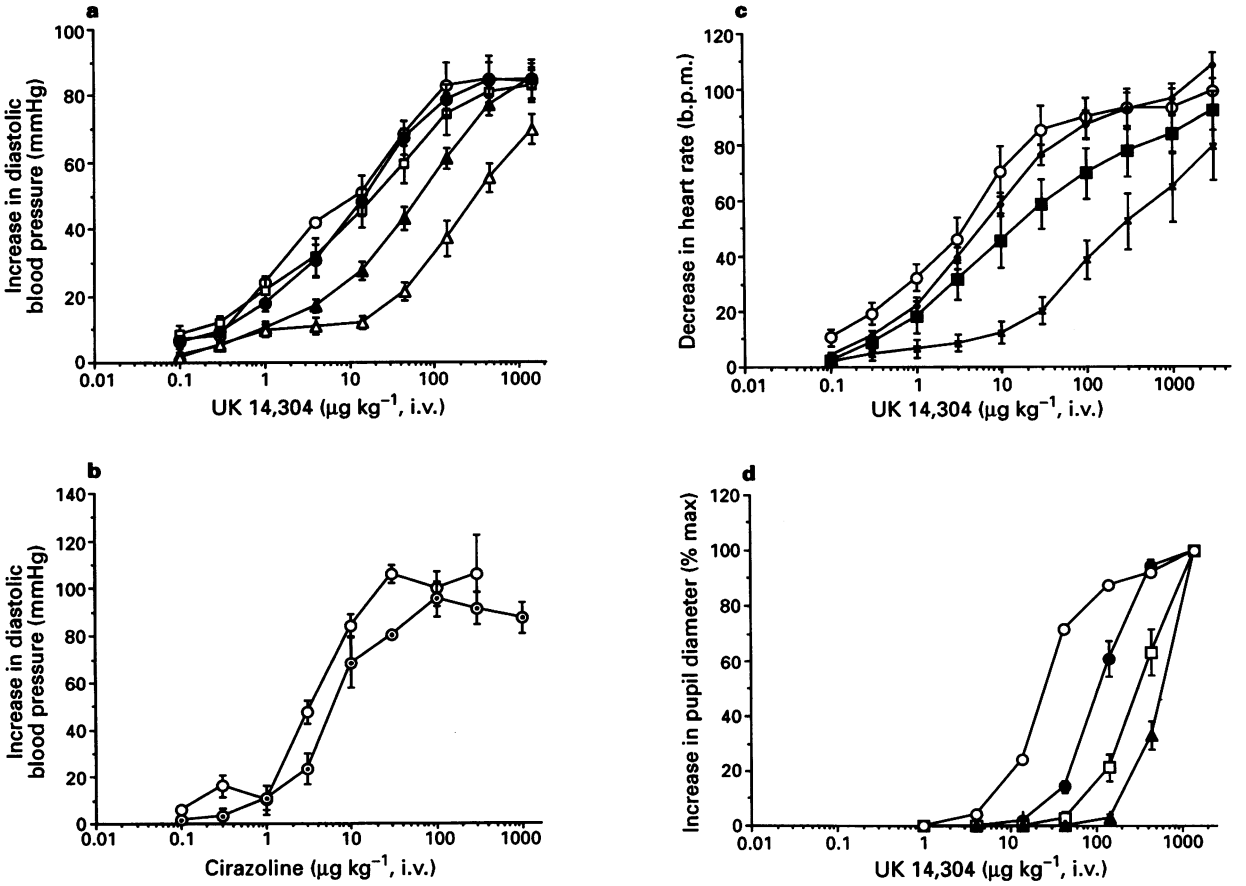

Figure 7 Displacement of agonist dose-response curves by RS-15385-197 *in vivo*. (a) Diastolic pressor response curves to cumulative doses of UK-14,304 in pithed rats pretreated with prazosin (100 μg kg^{-1}, i.v.); (b) diastolic pressor response curves to cirazoline in pithed rats; (c) bradycardiac response curves to UK-14,304 in pithed rats during stimulation of the cardiac sympathetic outflow; (d) mydriatic response curves to UK-14,304 in anaesthetized rats. (○) Control ($n = 6$–10); (●) RS-15385-197 9 μg kg^{-1}, i.v. ($n = 6$); (◆) 10 μg kg^{-1}, i.v. ($n = 6$); (■) 30 μg kg^{-1}, i.v. ($n = 6$); (□) 45 μg kg^{-1}, i.v. ($n = 6$); (×) 100 μg kg^{-1}, i.v. ($n = 6$); (▲) 225 μg kg^{-1}, i.v. ($n = 6$); (△) 1000 μg kg^{-1}, i.v. ($n = 6$); (○) 10000 μg kg^{-1}, i.v. ($n = 6$).

Displacement of agonist dose-response curves RS-15385-197 (9, 45 or 225 $\mu g\,kg^{-1}$, i.v.) caused a dose-related rightward displacement of the dose-response curve to UK-14,304 (Figure 7d). Idazoxan and yohimbine were less potent (data not shown). The dose-response curves in this assay were steeper than in the pithed rat pressor response assay and all three antagonists produced greater displacement of the agonist dose-response curve in the mydriasis assay than in the pithed rat pressor response tests.

Time course of antagonism following an oral dose At a dose of 1 $mg\,kg^{-1}$, p.o., RS-15385-197 had a dose-ratio of 46 after 1 h. This diminished over time, with an apparent effective half-life of around 2.5 h. Idazoxan, although given at a larger dose (30 $mg\,kg^{-1}$, p.o.), was less effective (dose-ratio 1 h after dosing was 40) and had a shorter duration of action (apparent effective half life $\sim$1.5 h).

Discussion

The present results demonstrate that RS-15385-197 is a highly potent α_2-adrenoceptor antagonist in a variety of test systems. The compound has exceptional affinity for the α_2-adrenoceptor in a range of tissues from different species with a $pK_i \geqslant 10$ in baboon cortex. The compound had essentially similar affinity in displacing [^{3}H]-yohimbine and [^{3}H]-idazoxan from α_2-adrenoceptors. RS-15385-197 has no affinity for imidazoline binding sites in hamster adipocytes, which provides further evidence that the α_2-adrenoceptor and the imidazoline binding site are very different recognition sites and probably represent different proteins. Furthermore, the structure-activity relationships for imidazolines at α_2-adrenoceptors and imidazoline binding sites are very different (Clark *et al.*, 1989b; 1991). RS-15385-197 did not differentiate between α_{2A} and α_{2B} sites, as represented by affinity for the human platelet and rat neonatal lung, consistent with previous data on [^{3}H]-RS-15385-197 binding in these preparations (MacKinnon *et al.*, 1992). These sites apparently have different genes (α_2 C10 represents α_{2A} whereas α_2 C2 represents α_{2B}; Lomasney *et al.*, 1990), yet RS-15385-197 had very similar affinity (pK_i 9.7 to 9.9) indicating that the recognition sites on these two subtypes show some similarities. However, RS-15385-197 did have significantly lower affinity (pK_i 8.38) for the α_{2A}-'like' subtype in hamster adipocytes, which provides further evidence that this site is different from the 'classical' α_{2A} subtype on human platelets (see also Brown *et al.*, 1990a). Yohimbine also shows lower affinity for this site (MacKinnon *et al.*, 1989). In this respect, high doses of RS-15385-197 (30 $\mu g\,kg^{-1}$, i.v.) are required to increase non-esterified fatty acid levels in dog plasma (unpublished observations).

The particular subtypes of α_2-adrenoceptors which mediate responses to α_2-adrenoceptor agonists in the guinea-pig ileum, rat vas deferens and dog saphenous vein are as yet unclear, and species differences may exist (Ruffolo *et al.*, 1991; Limberger *et al.*, 1992; Smith *et al.*, 1992). RS-15385-197 exhibited no selectivity between prejunctional (pA_2 in guinea-pig ileum 9.7) and postjunctional (pA_2 in dog saphenous vein 10.0) α_2-adrenoceptors *in vitro*. However, a slightly lower pA_2 value (9.3) with a slope less than unity (0.7) was obtained in the rat vas deferens. The reason for this is unknown, but it could be due to the presence of a mixed population of subtypes of prejunctional α_2-adrenoceptors, as has been suggested recently for this tissue (Harsing & Vizi, 1992; Smith *et al.*, 1992).

In contrast, RS-15385-197 had very low affinity for the α_1-adrenoceptor in rat cortex ([^{3}H]-prazosin binding) and in functional assays, with approximately 4000–14000 fold selectivity for α_2- over α_1-adrenoceptors. The α_1-adrenoceptor antagonist affinity against phenylephrine in rabbit aorta, and against cirazoline in dog saphenous vein were virtually identical; the α_1-adrenoceptor stimulated by cirazoline in this latter tissue has been considered to represent the α_{1A}-adrenoceptor subtype (Hicks *et al.*, 1991). In both binding and functional studies the −198 enantiomer was essentially devoid of α_2-adrenoceptor affinity, indicating a highly stereoselective interaction at the α_2-adrenoceptor. The structure-activity relationship for the interaction of various analogues of RS-15385 at the α_2-adrenoceptor has been described by Clark *et al.* (1991). It is quite possible that the residual activity of RS-15385-198 at the α_2-adrenoceptor may be due to contamination with the −197 isomer. The high performance liquid chromatography assay for RS-15385-198 only assures 99.9% purity and a small contamination with the active isomer could account for the activity observed with RS-15385-198. RS-15385-197 is therefore a potent α_2-adrenoceptor antagonist with greater selectivity over other receptors than previously available agents, having lower affinity for 5-HT receptors than yohimbine and rauwolscine and negligible affinity for imidazoline-preferring sites, unlike idazoxan. [^{3}H]-RS-15385-197 has recently been shown to be an excellent radioligand for the study of α_2-adrenoceptor subtypes (MacKinnon *et al.*, 1992).

In pithed rats, when a protocol of reversal of responses evoked by the selective α_2-adrenoceptor agonist, UK-14,304 was used, RS-15385-196 was a more potent antagonist than yohimbine, idazoxan, piperoxan or the peripherally-selective α_2-adrenoceptor antagonist, L-659,066. Using this protocol, we observed a small residual ($\sim$10%) pressor response to UK-14,304 which was still present after exclusion of both α_1-adrenoceptors (with prazosin) and α_2-adrenoceptors (with RS-15385-197, idazoxan, yohimbine or L-659,066). The cause of this small residual response to UK-14,304 is unknown; it is clearly not mediated by α-adrenoceptors and is therefore due to some unknown effect of UK-14,304. The residual response was excluded from the data analysis accordingly. When a range of doses of RS-15385-197 was tested for displacement of the full dose-response curve to UK-14,304 in pithed rats, it was estimated to give a dose-ratio of 2 at a dose of 45 $\mu g\,kg^{-1}$, i.v., indicating that it was 4 fold more potent than either idazoxan or yohimbine, and >5 fold more potent than L-659,066, which has previously reported to have a DR_2 of 264 $\mu g\,kg^{-1}$, i.v., in this test (Clineschmidt *et al.*, 1988). RS-15385-197 was highly selective *in vivo* for α_2-adrenoceptors compared with α_1-adrenoceptors over the dose-range tested, since even a very large dose (10 $mg\,kg^{-1}$, i.v.) showed minimal antagonism of the pressor responses induced by the selective α_1-adrenoceptor agonist, cirazoline.

In anaesthetized rats, RS-15385-197 was a potent antagonist of the mydriasis response induced by UK-14,304 or clonidine. The i.v. AD_{50} values obtained (5 and 7 $\mu g\,kg^{-1}$ respectively) corresponded to that obtained in the pithed rat experiments (7 $\mu g\,kg^{-1}$, i.v.), indicating that RS-15385-197 readily penetrates into the CNS. However, this equipotency was not confirmed by more detailed studies on displacement of the full dose-response curve to UK-14,304: RS-15385-197 was apparently more potent as an antagonist of central and of prejunctional respones to UK-14,304, than of the postjunctional pressor response. This is unlikely to be due to differences in receptor subtypes for the three responses as, in our binding studies, RS-15385-197 did not differentiate between the two α_2-adrenoceptor subtypes tested, i.e. α_{2A} (in human platelets) and α_{2B} (in rat neonate lung), and both idazoxan and yohimbine, which have different selectivities for these α_2-adrenoceptor subtypes, were also apparently more potent in displacing agonist dose-response curves in the mydriasis assay than in the pithed rat pressor response test. It is possible that RS-15385-197, yohimbine and idazoxan may be preferentially distributed in the brain, but a more likely explanation is that there is a non-adrenoceptor component in the pressor response to UK-14,304, as observed in the agonist reversal studies. Alternatively, the differences may reflect non-equilibrium states (Kenakin, 1987) and indicate the limitations of comparing different tests *in vivo*. This issue can only be resolved by further studies.

RS-15385-197 was also a potent α_2-adrenoceptor antagonist following oral administration, which was apparent both in experiments on reversal of agonist-evoked mydriasis and in the displacement of the agonist dose-response curves. These latter studies also showed that RS-15385-197 was relatively long-acting, compared to idazoxan, with an apparent biological half-life of around 2.5 h following oral administration.

In summary, RS-15385-197 is a potent, selective, competitive α_2-adrenoceptor antagonist *in vitro* and *in vivo*, which is orally active and readily penetrates the brain. It should prove to be a powerful tool with which to explore the various physiological roles of α_2-adrenoceptors in animals and man.

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Modulation of central noradrenergic function by RS-15385-197

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1 RS-15385-197, a highly potent and selective α_2-adrenoceptor antagonist, was examined in a variety of *in vitro* and *in vivo* functional tests to assess the selectivity of its interaction with central noradrenergic neurones in the rat.

2 In hypothalamic slices, RS-15385-197 was potent in augmenting K^+-evoked release of [^{3}H]-noradrenaline, with an EC_{50} of 9 nM. Idazoxan and yohimbine showed 100 fold less activity. This was due to its antagonist action at presynaptic α_2-adrenoceptors, as RS-15385-197 (10 μM), did not directly release [^{3}H]-noradrenaline from cortical slices unlike reserpine (10 μM), and did not inhibit noradrenaline re-uptake into cortical synaptosomes.

3 *In vivo*, RS-15385-197 (0.5 mg kg^{-1}, p.o.) increased levels of 3-methoxy-4-hydroxy-phenylglycol (MHPG) in the cerebral cortex without modifying levels of 5-hydroxyindoleacetic acid (5-HIAA). This dose, but not a lower dose (0.1 mg kg^{-1}, p.o.) caused β-adrenoceptor down-regulation in the cortex when administered once daily for 14 days whereas 5-HT_2 receptor number was unaltered, indicating a selective effect on noradrenergic transmission.

4 Selective depletion of cortical 5-HT by administration of *p*-chlorophenylalanine (PCPA; 100 mg kg^{-1}, i.p. for 14 days) or 5,7-dihydroxytryptamine (5,7-DHT; 150 μg i.c.v.) prevented the β-adrenoceptor down-regulation caused by RS-15385-197, indicating that a tonic 5-hydroxytryptaminergic input was required for it to elicit β-adrenoceptor down-regulation. It was not possible to prevent the loss of activity of RS-15385-197 in these 5-HT-depleted animals by co-administration with the 5-HT_{1A} partial agonist, 8-hydroxy-n-dipropyl aminotetralin (8-OH-DPAT, 0.3 mg kg^{-1}, i.p. twice daily for final 3 days).

5 At a dose (1 mg kg^{-1}, p.o.) which completely prevented the hypoactivity produced by clonidine (0.1 mg kg^{-1}, p.o.), RS-15385-197 did not affect behavioural stereotypy induced by 8-OH-DPAT (0.3 mg kg^{-1}, s.c.). Similarly, following chronic dosing with the racemate, RS-15385-196 (3 mg kg^{-1}, p.o., once daily for 14 days), there was no effect on the behavioural and hypothermic response to 8-OH-DPAT (0.5 mg kg^{-1}, s.c.). Therefore, RS-15385-197 was selective for central α_2-adrenoceptors over 5-HT_{1A} receptors in *in vivo* functional tests.

6 Thus, RS-15385-197 was highly selective in interacting with central noradrenergic neurones in the rat *in vitro* and *in vivo*. It is therefore currently the agent of choice for investigations of the role of α_2-adrenoceptors in the CNS.

Keywords: RS-15385-197; α_2-adrenoceptors; yohimbine; noradrenaline; 5-HT; β-adrenoceptor down-regulation

Introduction

RS-15385-197 is a highly potent and selective α_2-adrenoceptor antagonist (Clark *et al.*, 1989; 1991; Brown *et al.*, 1990a; 1993; MacKinnon *et al.*, 1991), which is >100 fold more selective for α_2-adrenoceptors versus other receptors tested, than previously used compounds such as yohimbine, rauwolscine and idazoxan. Previous α_2-adrenoceptor antagonists have been reported to cause a variety of central effects such as β-adrenoceptor down-regulation, a potential index of depression, but also anxiety and other behavioural effects (Holmberg & Gershon, 1961; Goldberg & Robertson, 1983; Charney *et al.*, 1983). However, because of the low selectivity of the agents used it is not clear whether these effects relate specifically to antagonism at α_2-adrenoceptors. Autoradiographic studies with [^{3}H]-rauwolscine and [^{3}H]-idazoxan have revealed complex patterns of distribution within the brain, with the ligands labelling different brain areas, and labelling apparently different populations of α_2-adrenoceptors (Boyajian *et al.*, 1987). While some of these differences may be consequent to different α_2-adrenoceptor subtypes, they may also be secondary to the lack of selectivity of the radioligands. For example, yohimbine has high affinity for some of the 5-HT receptor subtypes (<10 fold selectivity for α_2-adrenoceptors over 5-HT_{1A} and 5-HT_2 receptors; McGrath *et al.*, 1989), [^{3}H]-rauwolscine has been shown to label 5-HT_1-like sites in human and rabbit cerebral cortex (Convents *et al.*, 1988), and idazoxan has equivalent affinity for 'imidazoline binding sites' compared with affinity for α_2-adrenoceptors (Brown *et al.*, 1990b).

While RS-15385-197 is the most potent and selective α_2-adrenoceptor antagonist available, with some 4000–14,000 fold selectivity for α_1- vs. α_2-adrenoceptors (Brown *et al.*, 1993) it does not distinguish between α_{2A}- or α_{2B}-adrenoceptor subtypes, nor between pre- and postsynaptic α_2-adrenoceptors (MacKinnon *et al.*, 1992; Brown *et al.*, 1993). The compound may therefore be used to define the contribution of all α_2-adrenoceptors in a given experimental situation. In the present study we have investigated whether RS-15385-197 selectively increases noradrenergic activity in the brain and whether this results in selective effects on β-adrenoceptor number in cerebral cortex, compared with effects on 5-HT_2 receptor number. We show that β-adrenoceptor number is down-regulated following 14 days treatment with RS-15385-197, but requires a tonic 5-hydroxytryptaminergic input; this tonic input cannot be mimicked by a 5-HT_{1A} partial agonist. In addition, selective α_2-adrenoceptor antagonism with RS-15385-197 attenuates behavioural responses to clonidine without affecting 5-HT-related behaviour.

We have used predominantly the active -197 enantiomer in these studies, but also describe some studies with the racemate, RS-15385-196. A preliminary account of part of

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this work has been published as an abstract (Brown *et al.*, 1990a).

Methods

Animals

Male Sprague-Dawley rats (Charles River, U.K.) were used. They arrived in the animal unit at least 2 days before beginning the studies and were housed in pairs, on a 12 h light-dark cycle (on: 08 h 00 min; off: 20 h 00 min) with free access to food and water. Behavioural experiments were conducted at an ambient temperature of 19–22°C, with the identity of the treatments unknown to the observer. All dissections of brain tissue were carried out over ice.

K^+-evoked release of [^{3}H]-(−)-noradrenaline ([^{3}H]-NA) in hypothalamic slices

Tissue preparation The experiments on release of [^{3}H]-NA were carried out essentially as described by Middlemiss & Spedding (1985). Coronal slices (250 μm) of hypothalami from rats (300–350 g) were made with a McIlwain tissue chopper. The slices were suspended in 5 ml of Krebs-Ringer bicarbonate buffer (composition mM: NaCl 135, KCl 5, $NaHCO_3$ 25, $MgSO_4$, $MgSO_4$ 1.25, $CaCl_2$ 2, glucose 10, pH = 7.4, gassed with 95% O_2:5% CO_2) containing 10 μM pargyline and 0.1 μM [^{3}H]-NA (15 Ci mmol^{-1}) and incubated in a shaking waterbath for 15 min at 37°C. The slices were given three 5 ml washes with Krebs buffer and 50 μl aliquots transferred onto gauze filters in each of eight perfusion chambers. The slices were superfused with Krebs buffer containing 10 μM pargyline and 3 μM desmethylimipramine (DMI) for 30 min at a rate of 0.4 ml min^{-1} to allow the basal efflux of [^{3}H]-NA to stabilize prior to collection of fractions.

Stimulation of releases The protocol involved the collection of 19 consecutive 4 min fractions. Two identical 4 min pulses of high K^+ (25 mM) Krebs buffer, the Na^+ concentrations being reduced to 115 mM to maintain isomolarity, were timed to coincide with the start of fraction 3 and fraction 13. Drugs were added to the superfusate 20 min before the second stimulus and were present throughout the rest of the superfusion period. At the end of the experiment, the released [^{3}H]-NA in the superfusate fractions was measured by liquid scintillation spectrometry. The slices were sonicated in the perfusion chambers (setting 2 for 5 s) and a 50 μl aliquot taken for scintillation counting. Tritium content was assessed using 8 ml scintillation fluid ('Ready Safe', Beckman) with a 3 min count time, and d.p.m. calculated from an internal quench curve entered into the Packard counter.

Data analysis The tritium content of each superfusate fraction was expressed as a percentage of the radioactivity in the slices at the onset of the respective 4 min collection period. The additional tritium efflux evoked by K^+ depolarization was calculated by subtracting estimated basal values, assumed to decline linearly, from the total overflow of [^{3}H]-NA during fractions 3 to 7 for the first stimulus (S1) and fractions 13 to 17 for the second stimulus (S2). Drug effects were assessed by determining the S2/S1 ratio over a range of concentrations, where S1 is the control stimulation for each channel and S2 is the drug modulated stimulation (to at least $n = 4$). EC_{50} values were obtained graphically from concentration-response curves constructed by averaging S2/S1 ratios for each drug concentration and plotting mean ± s.e.mean for each drug concentration.

Direct release of [^{3}H]-(−)-noradrenaline in cortical slices

Cortical slices (250 × 250 μm) were prepared from rats (240–280 g) using a McIlwain tissue chopper. The slices were pre-loaded with 0.1 μM [^{3}H]-NA (39.4 Ci mmol^{-1}) in Krebs-Ringer bicarbonate buffer (composition as above) and incubated in a shaking waterbath for 15 min at 37°C. The slices were harvested, washed three times with buffer, and 50 μl of lightly packed slices were aliquoted into an 8-channel superfusion system linked to a perfusion pump and a fraction collector. A flow rate of 0.4 ml min^{-1} was maintained throughout the experiment. Buffer, containing 3 μM DMI, was perfused through the slices for 30 min to allow basal efflux of [^{3}H]-NA to stabilize prior to collection of 19 consecutive 5 min fractions. After obtaining a 5 min pre-drug fraction, reserpine (10 μM) or RS-15385-197 (10 μM) was added to the superfusion buffer. The radioactivity in each fraction was determined by liquid scintillation counting. In order to allow normalization of each individual channel of the superfusion, the results were calculated as:

$$\text{release ratio} = \frac{\text{radioactivity in drug fractions}}{\text{radioactivity in pre-drug fraction}}$$

Uptake of [^{3}H]-(−)-noradrenaline into cortical synaptosomes

Preparation of synaptosomes Crude synaptosomes (P_2) were prepared by the method of Gray & Whittaker (1962). Rat cerebral cortex was dissected out and homogenized in 0.32 M sucrose using 2–3 strokes of a Potter-S homogenizer with a Teflon pestle. The homogenate was centrifuged at 1000 *g* for 10 min at 4°C, the pellet discarded and the supernatant centrifuged at 12000 *g* for 20 min at 4°C. The supernatant was discarded and the pellet resuspended in the original volume of 0.32 M sucrose and again centrifuged at 12000 *g* for 20 min at 4°C. The resulting crude mitochondrial pellet (P_2), containing synaptosomal material, was resuspended in 0.32 M sucrose at approximately 1 ml g^{-1} of original weight of starting material.

Measurement of uptake of [^{3}H]-NA A modification of the method of Kilpatrich *et al.* (1986) was used. All additions to the assay, with the exception of the synaptosomes, were prepared in Tris-Krebs buffer of the following composition (mM): NaCl 136, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 10, ascorbate 1, Tris base 20, adjusted to pH 7.4 with HCl. The buffer was gassed with oxygen for 30 min before use. Uptake studies were carried out in a system containing 700 μl Tris-Krebs buffer, 100 μl competing drugs over the concentration range 0.1 nM to 1 mM, and 100 μl synaptosomes. Following 5 min pre-incubation at 37°C, uptake was initiated by the addition of 100 μl [^{3}H]-NA (40.4 Ci mmol^{-1}) to give a final concentration of 100 nM. Uptake was terminated after 2 min by the addition of 2.5 ml ice-cold Tris-Krebs buffer followed by rapid filtration over Whatman GF/B filters. The filters were washed twice with a further 2 ml Tris-Krebs buffer, dried and placed in 8 ml scintillation fluid. The radioactivity was determined by liquid scintillation counting. Each determination was carried out in duplicate.

Protein determinations Protein was measured by the method of Sorensen & Brodbeck (1986) using the Pierce BCA reagent and bovine serum albumin as standard.

Analysis of inhibition data Analysis of the inhibition curves for [^{3}H]-NA uptake was carried out with a non-linear least squares parametric curve-fitting programme capable of estimating the IC_{50}, i.e., the concentration of drug that inhibited the uptake of [^{3}H]-NA by 50%.

Determination of biogenic amines and amine metabolites

Rats (255–295 g) were dosed with RS-15385-197 (0.5 mg kg^{-1}, p.o., $n = 12$) or distilled water (5 ml kg^{-1}, p.o., $n = 12$). One or two hours later the animals were killed by stunning and cervical dislocation. The cerebral cortex was immediately

dissected, weighed, and frozen in liquid nitrogen. Samples were sonicated, frozen into 4 ml of 0.1 M perchloric acid using a Branson sonicator 450 (setting 2, 15 s). The resulting homogenates were centrifuged at 52,000 *g* for 15 min and the supernatant filtered through millipore HV4 filters (pore size 0.45 μm).

The levels of monoamines and metabolites in cortical tissue were measured by high performance liquid chromatography coupled to electrochemical detection. The separations used were a modification of the method of Mefford (1981). The supernatant was divided into two with 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) being measured using 'system A' and NA, 3-methoxy-4-hydroxy-phenylglycol (MHPG) and dopamine estimated using 'system B'. System A consisted of an Altex 110A pump, Kontron MS1 660 autosampler, ultrasphere reverse phase ion-pair column (25 cm × 4.5 mm i.d., 5 μm particle size) and a Bioanalytical systems LC-4A amperometric electrochemical detector. Buffer [composition: Na acetate (0.1 M), citric acid (0.1 M), Na_2 EDTA (0.5 mM); pH 4.1 and methanol (15% v/v)] was maintained at a flow rate of 1 ml min^{-1}. The amperometric, glassy carbon electrode was set at + 0.72V and a sensitivity of 2 nA was used. System B consisted of a Gilson 302 pump and 231 sample injector, ultrasphere reverse phase ion-pair column (25 cm × 4.5 mm i.d., 5 μm particle size) and an ESA coulometric electrochemical detector. Buffer [composition: Na acetate (0.09 M) citric acid (0.035 M), Na_2 EDTA (130 μM), Na 1-octane sulphonic acid (0.5 mM); pH 4.25 and methanol (8% v/v)] was pumped at 1 ml min^{-1} through porous graphite electrodes at the following potentials: guard cell + 0.45V, electrode 1, 0.001V and electrode 2, − 0.3V. Peak heights were measured with a Trio chromatography computing integrator and the level of the neurotransmitters and metabolites in the cortical samples calculated *via* interpolation of a standard curve constructed from a range of standards.

Cortical β-adrenoceptor down-regulation

Dosing protocol Rats were dosed with RS-15385-197 (0.1 or 0.5 mg kg^{-1}), RS-15385-196 (0.5, 1.0, 3.0 or 10 mg kg^{-1}) or distilled water by oral intubation once or twice daily for 3 or 14 days; 24 h after the last dose the animals were killed by cervical dislocation, the brain rapidly removed and the cerebral cortex frozen in liquid nitrogen. In a separate study using 14 days dosing with RS-15385-197 (0.5 mg kg^{-1}, p.o., once daily), three groups were prepared with modification to central 5-HT function:

Prolonged activation of 5-HT_{1A} receptors One group of rats received 8-hydroxy-n-dipropylaminotetralin (8-OH-DPAT, 0.3 mg kg^{-1}, i.p.) twice daily during the final 3 days of dosing with RS-15385-197.

Depletion of brain 5-HT with p-chlorophenylalanine (PCPA) One group of rats received PCPA (100 mg kg^{-1}, i.p.) once daily throughout the period of dosing with RS-15385-197. At the end of the study, cortical levels of noradrenaline and 5-HT were determined as described above. Animals treated with PCPA were excluded from the study if their cortical 5-HT level was within the range of the controls, or if their cortical NA level was outside the range of the controls.

Destruction of 5-HT neurones by intracerebroventricular (i.c.v.) injection of 5,7-dihydroxytryptamine (5,7-DHT) Rats weighing 160–260 g were used. Surgery was carried out under aseptic conditions. Each rat received an injection of DMI (25 mg kg^{-1}, i.p.) 60 min before the i.c.v. injection of 5,7-DHT or vehicle, to prevent uptake of the neurotoxin by noradrenergic neurones (Bjorklund *et al.*, 1975). 5,7-DHT was dissolved in a vehicle of 0.1% w/v ascorbic acid in distilled water, which had been gassed with nitrogen. Solutions were kept frozen in the dark between injections for a maximum of 2 h after preparation, and used within 10 min of thawing. Rats were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p.) and placed in a stereotaxic headholder in the orientation used in the atlas of Paxinos & Watson (1982). Core temperature was monitored by a rectal thermocouple and maintained at 36–37°C with a heating lamp. The neurotoxin was administered *via* a 50 μl glass syringe lowered by a micromanipulator so that the needle tip was 3 mm below the brain surface, at 0.8 mm posterior to Bregma and 1.4 mm to the right of the midline suture. The rats received an i.c.v. injection of either 5,7-DHT (150 μg in 20 μl vehicle) or 20 μl vehicle. In one rat, the injection of 20 μl Indian Ink confirmed that these coordinates were correct, the ink being distributed throughout the ventricles as far as the spinal canal. Animals were allowed to recover for 7–11 days before dosing with RS-15385-197. At the end of the study, cortical levels of noradrenaline and 5-HT were determined as described above. The same data exclusion criteria were imposed as used for PCPA.

Assay of cortical β-adrenoceptors and 5-HT_2 receptors Cortices were homogenized in 25 volumes of ice cold Tris HCl buffer (pH 7.4) using a polytron PT10 tissue disruptor and centrifuged at 49,000 *g* for 15 min. The resultant pellet was washed three times by repeated resuspension and centrifugation and the final pellet resuspended in 3 ml Tris HCl buffer (50 mM; pH 7.4) and frozen under liquid nitrogen; 200–300 μg membrane protein was incubated with increasing concentrations of [^{3}H]-dihydroalprenolol ([^{3}H]-DHA; 0.1–10 nM) or [^{3}H]-ketanserin (0.05–5 μM; 5-HT_2) in a final volume of 0.5 ml Tris HCl buffer (pH 7.4) for 30 min at 22°C. Non-specific binding was determined in the presence of 0.2 mM isoprenaline or 2 μM methysergide (5-HT_2). Bound ligand was separated from free by filtration over Whatman GFB filters on a Brandel 24 port Cell Harvester. The filters were suspended in 5 ml Ready Safe scintillation cocktail and counted in a Beckman scintillation counter. Protein determination was as described above.

Data analysis Analysis of saturation data was by use of the Scafit non-linear regression analysis programme LIGAND (Munson & Rodbard, 1980). Statistical significance was assessed by the unpaired Student's *t* test.

Antagonism of clonidine-induced hypoactivity

Thirty min after dosing with RS-15385-197 (0.1, 1 or 10 mg kg^{-1}, p.o.; $n = 6$ per group) or water (10 ml kg^{-1}, p.o.; $n = 6$) the rats (120–180 g) were given a dose of clonidine (0.1 mg kg^{-1}, p.o.). After a further 30 min they were placed singly in clear plastic cages (22 × 38 cm base, 15 cm high) positioned on an activity monitor (Ormed Instruments), and their general activity measured over 9 min. Comparison with the control clonidine group was made by Dunnett's *t* test.

5-HT_{1A} behavioural syndrome (acute dosing)

The experiments were conducted between 09 h 30 min–16 h 30 min. Thirty min after dosing with RS-15385-197 (1 mg kg^{-1}, p.o.; $n = 6$) or water (10 ml kg^{-1}, p.o.; $n = 6$) the rats (250–300 g) were dosed with 8-OH-DPAT (0.3 mg kg^{-1}, s.c.) and placed singly in clear plastic cages (22 × 38 cm, base, 15 cm high) positioned on an activity monitor (Ormed Instruments), and observed for 15 min. Total counts over this 15 min period were recorded. Observation sessions of 45 s per animal were begun 3 min after activating the activity detectors, and repeated every 3 min for a period of 15 min. Reciprocal forepaw treading, head weaving and flat body posture were assessed by a ranked intensity scale (Tricklebank *et al.*, 1985) where 0 = absent, 1 = equivocal, 2 = present and 3 = intense, and the total cumulative score over 15 min was

compiled (i.e. max score = 15 for each behavioural parameter). Data were compared by a Mann-Whitney U-test.

5-HT$_{1A}$ behavioural syndrome and hypothermia (chronic dosing)

Rats, weighing 140–180 g at the start of the study, were randomly assigned to two treatment groups: one group received RS-15385-196 (3 mg kg^{-1}, p.o.; $n = 9$) and the other group were given water (10 ml kg^{-1}, p.o.; $n = 9$). The rats were dosed by oral intubation (10 ml kg^{-1}) once daily (a.m.) for 14 days. The final weight range was 220–270 g. The experiments were conducted between 14 h 30 min–16 h 30 min on the day after the final dose, at an ambient temperature of 20–21°C. The rats had been brought to the testing room at least 2.5 h before beginning the measurements. The rats were placed in well-ventilated perspex holding cages (18 × 12 × 10 cm) and restrained by passing the tail through a 2 cm diameter aperture at the rear of the cage and taping it to a perspex rod which protruded out of the rear wall immediately above the opening. A plastic-covered thermocouple (Light Laboratories or RS Components) was inserted 5 cm past the anal sphincter. The rats were able to move their head and limbs easily and alter their posture, and usually became quiet and assumed a normal raised-hindquarters posture within a few minutes. One hour after placing the rats in the cages, observations were made at 5 min intervals of core temperature and of reciprocal forepaw treading, head weaving and flat body posture, using a ranked intensity scale as described above. Immediately after the third observation 8-OH-DPAT (0.5 mg kg^{-1}, s.c.) was injected, and the response followed over 1 h. Data were compared by a Mann-Whitney U-test.

Drugs

[^{3}H]-DHA (50–60 Ci mmol^{-1}), [^{3}H]-ketanserin (76 Ci mmol^{-1}) and [^{3}H]-NA (15–40.4 Ci mmol^{-1}) were purchased from DuPont UK Ltd. RS-15385-197 and RS-15385-196 were synthesized by Dr R. Clark, Syntex, Palo Alto, Ca, U.S.A. 8-OH-DPAT was purchased from Research Biochemicals, clonidine HCl, 5.7-DHT, isoprenaline bitartrate, pargyline HCl and PCPA from Sigma, reserpine from BDH, DMI HCl from Geigy Pharmaceuticals and pentobarbitone sodium from M & B.

Results

K$^+$-evoked release of [^{3}H]-(−)-noradrenaline in hypothalamic slices

RS-15385-197 potentiated the K$^+$-evoked release of [^{3}H]-NA in a concentration-dependent manner, with an EC_{50} of 1 nM ($n = 7$). Idazoxan ($EC_{50} = 0.1$ μM; $n = 4$) and yohimibine ($EC_{50} = 0.1$ μM; $n = 6$) showed 100 fold less activity.

Direct release of [^{3}H]-(−)-noradrenaline in cortical slices

Reserpine (10 μM) caused a time-dependent increase in the efflux of [^{3}H]-NA from pre-labelled slices of rat cerebral

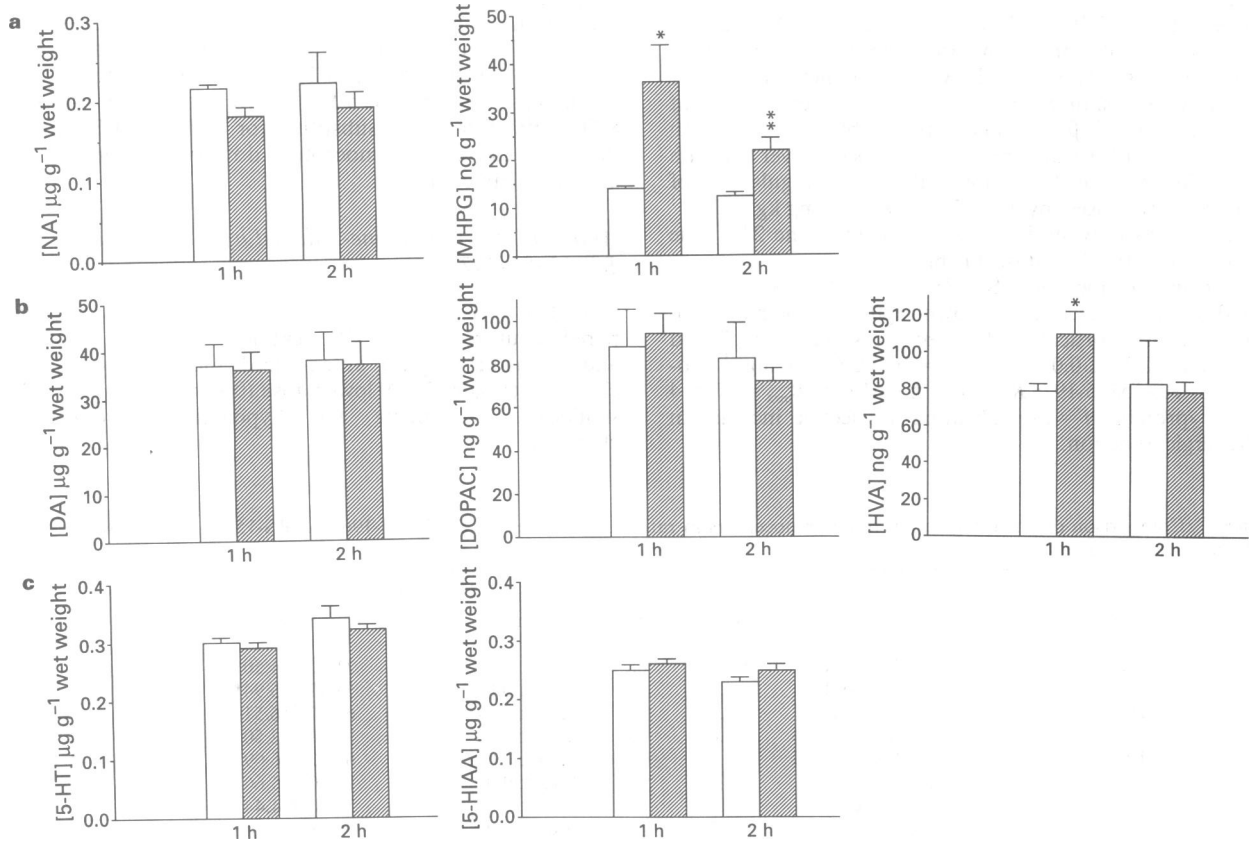

Figure 1 Effects of RS-15385-197 on levels of monoamine neurotransmitters and their metabolites in rat cerebral cortex. Rats were dosed with either water (5 ml kg^{-1}, p.o., $n = 12$; open columns) or RS-15385-197 (0.5 mg kg^{-1}, p.o., $n = 12$; hatched columns) 1–2 h before removal of brain tissue following stunning and cervical dislocation. (a) Noradrenaline (NA) and 3-methoxy-4-hydroxy-phenylglycol (MHPG); (b) dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA); (c) 5-hydroxytryptamine (5-HT) and 5-hydroxyindole acetic acid (5-HIAA). *$P < 0.05$; **$P < 0.01$; control vs. drug treated (Student's t test).

cortex reaching a maximum (2–3 fold) by 60 min (3 determinations). In contrast, RS-15385-197 (10 μM) failed to stimulate the efflux of [^{3}H]-noradrenaline over this time period (3 determinations).

Uptake of [^{3}H]-(−)-noradrenaline into cortical synaptosomes

The uptake of [^{3}H]-NA into isolated synaptosomes from rat cerebral cortex was inhibited by DMI in a concentration-dependent manner, with an IC_{50} of 28 nM. The potency of DMI in inhibiting uptake was similar to that observed by Blackburn *et al.* (1978) for the inhibition of noradrenaline uptake into rat hypothalamic synaptosomes, who reported an IC_{50} value of 15 nM. RS-15385-197 at concentrations up to 10 μM did not inhibit the uptake of [^{3}H]-NA into synapotosomes isolated from rat cerebral cortex.

Neurochemical selectivity of RS-15385-197 in vivo

RS-15385-197 (0.5 mg kg^{-1}, p.o. given 60 or 120 min before the assay) markedly increased brain levels of the noradrenaline metabolite, MHPG (Figure 1a) without modification of the brain levels of noradrenaline, 5-HT or the 5-HT metabolite, 5-HIAA (Figure 1c) or brain levels of dopamine or DOPAC. However, there was a small but significant increase in HVA levels 1 h after dosing (Figure 1c). This dose of RS-15385-197 is associated with marked α_2-adrenoceptor antagonistic effects (Brown *et al.*, 1993), and these findings indicate that RS-15385-197 is relatively selective for the noradrenergic system in rat brain.

β-Adrenoceptor down-regulation by RS-15385-197

Administration of RS-15385-197 (0.5 mg kg^{-1}, p.o.) or the racemate, RS-15385-196 (0.5 or 1.0 mg kg^{-1}, p.o.), caused a down-regulation of cortical β-adrenoceptors on prolonged (14 days) dosing (Table 1). However, neither higher nor lower doses, nor shorter dosing periods caused significant down-regulation of β-adrenoceptors (Table 1). Although there was a trend for down-regulation to occur over the 24 h period following the final dose, the down-regulation of β-adrenoceptors caused by RS-15385-197 (0.5 mg kg^{-1} day^{-1} for 14 days) was only statistically significant at the 24 h time point following the last dose (Table 2).

This dosing regime for RS-15385-197 (0.5 mg kg^{-1} day^{-1} for 14 days) was selective for down-regulation of β-adrenoceptors, because cortical 5-HT_2 receptors were unaffected (control: $K_d = 0.32 \pm 0.02$ nM, $B_{max} = 103 \pm 8$ fmol mg^{-1} protein, $n = 8$; RS-15385-197: $K_d = 0.35 \pm 0.01$ nM, $B_{max} = 97 \pm 4$ fmol mg^{-1} protein, $n = 8$), indicating a selective increase in noradrenergic function.

Table 2 Time course of down-regulation of β-adrenoceptors in rat cerebral cortex following 14 days once-daily dosing with RS-15385-197 (0.5 mg kg^{-1}, p.o.)

		[^{3}H]-dihydroalprenolol binding	
Time	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)
Control	9	0.41 ± 0.03	70.69 ± 2.82
2 h	10	0.36 ± 0.01	64.11 ± 2.32
6 h	10	0.35 ± 0.02	62.42 ± 2.47
24 h	10	0.34 ± 0.03	51.17 ± 4.44*
48 h	9	0.43 ± 0.02	76.33 ± 6.16

β-Adrenoceptor density was measured in ligand binding experiments with [^{3}H]-DHA as described in methods. The number of animals per group is indicated by *n*. The result represents the mean ± s.e.mean of *n* experiments performed in triplicate.
*$P < 0.05$ different from control.

Influence of 5-hydroxytryptamine on β-adrenoceptor down-regulation

Selective 5-hydroxytryptaminergic denervation was effected by central injection of 5,7-DHT (150 μg, i.c.v.): this procedure selectively reduced cortical 5-HT levels to 18% of control without modification of noradrenaline levels. Reduction of cortical 5-HT levels did not affect β-adrenoceptor number directly but prevented the down-regulation of β-adrenoceptors caused by RS-15385-197 (0.5 mg kg^{-1} day^{-1} for 14 days; Table 3). Depletion of 5-HT with PCPA to 6% of control also prevented the decrease in cortical β-adrenoceptor density induced by RS-15385-197 without directly affecting cortical β-adrenoceptor density. Administration of the 5-HT_{1A} agonist, 8-OH-DPAT (0.3 mg kg^{-1}, i.p. twice daily during the final 3 days of study) did not modify β-adrenoceptor number directly but prevented the down-regulation of β-adrenoceptors by RS-15385-197. When given the same way to animals in which the 5-HT neurones had been destroyed, 8-OH-DPAT failed to substitute for 5-HT, as RS-15385-197 did not cause β-adrenoceptor down-regulation in these animals (Table 3).

Antagonism of clonidine-induced hypoactivity by RS-15385-197

Clonidine (0.1 mg kg^{-1}, p.o.) reduced general activity compared to untreated rats. Pretreatment with RS-15385-197 (1 and 10 mg kg^{-1}, p.o.) antagonized the sedative effects of clonidine (Figure 2). A lower dose (0.1 mg kg^{-1}, p.o.) did not significantly attenuate the hypoactivity produced by clonidine.

Table 1 Comparison of down-regulation of β-adrenoceptors in rat cerebral cortex by RS-15385-196 and RS-15385-197 following two different dosing regimes

				[^{3}H]-dihydroalprenolol binding	
Isomer	*Duration*	*Dose*	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)
Control	14 days o.d.	Water	68	0.51 ± 0.05	71.70 ± 4.50
196	14 days o.d.	0.5	10	0.48 ± 0.04	57.17 ± 5.94*
196	14 day o.d.	1.0	9	0.49 ± 0.04	61.97 ± 6.51
196	14 day o.d.	3.0	8	0.51 ± 0.02	71.50 ± 2.50
196	14 day o.d.	10	9	0.50 ± 0.05	67.62 ± 6.09
197	14 day o.d.	0.1	10	0.45 ± 0.04	79.30 ± 5.60
197	14 day o.d.	0.5	10	0.34 ± 0.03	51.17 ± 4.44*
197	3 day o.d.	0.5	10	0.41 ± 0.02	76.18 ± 3.14
196	3 day b.i.d.	1.0	8	0.59 ± 0.07	76.16 ± 4.96
196	14 day b.i.d.	1.0	8	0.51 ± 0.04	59.20 ± 2.57*

Rats were dosed by oral intubation, either once (o.d.) or twice (b.i.d.) daily. β-Adrenoceptor density was measured in ligand binding experiments with [^{3}H]-DHA as described in methods. The number of animals per group is indicated by *n*. The result represents the mean ± s.e.mean of *n* experiments performed in triplicate.
*$P < 0.05$ different from control.

Table 3 Effects of depletion of brain 5-hydroxytryptamine (5-HT), destruction of its neurones, or prolonged activation of 5-HT_{1A} receptors, on down-regulation of β-adrenoceptors in rat cerebral cortex following 14 days once-daily dosing with RS-15385-197 (0.5 mg kg^{-1}, p.o)

Treatment group	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)
Sham control	7	0.41 ± 0.04	82.75 ± 4.38
Lesioned control	3	0.34 ± 0.03	80.55 ± 7.37
Sham/RS-15385-197	8	0.32 ± 0.04	64.59 ± 4.58*
Lesioned/RS-15385-197	5	0.34 ± 0.03	78.43 ± 3.68
PCPA/RS-15385-197	8	0.37 ± 0.03	82.02 ± 6.4
Sham/8-OH-DPAT/ RS-15385-197	6	0.33 ± 0.05	78.54 ± 7.39
Lesioned/8-OH-DPAT RS-15385-197	7	0.32 ± 0.03	78.15 ± 4.85

β-Adrenoceptor density was measured in ligand binding experiments with [^{3}H]-DHA as described in methods. The number of animals per group is indicated by *n*. The result represents the mean ± s.e.mean of *n* experiments performed in triplicate. 'Sham': ascorbate vehicle (20 μl, i.c.v. before start of study); 'lesioned': 5-7-dihydroxytryptamine, 150 μg, i.c.v. before start of study; PCPA: *p*-chlorophenylalanine, 100 mg kg^{-1} i.p. once daily during study; 8-OH-DPAT: 8-hydroxy-n-dipropylaminotetralin, 0.3 mg kg^{-1} s.c. twice daily during final 3 days of study.
*$P < 0.02$ different from control.

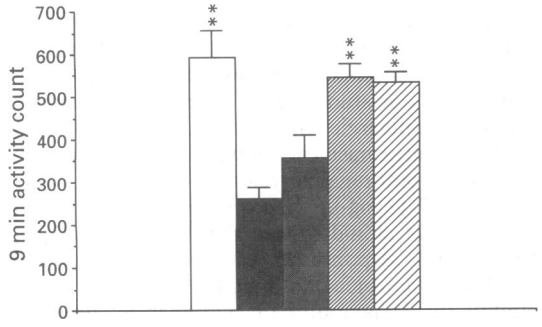

Figure 2 Effects of RS-15385-197 on reduction in spontaneous locomotor activity induced by clonidine. Rats ($n = 6$ per group) were dosed with RS-15385-197 or the same volume of water (10 ml kg^{-1}, p.o.) 30 min before administration of clonidine (0.1 mg kg^{-1}, p.o.). Columns are (from left to right): water + water; water + clonidine; RS-15385-197 (0.1 mg kg^{-1}, p.o.) + clonidine; RS-15385-197 (1 mg kg^{-1}, p.o.) + clonidine; RS-15385-197 (10 mg kg^{-1}, p.o.) + clonidine. **$P < 0.01$ vs. water + clonidine group (Dunnett's *t* test).

5-HT_{1A} behavioural syndrome (acute dosing)

8-OH-DPAT (0.3 mg kg^{-1}, s.c.) rapidly evoked the 5-HT_{1A} behavioural syndrome comprising hyperlocomotion, reciprocal forepaw treading, a flattened body posture and head-weaving. This was unaffected by pretreatment with a single dose of RS-15385-197 (1 mg kg^{-1}, p.o.; $n = 6$ per group; data not shown).

5-HT_{1A} behavioural syndrome and hypothermia (chronic dosing)

8-OH-DPAT (0.5 mg kg^{-1}, s.c.) rapidly evoked the 5-HT_{1A} behavioural syndrome comprising reciprocal forepaw treading and a flattened body posture, but headweaving was not observed. This response was unaffected by chronic pretreatment with RS-15385-196 (3 mg kg^{-1}, p.o., once daily for 14 days; $n = 6$ per group; Figure 3). This dose of 8-OH-DPAT also elicited a decrease in core (rectal) temperature, which in the control group fell from 38.0 ± 0.2°C to 35.4 ± 0.1°C ($P < 0.001$), measured at the nadir 30 min after injection. This was not significantly altered by chronic pretreatment with RS-15385-196 (Figure 3).

Discussion

RS-15385-197, a potent and selective α_2-adrenoceptor antagonist which readily penetrates the CNS (Brown *et al.*, 1993) was examined for effects on central noradrenergic neurotransmission in the rat, *in vitro* and *in vivo*. In slices taken from the hypothalamus, an area rich in α_2-adrenoceptors (Brünig *et al.*, 1981; Unnerstall *et al.*, 1984; Boyajian *et al.*, 1987), RS-15385-197 augmented K^+-evoked release of noradrenaline in a concentration-related manner, with nanomolar potency. This was also achieved with the α_2-adrenoceptor antagonists, idazoxan and yohimbine, but in the micromolar range. This effect of RS-15385-197 was not due to direct displacement of noradrenaline from its storage vesicles as, even at a concentration of 10 μM, RS-15385-197 did not directly release noradrenaline from cortical slices whereas the catecholamine-depleting agent, reserpine, was clearly demonstrated to do so. Also, the augmentation of K^+-evoked release of noradrenaline by RS-15385-197 was not due to inhibition of noradrenaline re-uptake: in synaptosomes isolated from rat cerebral cortex, RS-15385-197 (10 μM) did not inhibit noradrenaline uptake whereas DMI was demonstrated to be a potent uptake inhibitor. Instead, RS-15385-197 is most probably acting as an antagonist at presynaptic α_2-adrenoceptors which provide feedback inhibition of noradrenaline release (Langer, 1974; Starke, 1977). Such augmentation of noradrenaline release is likely to be involved in the mechanism of action of RS-15385-197 in the CNS *in vivo*.

In vivo, RS-15385-197 increased the levels of MHPG and to a lesser extent, HVA, in rat brain without modifying 5-HIAA levels, indicating that the compound was selective for noradrenergic function and would increase noradrenaline turnover without affecting global 5-HT function. In view of the *in vitro* data, the increased turnover of noradrenaline probably indicates increased release of noradrenaline per unit impulse, although an increase in firing rate of noradrenergic neurones is also a possibility. The same dose of RS-15385-197 found to increase MHPG levels (0.5 mg kg^{-1}, p.o.) caused cerebral β-adrenoceptor down-regulation over a 14 day period. This was a dynamic phenomenon, because although there was a trend for down-regulation to occur over the 24 h period following the last dose, this was only significant at the 24 h time point. In comparison, significant β-adrenoceptor down-regulation was not observed following treatment with idazoxan (10 mg kg^{-1}, p.o. once-daily for 14 days; unpublished observations). Yohimbine (2–5 mg kg^{-1}, i.p. twice-daily for 14 days) also causes no significant down-regulation of cortical β-adrenoceptors, but accelerates the β-adrenoceptor down-regulation produced by DMI, amitriptyline, mianserin and pargyline (Johnson *et al.*, 1980; Scott & Crews, 1982). The extent of β-adrenoceptor down-regulation achieved with RS-15385-197 was similar to that observed with DMI, indicating potential utility for RS-15385-197 as an

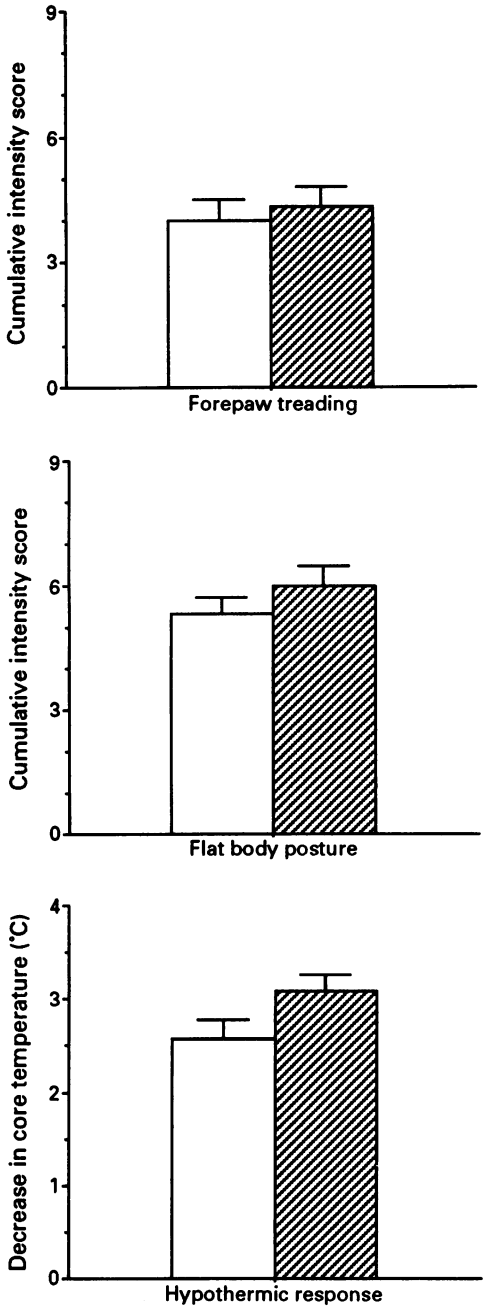

Figure 3 Effects of chronic treatment with RS-15385-196 (3 mg kg^{-1}, p.o., twice daily for 14 days) on behavioural and hypothermic responses to 8-hydroxy-n-dipropylaminotetralin (0.5 mg kg^{-1}, s.c.). Open columns: water-treated controls ($n = 9$); hatched columns: RS-15385-196-treated ($n = 9$). There were no significant differences between the two groups (Mann-Whitney U-test).

antidepressant agent (MacDonald *et al.*, 1988). However, selective 5-HT uptake inhibitors (Cross & Horton, 1988) and other antidepressants (Charney *et al.*, 1981) also down-regulate 5-HT_2 receptors in the rat cortex on chronic dosing, but the dosing schedule used with RS-15385-197 did not modify 5-HT_2 receptor density, consistent with a selective effect on noradrenergic neurones.

It would appear from our study that a tonic release of 5-HT is required for β-adrenoceptor down-regulation, in that depletion of brain 5-HT or destruction of its neurones prevented the down-regulation by RS-15385-197. Concurrent treatment of rats with 8-OH-DPAT had the same effect as depletion or destruction of 5-HT neurones, suggesting that it was acting at 5-HT_{1A} autoreceptors on 5-hydroxytryptaminergic cell bodies to reduce firing rate (Sprouse & Aghajanian, 1987) and thereby reduce 5-HT release from their axon terminals (Sharp *et al.*, 1989). These findings confirm and extend earlier studies which showed that β-adrenoceptor down-regulation in the rat cortex following chronic treatment with established antidepressants requires a tonic increase in noradrenergic function (Vetulani & Sulser, 1975; Frazer, 1981; Janowsky *et al.*, 1982) together with an intact, tonic 5-HT input (Brunello *et al.*, 1982; Manier *et al.*, 1983). Furthermore, 5-HT_{1A} partial agonists cannot substitute for 5-HT depletion, as 8-OH-DPAT did not restore the down-regulation of β-adrenoceptors by RS-15385-197 in 5-HT-depleted rats in our study. Thus, in the presence of an intact 5-hydroxytryptaminergic innervation, a very selective α_2-adrenoceptor antagonist can down-regulate β-adrenoceptors by increasing noradrenaline release.

RS-15385-197 (1 mg kg^{-1}, p.o.) completely prevented the sedative effects of clonidine, but the same dose failed to alter the behavioural response to 8-OH-DPAT. Similarly, RS-15385-196 given chronically (3 mg kg^{-1}, p.o. for 14 days) did not modify the behavioural or hypothermic response to 8-OH-DPAT. This indicates that acute or chronic blockade of central α_2-adrenoceptors does not influence responses mediated *via* 5-HT_{1A} receptors.

In summary, the selective α_2-adrenoceptor antagonist, RS-15385-197, enhances neuronal release of noradrenaline *in vitro*, most probably by its antagonist action at presynaptic α_2-adrenoceptors. This action probably accounts for the increased turnover of cortical noradrenaline observed *in vivo*, and for the down-regulation of cortical β-adrenoceptors which occurs upon chronic treatment with this agent. Although RS-15385-197 was shown not to interact directly with central 5-HT neurotransmission, this neuronal system plays a permissive role in the down-regulation of cortical β-adrenoceptors by RS-15385-197. Because of its relative promiscuity, yohimbine has only limited value in defining the role of α_2-adrenoceptors in central noradrenergic function and idazoxan has affinity for other receptor binding sites, most notably imidazoline sites. The high selectivity and potency of RS-15385-197 indicate that this compound is currently the drug of choice for definition of the role of α_2-adrenoceptors in the CNS.

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Electropharmacological effects of berberine on canine cardiac Purkinje fibres and ventricular muscle and atrial muscle of the rabbit

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1 Conventional microelectrode techniques were used for intracellular recordings of the transmembrane electrical potentials, the effects of berberine were studied on canine cardiac Purkinje and ventricular muscle fibres and on rabbit atrial fibres.

2 Berberine (3–30 μM) increased in a concentration-dependent manner, the action potential duration (APD) in canine Purkinje and ventricular muscle without affecting other parameters of the action potential.

3 The berberine-induced enlargement of the APD showed reverse use-dependence, so that the effect was greater at lower rates of stimulation.

4 Preparations perfused with berberine (30 μM) and driven at rates below 0.5 Hz exhibited early after depolarizations which persisted 3–4 h after washing.

5 The early afterdepolarizations were reversibly abolished by perfusion with lignocaine (3 μM) or by the increase in the rate of stimulation.

6 The effective refractory period (ERP) of Purkinje fibres was greatly increased by berberine (30 μM); however, the ratio ERP/APD was not significantly affected.

7 Berberine (10–100 μM) decreased in a concentration-dependent manner the spontaneous frequency of rabbit sinoatrial cells. The decrease in frequency was accompanied by a depression of the phase 4 depolarization, without significant changes in other parameters of the nodal action potential.

8 Atropine (2.5 μM) did not affect the bradycardic effect of berberine. On the other hand, berberine (30 μM) did not alter the chronotropic effect of isoprenaline.

9 Berberine (30 μM) also increased the duration of slow responses in K-depolarized rabbit atrial muscle fibres, other parameters being unaffected.

10 It is suggested that berberine exerts Class III antiarrhythimic and proarrhythmic actions in cardiac muscle of the dog *in vitro*.

Keywords: Berberine; canine myocardial fibres; rabbit atria; Class III antiarrhythmic agents; early afterdepolarization

Introduction

Berberine, an alkaloid found in numerous plants of the genera *Berberis* and *Coptis*, has been used as a medicinal agent for many centuries. It has been reported that berberine and some of its derivatives are potent α-adrenoceptor antagonists (Ko & Lim, 1980; Vizi *et al.*, 1986). Berberine has also shown positive inotropic activity in guinea-pig (Shaffer, 1985) and man (Marin Neto *et al.*, 1988). Antiarrhythmic (Krol *et al.*, 1982) and pro-arrhythmic (Marin Neto *et al.*, 1988) effects have also been found after i.v. administration of berberine in dogs and men. In the present investigation, by use of conventional microelectrode techniques, the electro-pharmacological effects of berberine were studied on segments of dog and rabbit hearts.

Methods

Adult mongrel dogs (10–15 kg) were anaesthetized with sodium thiobarbitone (50 mg kg^{-1}, i.v.) and their hearts were quickly removed through a left thoracotomy. The ventricles were opened and various pieces of muscle with attached false tendons were dissected and stored in cool oxygenated Tyrode solution. One piece was mounted in a 15 ml tissue chamber with a Sylgard-lined base. Rabbit atrial preparations were obtained and studied as described previously (Riccioppo Neto, 1982).

Tyrode solution aerated with 95% O_2 (pH 7.4) was kept at a temperature of 36 ± 0.5°C and flowed over the preparation at a rate of 12–15 ml min^{-1}. The Tyrode solution had the following composition (mM): NaCl 137, $CaCl_2$ 1.8, KCl 4 (for rabbit atrial tissue: KCl 5.4), $MgCl_2$ 0.45, $NaHCO_3$ 12, NaH_2PO_4 0.32 and glucose 5.5. In order to obtain the slow response an equimolar concentration of NaCl was substituted by KCl (25 mM) and isoprenaline (10^{-6} M) was added to the perfusion fluid.

Transmembrane potentials were recorded with conventional glass microelectrodes filled with 3 M KCl having resistances of 10–20 MΩ and displayed, via an input capacity neutralization preamplifier (Grass P16) on an oscilloscope (Tektronix 5113). The maximum rate of rise of the action potential was determined electronically with an OP-AMP (Analog 118 A). Oscilloscope traces were photographed on 35 mm film with a Nihon-Kohden PC-3A camera. For analysis of the sinoatrial rate, bipolar surface electrograms were recorded from the crista terminalis and the signal was simultaneously displayed on the oscilloscope.

Square wave pulses were obtained either from a Grass stimulator (S4-SIU4) or from a specially built stimulator so that an extra-stimulus (S2) could be delivered, with variable delay and amplitude, after the eight basic pulse (S1). S1 pulses (1.5 × threshold, 1 ms duration) and S2 pulses (3 × threshold, 1 ms duration) were delivered to the preparation

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through the same pair of Teflon coated silver wire electrodes. For studies of membrane refractoriness two recording microelectrodes were used. The effective refractory period (ERP) was measured as the shortest interval between S1 and S2 pulses needed to elicit an extrasystole that propagated to the distal microelectrode. The normal frequency of stimulation was 1.5 Hz.

Action potential characteristics were analysed by hand after enlargement of the film (7 ×) and the following parameters were measured: maximum diastolic potential (MDP), action potential amplitude (APA), maximum rate of rise of action potential (V_{max}), duration of the action potential from its peak to 50% (APD_{50}) and 90% (APD_{90}) repolarization, and sinoatrial rate.

Drugs used were: atropine sulphate (Merck); berberine chloride (Sigma); (−)-isoprenaline hydrochloride (Sigma); lignocaine hydrochloride (K & K). Unless otherwise stated, the results describe effects of drugs applied cumulatively during a single stable impalement.

Data are expressed as mean values ± s.e.mean and comparisons between two means were made by Student's paired *t* test. *P* values of less than 0.05 were considered to indicate significant differences.

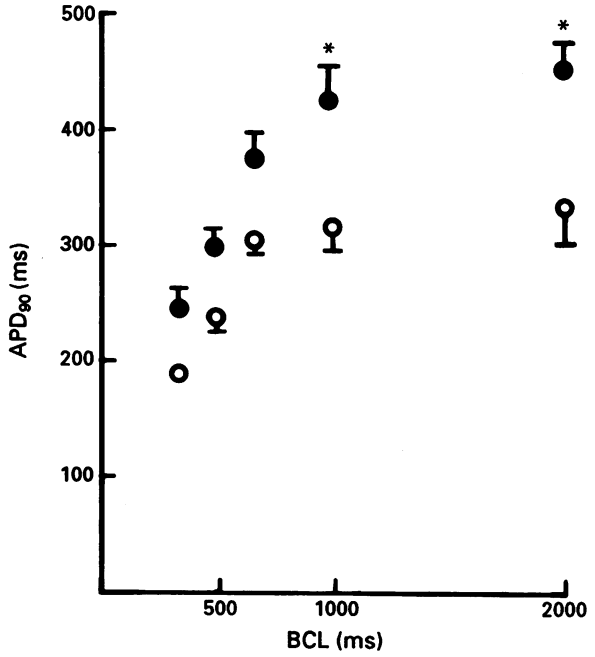

Figure 1 Action potential duration at 90% repolarization (APD_{90}) of Purkinje fibres ($n = 5$) driven at different rate before (○) and after berberine (30 μM) perfusion (●). BCL = basic cycle length. $^*P < 0.05$ compared with control at the same frequency.

Results

Effects on action potential parameters of dog Purkinje and ventricular muscle fibres

Berberine at concentrations between 1 and 30 μM prolonged the action potential duration (APD) of both Purkinje and ventricular muscle fibres in a concentration-dependent manner, without affecting the other action potential characteristics (Table 1). Concentrations up to 100 μM did not further increase the APD when tested upon Purkinje fibres. The prolongation of the APD induced by berberine was irreversible. The APD even increased slightly during washing in drug-free Tyrode solution and remained prolonged after 2 h of observation.

Effects of berberine on Purkinje fibres driven at different rates

Five Purkinje fibre preparations were initially stimulated at a frequency of 0.5 Hz and after consecutive intervals of 3 min to allow stabilization, the frequency of stimulation was increased to 1; 1.5; 2 and 3 Hz, respectively. Berberine (30 μM) was then perfused during 40 min and the stimulation at various frequencies repeated. As shown in Figure 1, the berberine-induced enlargement of the APD varied inversely with the rate of stimulation. Below 0.5 Hz four of the preparations studied showed oscillations of the membrane potential around −40 mV (Figure 2). These early afterdepolarizations (EADs) were reversibly blocked by the increase in the frequency of stimulation. In two preparations, the addition of a small concentration of lignocaine (3 μM) blocked reversibly the early afterdepolarizations (not shown). Berberine-induced EADs could be elicited during 3–4 h after washing off the drug, the only requirement being slow rate of stimulation.

Effects on membrane refractoriness

The effective refractory period (ERP) of Purkinje fibres treated with berberine (30 μM) was significantly increased in four experiments from 255 ± 12 ms (control) to 321 ± 15 ms ($P < 0.01$). However, since the total APD in the preparations was increased from 320 ± 10 ms (control) to 432 ± 9 ms (berberine-treated) the ratio ERP/APD decreased, although not significantly.

Table 1 Dose-related effects of berberine on the action potential parameters of the dog myocardium

	Berberine (μM)				
Purkinje fibres	0	3	10	30	100
APA (mV)	123 ± 2	122 ± 3	123 ± 3	121 ± 3	122 ± 3
MDP (mV)	90 ± 2	88 ± 3	88 ± 3	87 ± 3	88 ± 4
V_{max} (Vs^{-1})	690 ± 26	693 ± 23	700 ± 20	692 ± 28	686 ± 31
APD_{50} (ms)	226 ± 13	230 ± 16	267 ± 11	290 ± 17*	288 ± 16*
APD_{90} (ms)	275 ± 8	278 ± 6	316 ± 12*	408 ± 13**	410 ± 15**
Ventricular muscle					
APA (mV)	110 ± 3	111 ± 3	108 ± 2	109 ± 2	107 ± 3
MDP (mV)	85 ± 2	85 ± 2	87 ± 2	85 ± 3	84 ± 2
V_{max} (Vs^{-1})	440 ± 22	430 ± 29	425 ± 20	433 ± 24	428 ± 26
APD_{50} (ms)	118 ± 9	120 ± 8	135 ± 7	180 ± 13**	177 ± 10**
APD_{90} (ms)	178 ± 8	182 ± 10	199 ± 6	230 ± 10**	288 ± 9**

The data are presented as means ± s.e.mean of five preparations.
$^*P < 0.05$; $^{**}P < 0.01$, as compared with predrug control values.

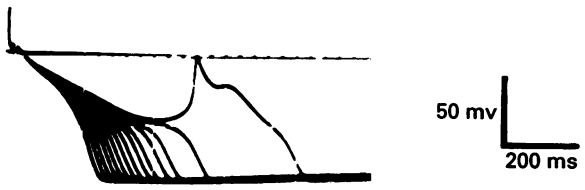

Figure 2 Superimposed action potentials of a berberine-treated (30 μM) Purkinje fibre stimulated at a decreasing frequency from 1.5 Hz (shortest action potential duration) to 0.1 Hz, in which the early afterdepolarization appeared.

Effects on the sinoatrial node of rabbits

In four preparations in which stable impalements could be maintained throughout, berberine was applied cumulatively (10–100 μM). There was a decrease in the rate of firing of nodal cells due to a depression of the phase 4 depolarization without major effects on other action potential parameters (Figure 3, inset).

In eight other preparations, atropine (2.5 μM) did not significantly reduce the negative chronotropic effect of berberine. On the other hand, the positive chronotropic effect of isoprenaline was not influenced by concomitant treatment with berberine (30 μM) (Figure 3).

Effects of berberine on the slow-response of rabbit atrium

Slow responses were obtained from four left atrium trabeculae driven at a rate of 0.3 Hz. After 40 min perfusion with berberine (30 μM) there was a consistent delay in the repolarization phase of the slow responses, the other parameters being unchanged. In these preparations APD_{50} and APD_{90} increased 55 ± 9% and 45 ± 6%, respectively, after berberine perfusion.

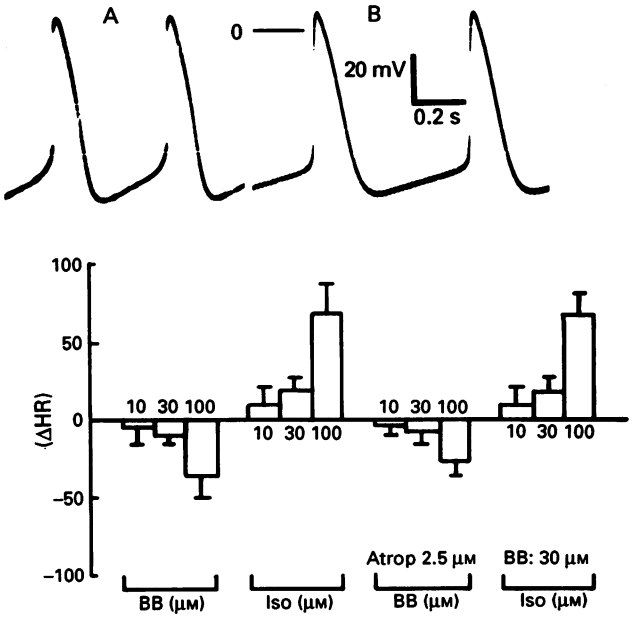

Figure 3 Variations in the spontaneous sinoatrial frequency (Δ HR) of isolated right atria of rabbits treated with berberine (BB; $n = 8$), isoprenaline (Iso; $n = 4$), berberine during atropine (Atrop; $n = 4$) and isoprenaline during berberine ($n = 4$). Inset: Effect of berberine (100 μM) on the action potentials of a S-A node cell; (A) control, (B) berberine, 30 min.

Discussion

Berberine induced a marked prolongation of the repolarization phase of the action potential on dog isolated ventricular myocardium without affecting the other action potential characteristics. The increase in the action potential duration (APD) exhibited reverse use-dependence (Hondeghem & Synders, 1990), a property common to drugs that have the greatest effect on prolonging repolarization at lower frequencies of stimulation like quinidine (Roden & Hoffman, 1985), sotalol (Strauss *et al.*, 1970) etc. Like these agents, berberine was also capable of inducing early after depolarizations (EADs) in preparations driven at slower rates. Manoeuvres such as lignocaine perfusion or an increase in the rate of stimulation were able to suppress reversibly the berberine-induced EADs as has been observed for EADs caused by quinidine (Valois & Sasyniuk, 1988; Davidenko *et al.*, 1989) and hypoxia plus acidosis (Rozanski & Witt, 1991).

Since no effect on V_{max} was detected in the range of concentrations used and the ratio ERP/APD was unaltered after berberine, the increase in the membrane refractoriness is probably the result of the increase in the APD. Local anaesthetic properties have been reported for berberine at higher concentrations, (Sabir & Bhide, 1971); no depression of the action potential amplitude of the rabbit vagus nerve was however found for berberine up to 0.5 mM (Riccioppo Neto, unpublished).

It seems reasonable, therefore, to attribute Class III action (Vaughan Williams, 1984) to berberine. Berberine has indeed been able to antagonize arrhythmias induced in dogs (Krol *et al.*, 1982; Kwiezycka *et al.*, 1983) and in rats (Ribeiro *et al.*, 1982), effects that could be accounted for by the drug-induced increase in refractoriness observed in the present experiments. Proarrhythmic effects such as the development of ventricular tachycardia with torsades de pointes morphology were also detected in men after i.v. infusion of berberine (Marin Neto *et al.*, 1988). There has been an increasing wealth of evidence suggesting that EAD is the basic cellular mechanism of torsades de pointes (El-Sherif *et al.*, 1989; Carlsson *et al.*, 1990) and the berberine-induced EADs observed *in vitro* could be one of the operative mechanisms responsible for the arrhythmia that ocurred in men.

The techniques employed in the present work do not permit a distinction of the underlying ionic mechanism(s) responsible for the lengthening of the APD caused by berberine. Although this can be achieved by a relative increase in inward over outward currents, it is believed that the great majority of Class III antiarrhythmic agents act primarily by blocking cardiac potassium channels which normally permeate outward currents (Carmeliet, 1985; Balser *et al.*, 1987).

In spontaneously beating right atria from rabbits, berberine caused a concentration-dependent decrease in the rate of diastolic depolarization. The consequent bradycardia was not blocked by atropine suggesting that muscarinic receptors were not involved. An atropine-resistant bradycardia after berberine has been previously described by Chun *et al.* (1979) in rats and by Shaffer (1985) in guinea-pig isolated right atria. A berberine-induced increase in spontaneous rate of isolated right atria from rabbits, bathed in Ringer-Locke solution at 30°C, is referred to by Sabir *et al.* (1978). Berberine has not been shown to possess adrenoceptor blocking action since it did not affect the chronotropic effect of isoprenaline in rabbit right atria. It has been recently reported that Class III antiarrhythmic agents like sotalol, bretylium and amiodarone at lower concentrations (10^{-6} M), prolonged significantly the cycle length of rabbit S-A node preparations without affecting other action potential characteristics (Satoh, 1991). In voltage-clamped preparations, despite some differences in potency and in the type of current affected, all three agents decrease the hyperpolarization-activated inward current, an effect that would result in a

decrease in the rate of spontaneous discharge. It remains an open question whether berberine could exert a similar action.

It is interesting to note that in guinea-pig atria, berberine caused positive inotropism not prevented by reserpinization or propranolol (Shaffer, 1985) and that in dogs with experimental heart failure berberine improved left ventricular function (Vik-Mo *et al.*, 1983). Selective lengthening of the APD may increase the transsarcolemmal calcium current, elevate the release of calcium from the sarcoplasmic reticulum and, as a consequence, increase the free calcium concentration close to the contractile elements (Reiter, 1988; Carlsson *et al.*, 1991). On the other hand it should be noted that an increase in tension development has been associated with either shortening or lengthening of the cardiac APD (Allen, 1977).

In isoprenaline-induced slow action potentials berberine produced an increase in APD. However, on slow-responses elicited by the application of histamine in potassium-depolarized guinea-pig papillary muscles, berberine (25 μM) increased significantly not only APD and the ERP, but also the action potential amplitude and V_{max} (Huang *et al.*, 1990). Since these two last effects were blocked by a very high concentration of propranolol (34 mM) it was argued that the inotropic effect of berberine could be the result of a β-receptor stimulating action (Huang *et al.*, 1990). It is, however, difficult to exclude nonspecific depressor effects of such an elevated concentration of propranolol upon the inotropic effect of berberine.

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Interference of a neutrophil recruitment inhibitory factor upon the accumulation of inflammatory cells and airway hyperreactivity in sensitized guinea-pigs after intranasal antigen challenge

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1 A neutrophil recruitment inhibitory factor (NRIF) recovered from the crude supernatant of lipopolysaccharide (LPS)-stimulated macrophages inhibited neutrophil migration following both intratracheal and intravenous administration of LPS, but did not alter the pattern of leukopenia/leucocytosis induced by intravenous LPS.

2 The correlation between airway infiltration by inflammatory cells and hyperreactivity in lungs from actively sensitized and challenged guinea-pigs was investigated by use of NRIF.

3 Increased eosinophil counts were found in the bronchoalveolar lavage fluid from guinea-pigs sensitized with 10 μg ovalbumin and challenged at day 14 by the intranasal administration of the antigen. The increase was evident 5 h after challenge and persisted at 24 h. Neutrophil numbers were also increased at this time. Pretreatment with NRIF suppressed the leucocyte increase in the bronchoalveolar lavage fluid.

4 Bronchoconstriction and histamine release induced by 3 ng PAF injected into the isolated lungs were increased in challenged guinea-pigs as compared to sensitized but unchallenged controls. Pretreatment of the animals with NRIF did not interfere with this response, but significantly reduced the bronchoconstriction induced by ovalbumin injection.

5 Even though the increased number of inflammatory cells in bronchoalveolar lavage and airway hyperresponsiveness were concomitant, NRIF inhibited cellular infiltration but failed to alter airway hyperreactivity to PAF, demonstrating that these events may occur independently. Conversely, the inhibition of antigen-induced bronchoconstriction by NRIF suggests that this response is dependent upon the emigration of granulocytes.

Keywords: Neutrophil recruitment inhibitory factor; intranasal antigen challenge; cell migration; hyperreactivity; sensitized guinea-pigs

Introduction

Asthma is characterized by a reversible obstruction to airflow and by nonspecific bronchial hyperresponsiveness (Boushey *et al.*, 1980). The mechanisms involved in bronchopulmonary hyperreactivity are not well understood (Boushey *et al.*, 1980), and it has been suggested that inflammation of the bronchial mucosa and submucosa is a key factor for the enhancement of reactivity (Payne & Cheng, 1990). Active sensitization of guinea-pigs followed by a booster injection of antigen leads to *ex vivo* bronchopulmonary hyperresponsiveness to different agonists, including platelet-activating factor (PAF). Such hyperresponsiveness appears to depend on lung infiltration by inflammatory cells, mainly eosinophils (Pretolani *et al.*, 1988).

Leucocytes and particularly neutrophils are implicated in lung injury during shock, as indicated by depletion studies (Worthen *et al.*, 1986; Chang *et al.*, 1987). We reported that the intravenous administration to rats of supernatants from lipopolysaccharide (LPS)-stimulated macrophages inhibited neutrophil migration into the inflamed peritoneal cavity (Cunha *et al.*, 1989). In contrast, the same supernatant did not inhibit mononuclear cell migration to rat peritoneal cavities induced by thioglycollate (Tavares *et al.*, 1989). The substance responsible for this activity, which was recovered from macrophage conditioned medium, was referred to as neutrophil recruitment inhibitory factor (NRIF) (Tamashiro *et al.*, 1992). Inhibition was not accounted for by the presence of known cytokines such as interleukin-1 (IL-1), IL-8 or tumour necrosis factor-α (TNF-α) (Tamashiro *et al.*, 1992). In addition, the factor was not active against eosinophil recruitment into the rat peritoneal cavity (Oliveira *et al.*, unpublished).

In the present work we first investigated whether intravenous administration of NRIF, obtained from rat macrophages, was able to inhibit the neutrophil emigration to the inflammatory exudate induced by intratracheal or intravenous administration of LPS in the guinea-pig. Because of its effectiveness, we used NRIF as a tool to correlate airway infiltration by inflammatory cells, as assessed by bronchoalveolar lavage, with bronchial hyperreactivity to spasmogens and with histamine and thromboxane B_2 release in lungs from sensitized guinea-pigs after intranasal challenge with antigen. This new model of challenge of actively sensitized guinea-pigs is very convenient, since it requires only 1 day for the expression of hyperresponsiveness.

Methods

Sensitization procedure

Male Hartley guinea-pigs (Elevages Lebau, France, 400–750 g) were actively sensitized at day 0 by a subcutaneous (s.c.)

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injection of 0.5 ml of 0.9% NaCl solution (saline), containing 10 μg of ovalbumin (OA) dispersed in 1 mg $Al(OH)_3$ (modified from Andersson, 1980). The animals were challenged at day 14 by the intranasal (i.n.) administration of 300 μl of sterile saline containing OA (1 mg). All guinea-pigs were pretreated 1 h before challenge, with an intraperitoneal (i.p.) injection of 500 μg kg^{-1} of the antihistamine mepyramine, to prevent early bronchoconstriction and death. One group of animals received intravenous (i.v.) injection of NRIF (S-300 P2 fraction, 0.2 ml, equivalent to material released from approximately 5×10^6 macrophages) (Tamashiro *et al.*, 1992), into the exposed saphenous vein, 15 min before and 5 h after the challenge. Controls were actively sensitized guinea-pigs that received saline either i.v. (in place of NRIF) or i.n. (in place of OA). The animals were used 5, 24 or 96 h after challenge.

In vivo *preparation*

Non-immunized guinea-pigs were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.p.) and the tracheae were cannulated. Spontaneous breathing was suppressed with pancuronium (2 mg, i.v.). A jugular vein was catheterized for i.v. injections and one carotid artery was cannulated for blood sampling.

In a first series of experiments, guinea-pigs received 1 mg of LPS diluted in saline (0.1 ml) by the intratracheal (i.t.) route, at $t = 0$. One group of animals was pretreated with NRIF (0.2 ml, i.v.) 15 min before LPS administration. Controls received saline by i.v. or i.t. routes. Bronchoalveolar lavages were performed 3 h after LPS administration.

In a second series of experiments, animals received an i.v. injection of LPS (100 μg kg^{-1} in 0.2 ml) 4 h after anaesthesia. The experimental group received NRIF (0.2 ml, i.v.) 15 min before LPS injection, whereas controls received saline i.v. Blood samples were collected from the carotid artery before injections and 10, 20, 30, 60, 90, 120, 150 and 180 min after LPS, for total leucocyte counts.

In a separate experiment, non-anaesthetized guinea-pigs were injected i.v. with 0.2 ml of saline containing LPS (100 μg kg^{-1}). Experimental groups received NRIF (0.2 ml, i.v.) 15 min before LPS injection and controls received saline. Bronchoalveolar lavages and total leucocyte counts in blood were performed 24 h after LPS injection. In this case, blood was obtained from superficial ear veins of non-anaesthetized guinea-pigs, before bronchoalveolar lavage.

Blood cell counts

Blood samples (40 μl) were collected from the carotid artery of superficial ear veins and mixed with 10 ml of a balanced electrolyte solution (Isoton II). Erythrocytes were then lysed with Zapoglobin and the total leucocyte counts assessed with a Coulter ZBI. Blood samples were obtained as described above.

Neutrophil recruitment inhibitory factor production

The method for obtaining crude macrophage supernatants containing NRIF was described in detail by Cunha *et al.* (1989). Briefly, rat macrophages were harvested from peritoneal cavities elicited 4 days earlier with 10 ml of 3% thioglycollate (w/v) and incubated in plastic tissue culture dishes for 1 h at 37°C, in an atmosphere of air containing 5% CO_2. The adherent monolayers (95% macrophages) were washed three times with phosphate-buffered saline (PBS, pH 7.4) and incubated with LPS (10 μg ml^{-1} in RPMI) for 15 min at 37°C. The cells were again washed three times with PBS followed by a final incubation with 5 ml of LPS-free medium, for 1 h at 37°C. The cell-free incubation fluids were subsequently ultradiafiltered through a YM-10 membrane (Amicon, Corp, Lexington, MA, U.S.A.). The final volume (corresponding to 5% of the original volume) was filtered through 0.22 μm membrane (Millipore, Bedford, MA, U.S.A.). According to the procedure described by Tamashiro *et al.* (1992), 1 ml samples of this material, released by 2.5×10^8 adherent cells, were dialysed against 0.5 M NaCl solution buffered with 0.02 M phosphate pH 7.4 and chromatographed on Sephacryl S-300 column (2.6 × 70 cm, Pharmacia, Upsala, Sweden). The S-300 column was calibrated with a molecular weight standard kit (Pierce Chemical Co., Rockford, IL, U.S.A.). The P2 fraction, containing the original inhibitory activity (Tamashiro *et al.*, 1992), desalted on a PD10 column (Pharmacia) was used for the inhibitory assays, and is designated as NRIF.

Sampling of bronchoalveolar cells

The cell composition of the bronchoalveolar lavage fluid from actively sensitized unchallenged guinea-pigs was compared to that of fluids collected 5, 24 and 96 h following challenge with OA. Since marked effects were noted after 24 h, this interval was used to study the effect of NRIF upon the influx of inflammatory cells into the bronchoalveolar lavage. To do so, 5 ml of sterile saline was instilled, at room temperature, through a polyethylene cannula introduced into the trachea, and recovered immediately. This was followed by nine repeated lavages with 5 ml aliquots of sterile saline until almost all of the 50 ml administered was recovered. Total leucocyte counts were evaluated with a Malassez haemacytometer and cell viability was estimated with the trypan blue exclusion test. The suspension containing 120,000 cells ml^{-1} was then centrifuged in a Shandon-Elliott cytometer and the cells were counted and differentiated by May-Grunwald-Giemsa staining (Diff-Quick, American Scientific Products, McGraw Park, IL, U.S.A). Results are expressed as leucocyte numbers × 10^6 ml^{-1} of bronchoalveolar lavage fluid.

Lung perfusion

For this experiment guinea-pigs were used 24 h after intranasal antigen challenge. Guinea-pigs were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.p.), tracheae were cannulated and the animals ventilated (60 strokes min^{-1}, 10 ml kg^{-1}) with a Palmer miniature respiratory pump. A thoracotomy was performed and the lungs were removed as described by Lefort *et al.* (1984), placed in a plastic chamber, ventilated (60 strokes min^{-1}, 10 ml kg^{-1}) and perfused intra-arterially (10 ml min^{-1}, 37°C) with a gassed (95% O_2/5% CO_2) Krebs solution containing 0.25% (wt/vol) bovine serum albumin (BSA) and 2 μM mepyramine, to abolish the histamine component of the bronchoconstrictor effect of PAF in lungs from actively sensitized guinea-pigs (Pretolani *et al.*, 1988). After a 10 min equilibration period, increasing doses of PAF (3 and 30 ng in 100 μl of saline containing 0.1% BSA) were sequentially injected into the pulmonary artery of each perfused lung, at 10 min intervals. Bronchoconstrictor responses were recorded by a pressure transducer located between the trachea and the respiratory pump, and expressed as cm^2 of area of the tracing of the paper record over the base line. Before the end of each experiment, 10 μg OA was administered to assess the anaphylactic response in lungs from the sensitized animals. One minute fractions of each lung effluent were collected before, during the first 3 min and between the 3rd and 6th min after the successive injections of the two doses of PAF and OA, for the determination of thromboxane B_2 (TxB_2) (Sors *et al.*, 1978) and histamine (Lebel, 1983). Results were expressed as histamine or TxB_2 released (ng ml^{-1}).

Evaluation of thromboxane and histamine in the lung effluent

Before storage at − 20°C, aliquots (1 ml) of the lung perfusate were maintained at room temperature for 60 min to

allow the conversion of TxA_2 into its stable metabolite TxB_2. Under such conditions, the conversion of TxA_2 in TxB_2 approaches 100% in 30 min (Charpentier *et al.*, 1986). To perform the radioimmunoassay, 100 µl of each sample were incubated overnight at 4°C with ^{125}iodine-labelled TxB_2 and anti-TxB_2 antiserum, in a phosphate buffer (10 mM, pH 7.4) containing bovine gamma-globulins (0.3% w/v). The next day, bound and free ligand were separated by the addition of a solution of polyethyleneglycol 6000 (30% in distilled water), followed by centrifugation for 10 min at 1500 *g* and at 4°C. The supernatants were decanted and radioactivity present in the pellet, which corresponds to the bound fraction, was counted for 1 min in a gamma counter. The monoclonal antibodies employed were less than 0.02% crossreactive with prostaglandin D_2 (PGD_2), PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and arachidonic acid for the anti-TxB_2 antiserum. The sensitivity of the assay was approximately 2 pg of immunoreactive TxB_2, in 0.1 ml sample.

For the histamine assay, 1 ml-aliquots of the collected fractions were mixed with 1 ml of 0.8 N perchloric acid solution. After centrifugation for 10 min at 1200 *g* and 4°C, the supernatants were stored at 4°C. An automatic spectrofluorimetric assay for histamine was performed according to Shore *et al.* (1959), as modified by Lebel (1983).

Materials

The composition of the Krebs solution was (in mM): NaCl 118, KCl 4.7, $CaCl_2.2H_2O$ 2.5, $MgSO_4$ $7H_2$ 1.2, $NaHCO_3$ 25 and glucose 5.6 The drugs used were: chicken ovalbumin (Milles, Naperville, IL, U.S.A.); endotoxin (*E. coli* O_{111}: B_4 lipopolysaccharide) from Sigma Chemical Company; PAF-acether (1-O- hexadecyl-2-acetyl-sn-glycero-3-phosphocoline, obtained from Bachem, Switzerland); perchloric acid (Merck, Darmstadt, Germany); mepyramine maleate (Rhône-Poulenc, Vitry/Seine, France); pentobarbitone (sodium pentobarbital, Clin-Midy, Montpellier, France); zapoglobin and isoton II (Coultronics, France); pancuronium (Pavulon, Organon, France); $Al(OH)_3$; BSA fraction V (Sigma); RPMI 1640 culture medium (Flow Laboratories, McLean, VG, U.S.A.); thioglycollate medium (Difco). The antibodies and radiolabelled ligand for radioimmunoassay of TxB_2 were obtained from the URIA, Institut Pasteur-INSERM U207, France.

Data analysis

A one way analysis of variance (ANOVA) was used to compare the changes between different treatments. If significance was determined, individual comparisons were subsequently tested with Dunnett's *t* test for unpaired values (Dunnett, 1964). For the analysis of $T \times B_2$ and histamine release in individual comparisons the Mann Whitney test was used, since the observations were drawn from not normally distributed populations (Siegel, 1956). Differences were considered statistically significant when $P<0.05$.

Results

Interference of NRIF with the neutrophil recruitment into guinea-pig bronchoalveolar lavage fluid induced by the intratracheal or intravenous administration of LPS

The intravenous injection of NRIF inhibited the neutrophil migration to the bronchoalveolar lavage fluid in naive guinea-pigs challenged 3 h after intratracheal LPS (Figure 1a) or 24 h after its intravenous injection (Figure 1b). There was no significant change in the eosinophil counts.

Lack of effect of NRIF on leukopenia or leucocytosis induced by intravenous LPS (100 µg kg^{-1})

Leukopenia induced by intravenous LPS was maximal 30 min after its injection, persisted for 3 h and was not modified by NRIF (Figure 2).

Similarly, pretreatment with NRIF failed to modify leucocytosis induced 24 h after intravenous injection of LPS. Leucocyte counts were: control = $3.9 \pm 0.13 \times 10^3$ µl^{-1}; LPS = $8.3 \pm 1.48 \times 10^3$ µl; NRIF + LPS = $9.01 \pm 0.94 \times 10^3$ µl^{-1} (mean ± s.e.mean, $n = 5-6$ animals).

Protection by NRIF against leucocyte recruitment induced by the intranasal challenge of sensitized guinea-pigs with ovalbumin

As seen in Figure 3, the number of eosinophils in the bronchoalveolar lavage fluid increased 5 h after the intranasal

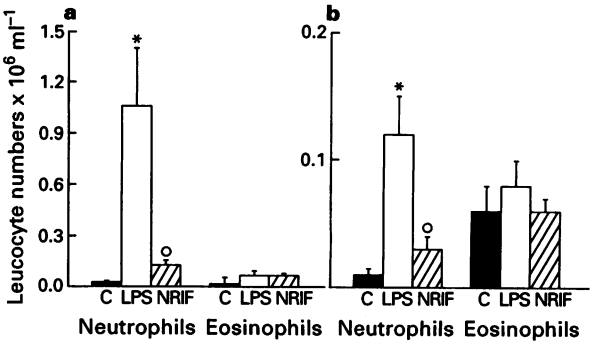

Figure 1 Neutrophil recruitment into the bronchoalveolar lavage fluid induced 3 h after the intratracheal (a, 1 mg) or 24 h after the intravenous (b, 100 µg kg^{-1}) administration of lipopolysaccharide (LPS) (open columns) to normal guinea-pigs. Interference of NRIF i.v. injected 15 min before LPS (hatched columns). Controls (C) received saline. Columns represent the means ± s.e.mean (vertical bar) of the leucocyte numbers for 5 to 7 animals. $*P<0.05$ as compared to controls; $°P<0.05$ as compared to LPS.

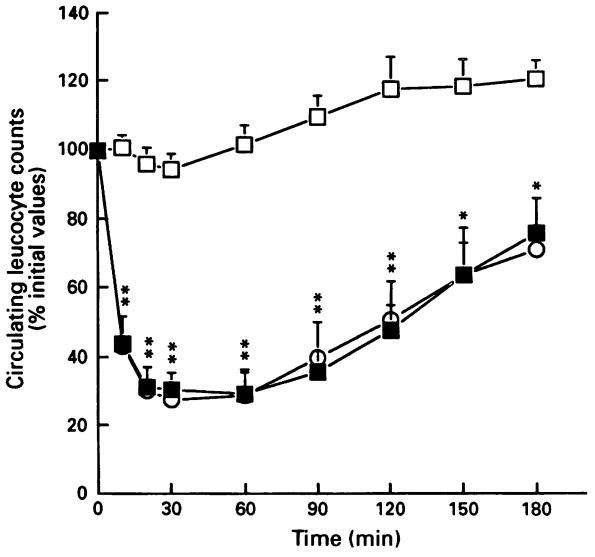

Figure 2 Leukopenia induced at different times after i.v. administration of lipopolysaccharide (LPS, 100 µg kg^{-1}, ○) to normal guinea-pigs. Lack of effect of NRIF i.v. injected 15 min before LPS (■). Controls received saline (□). Blood samples were collected from the carotid artery 4 h after anaesthesia with sodium pentobarbitone. Results are shown as the means ± s.e.mean (vertical bars) of the leucocyte counts of 4 to 5 animals. $**P<0.01$; $*P<0.05$ as compared to controls.

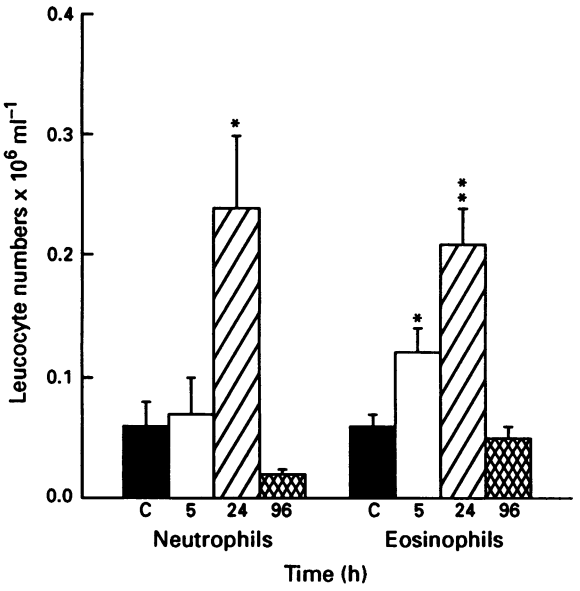

Figure 3 Leucocyte recruitment into bronchoalveolar lavage fluid at different times (5, 24 and 96 h) after the intranasal challenge with ovalbumin (OA, 1 mg) on the 14th day after sensitization. Controls received saline ($n = 12$). Results represent the means ± s.e.mean (vertical bars) of the leucocyte numbers in 5 h ($n = 5$), 24 h ($n = 6$) and 96 h ($n = 6$) group of animals. $^*P<0.05$; $^{**}P<0.01$ as compared to controls.

challenge of sensitized animals with OA. This augmentation of eosinophil counts persisted after 24 h and was associated with a significant increase in neutrophil numbers. At no time were mononuclear cell counts altered (data not shown).

For the assessment of potential inhibition, the optimal interval of 24 h was used. Pretreatment with NRIF blocked the OA-induced increase in neutrophil and eosinophil counts (Figure 4).

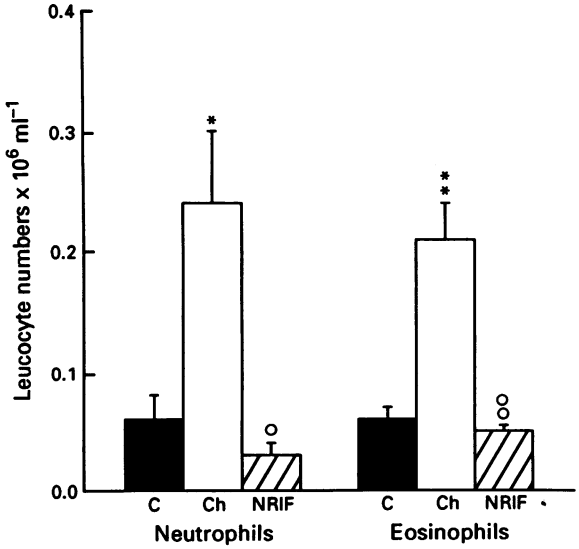

Figure 4 Leucocyte recruitment induced into the bronchoalveolar lavage fluid 24 h after the intranasal challenge with ovalbumin (Ch, 1 mg, $n = 6$). Protection by NRIF i.v. injected 15 min before and 5 h after challenge (hatched columns, $n = 6$). Controls (C) received saline as intranasal challenge ($n = 12$). Columns represent the means ± s.e.mean (vertical bars) for the leucocyte numbers of the animals. $^*P<0.05$; $^{**}P<0.01$ as compared to controls; $^\circ P<0.05$; $^{\circ\circ}P<0.01$ as compared to challenge.

Interference of NRIF with PAF and antigen-induced bronchoconstriction in lungs from sensitized guinea-pigs

Guinea-pigs were anaesthetized and the lungs removed and perfused as described in the Methods section. After a 10 min equilibration period, 3 and 30 ng doses of PAF were sequentially injected, at 10 min intervals, into the pulmonary artery of each perfused lung preparation. At the end of each experiment, 10 μg OA was administered to assess the anaphylactic response in lungs from sensitized animals. The injection of 3 and 30 ng PAF into lungs from actively sensitized but unchallenged guinea-pigs resulted in dose-dependent bronchoconstriction (Figure 5, solid columns). The first administration of 3 ng PAF to lungs from challenged animals evoked a significant increase in bronchoconstriction, which was similar to that induced by 30 ng of the phospholipid (Figure 5, open columns). After the administration of PAF, an injection of 10 μg OA produced a further increase of bronchoconstriction, but this did not differ significantly in area from that induced by the highest dose of PAF. No alteration was found in the intensity of bronchoconstriction induced by PAF in guinea-pigs pretreated with NRIF, although a significant inhibition of bronchoconstriction induced by the antigen was observed (Figure 5, hatched columns).

Failure of NRIF to inhibit PAF-induced histamine release from lungs of challenged guinea-pigs

One minute fractions of each lung effluent were collected before, during the first 3 min and between the 3rd and 6th min after the successive injections of the two doses of PAF and OA, for histamine and TxB_2 determination, as described in the Methods section. The basal release of histamine from lungs of challenged guinea-pigs was above that of control unchallenged lungs. Histamine release was enhanced after the injection of 3 ng PAF into lungs from challenged guinea-pigs (Figure 6, open columns). This release reached greater values in the first 3 min after PAF, but the effect persisted at 6 min. Pretreatment with NRIF failed to alter this histamine release (Figure 6, hatched columns), or that induced by 10 μg OA

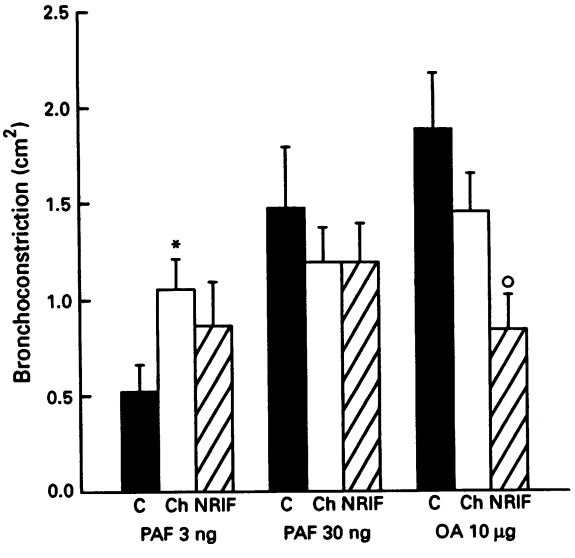

Figure 5 Bronchoconstrictor responses induced by the intra-arterial administration of 3 and 30 ng PAF and 10 μg ovalbumin (OA) into perfused lungs from sensitized and challenged guinea-pigs (Ch, $n = 11$). Controls (C) received saline as a challenge ($n = 9$). NRIF was injected i.v. 15 min before and 5 h after antigen challenge (cross hatched columns, $n = 8$) and experiments were performed 24 h later. Columns represent means ± s.e.mean (vertical bars) of the bronchoconstrictor effect. $^*P<0.05$ as compared to control; $^\circ P<0.05$ as compared to challenge.

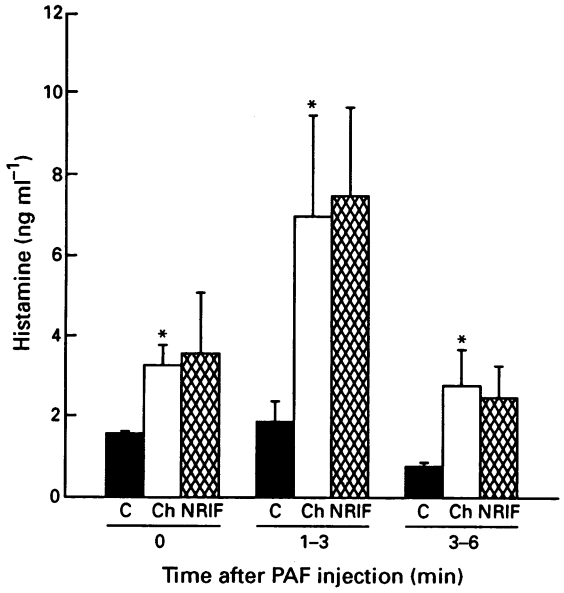

Figure 6 Release of histamine from isolated lungs of challenged (Ch) guinea-pigs injected with 3 ng PAF. One minute fractions of the lung effluent were collected before, during the first 3 min and between the 3rd and 6th min after injection of PAF, for the determinations of histamine. Controls (C) received saline. Lack of effect of pretreatment with NRIF (cross hatched columns). Experiments were performed 24 h after intranasal challenge. Results are expressed as the mean values ± s.e.mean (vertical bars) of histamine release for 6 to 8 experiments. $*P<0.05$ as compared to controls.

(data not shown). Similarly, no statistically significant differences were noted in TxB_2 release from control (1.5 ± 0.77 ng ml^{-1}), challenged (3.4 ± 1.2 ng ml^{-1}) and NRIF + challenged (5.33 ± 2.39 ng ml^{-1}) isolated perfused lungs, during the first 3 min after 3 ng PAF.

Discussion

In the present study, NRIF obtained from rat macrophages was effective in inhibiting neutrophil migration into the bronchoalveolar lavage fluid following the intratracheal or intravenous administration of LPS to naive guinea-pigs. Since NRIF is not species-specific (it also blocks inflammatory neutrophil migration in mice, Tavares-Murta *et al.*, unpublished), it can be used as a tool for correlating the presence of emigrated leucocytes with tissue events. In contrast to the inhibitory effect upon neutrophil recruitment to the inflamed site, NRIF did not alter the biphasic effect of intravenous LPS, i.e. leucopenia and the subsequent increase in leucocyte numbers in blood. Thus, it is not by acting upon the motility of circulating leucocytes (or as an LPS-binding protein) that NRIF inhibits neutrophil migration. It has also been demonstrated that the inhibitory effect cannot be attributed to neutropenia or to hypotension, since crude macrophage supernatants containing NRIF failed to alter either when given intravenously to conscious rats (Cunha *et al.*, 1989). Moreover, NRIF does not modify the *in vitro* chemotactic responses of neutrophils (Tamashiro *et al.*, 1992) or eosinophils (Coëffier *et al.*, unpublished). Alternatively, it has been hypothesized that NRIF reduces neutrophil adhesion to the endothelium (Cunha *et al.*, 1989; Tamashiro *et al.*, 1992). Previous studies have suggested that intercellular adhesion molecule-1 may be pivotal in the pathogenesis of asthma (Wegner *et al.*, 1990). The hypothesis that NRIF may act by reducing the expression of adhesion molecules is attractive, but remains to be further elucidated.

Sensitized animals of different species develop airways obstruction immediately or several hours after immune challenge (Larsen, 1988), as well as increased leucocyte numbers in the bronchoalveolar lavage fluid (Lellouch-Tubiana *et al.*, 1988; Larsen *et al.*, 1991). A delay of 7 days after the booster injection of antigen for the full expression of bronchoconstrictor hyperresponsiveness has been described by Pretolani *et al.* (1988), whereas in the present work the lungs already showed hyperresponsiveness 24 h after challenge. Although we did not study the duration of the increased responsiveness, as compared with other methods, the short period necessary for sensitization makes this procedure suitable for the study of short-acting substances, such as peptides and protein hormones.

We also investigated the cell composition of the bronchoalveolar lavage fluid after immune challenge by the intranasal route. The number of eosinophils was markedly increased 5 and 24 h after challenge, whereas neutrophil counts were also increased, but were delayed, being significant only at 24 h. Others have found even greater neutrophil counts in rats as compared to eosinophils, 24 h after aerosolized antigen (Kips *et al.*, 1992). One possible explanation for the large neutrophilic effect in our experiments involves the direct nasal instillation of the antigen. With a slightly different procedure of active sensitization, antigen challenge also caused a significant early increase in the number of eosinophils, without a change in neutrophil counts (Desquand *et al.*, 1991). Thus, our present results support the concept that the signal for antigen-induced eosinophil and neutrophil recruitment involves different pathways, which are enhanced in sensitized guinea-pigs.

Isolated lungs from challenged guinea-pigs compared with those of control saline-treated animals showed an increased bronchoconstriction and an enhanced histamine release into the perfused fluid after the first PAF injection. A protocol involving the sequential injections of two doses of PAF and a final challenge with OA was used, in order to assess the possible interference of NRIF with submaximal provocations with PAF and with the responses to antigen. Indeed, sensitization followed by a booster injection of antigen was critical for the development of bronchopulmonary hyperresponsiveness in lungs from actively sensitized guinea-pigs (Pretolani *et al.*, 1988). The fact that we did not find an increased bronchoconstriction with the second injection of PAF or with ovalbumin in lungs from challenged animals may result from our experimental conditions, since the effects of PAF were already supramaximal at its lowest dose. In contrast to Pretolani *et al.* (1988), we did not find an enhanced thromboxane B_2 release after PAF, indicating that in this model, bronchoconstriction and release of this mediator occur independently. It is also of interest that lungs from NRIF-pretreated animals were less responsive to challenge with ovalbumin than controls, suggesting that inhibition by NRIF may extend to some effects of antigen, particularly bronchoconstriction.

Simultaneous accumulation of granulocytes, as assessed by bronchoalveolar lavage counts, and hyperreactivity of isolated lungs to PAF may be triggered by independent mechanisms, since NRIF inhibited neutrophil and eosinophil recruitment but failed to modify the enhanced airway reactivity to PAF. In confirmation, Sanjar *et al.* (1990) detected an increased airway content of neutrophils and eosinophils accompanied by enhanced airway reactivity, which peaked 8 to 24 h after exposure of sensitized guinea-pigs to antigen. Although these events were associated, no significant correlation between them was found, as assessed by drug inhibition. Moreover, one group of sensitized animals responded to antigen exposure with severe bronchial eosinophilia unaccompanied by increased airway reactivity. Pretolani *et al.* (unpublished) obtained similar results in guinea-pigs made hypereosinophilic with repeated exposure to antigen by aerosol, since bronchoconstriction following intratracheal methacholine was not modified. Finally, Elwood *et al.* (1992) found in-

creased granulocyte and lymphocyte counts in bronchoalveolar lavage fluid, 18 to 24 h after exposure of actively sensitized rats to aerosolized ovalbumin. Although this was accompanied by increased bronchial responsiveness to inhaled acetylcholine, cyclosporin A failed to prevent airway hyperresponsiveness while producing a significant inhibition of the inflammatory cell influx.

NRIF inhibited leucocyte migration into the lungs without interfering with bronchial hyperresponsiveness to PAF, even though it reduced the bronchoconstrictor effect of OA. Thus, recruited cells may contribute to OA-induced bronchoconstriction. In this context, we have already shown that NRIF inhibits carrageenan-induced rat paw oedema, which depends upon migrating cells, but failed to modify oedema induced by dextran, which depends upon resident mastocytes (Tamashiro *et al.*, 1992; Lo *et al.*, 1982). NRIF also blocks antigen-induced oedema in rats, suggesting that here also migrating leucocytes are involved (Tamashiro *et al.*, 1992).

Bronchial asthma is a multicellular-dependent process. It seems that the immunological stimulus leads to secretion of substances that produce different effects on airway function. This study shows coincidence between the increased number of inflammatory cells in bronchoalveolar lavage fluid and airway hyperresponsiveness, since they occurred following the same stimulus, but the studies using NRIF demonstrated that the two events can be dissociated.

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Investigations into the mechanism of vasoconstrictor action of the topical steroid betamethasone-17-valerate in the rat

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1 The effect of topical betamethasone upon skin blood flow was investigated in the rat. Two types of vasodilator stimuli were used; local heating to the surface of the skin and intradermal application of inflammatory agents. Blood flow was measured by laser doppler velocimetry.

2 Topical betamethasone-17-valerate (1 g with an 18 h pretreatment) significantly inhibited the heat-induced vasodilatation in the rat skin, as also did systemically administered betamethasone (1 mg kg^{-1}, 3 h pretreatment).

3 Angiotensin converting enzyme (ACE) inhibitors (captopril, 5 mg kg^{-1} and enalapril, 1 mg kg^{-1}, 30 min pretreatments) were the only drugs out of several different types of systemically administered inhibitors and antagonists that were tested which also inhibited the heat-induced vasodilatation. Aprotinin (100,000 KIU kg^{-1}, 5 min pretreatment) a serine protease inhibitor, significantly potentiated the heat-induced response.

4 Bradykinin (50 nmol per site), des-Arg^9-bradykinin (5 nmol per site), substance P (0.1 nmol per site) and capsaicin (1 μmol per site) induced an increase in skin blood flow.

5 Topical betamethasone treatment resulted in a significant inhibition of the vasodilator response to des-Arg^9-bradykinin, whereas captopril treatment inhibited the responses to substance P, capsaicin, bradykinin and des-Arg^9-bradykinin.

6 Intradermal application of captopril (10–100 μg) also caused a dose-dependent inhibition of the heat-induced vasodilatation.

7 These results suggest that topical betamethasone may be acting in a manner similar to that of the ACE inhibitors to produce an inhibition of the flow responses in the skin and that this effect may be brought about by interfering with the action of vasodilator peptide(s) or protein(s).

Keywords: Topical steroids; vasoconstriction; skin blood flow; ACE inhibitors

Introduction

Topical steroids have been used in the treatment of skin disease for many years. Their effectiveness and the availability of efficient assay systems have aided their ever-increasing popularity. Although these drugs are used extensively in the treatment of skin disease their mechanism of action remains unknown.

One of the properties of the topical steroids is their ability to induce a blanching of the skin after topical application, otherwise described as vasoconstriction. In fact, it is upon this property that the most commonly used assay system for determination of anti-inflammatory potency is based (McKenzie & Stoughton, 1962). Although this blanching is used as a marker of anti-inflammatory potency, no direct link between these properties has ever been made. It is not known exactly how this blanching is brought about or what role, if any, it may have to play in providing the anti-inflammatory effects of the topical steroids.

Vasodilatation is one of the primary features of inflammation and its importance was outlined by Williams & Peck in 1977, when describing the two-mediator hypothesis. This theory indicates that oedema is dependent upon two factors, firstly the presence of a vasodilator and secondly an agent which incudes venular leakage, both of which are normally present at a site of inflammation. They also stated that these two factors when present together will synergize to produce a greater oedema than when either is given alone. Thus, it seems correct to assume that vasodilatation is an important factor in the development and presentation of an inflammatory response.

As indicated earlier, topical steroids will cause a vasoconstriction of the skin as shown by blanching, although whether this occurs through the induction of a vasoconstrictor substance or through the inhibition of formation or enhanced breakdown of a vasodilator(s) is unknown. It seems plausible that the vasoconstrictor property of the steroids contributes to the anti-inflammatory behaviour of these drugs, since vasodilatation plays such an active role in the development of an inflammation. If we can gain an understanding of exactly how this vasoconstriction is produced then its relevance to the anti-inflammatory effects of the topical steroids may be eluicidated.

Laser Doppler velocimetry (LDV) is one of the techniques widely employed to measure both basal and stimulus-induced changes in cutaneous blood flow (Bircher *et al.*, 1990). In 1983, Amantea *et al.* used the LDV technique to measure the effects of steroids upon human skin blood flow. They compared basal flow to flow after treatment with the steroid, fluocinonide, and found no measurable decrease in flow although blanching was visible to the naked eye. Therefore they went on to investigate the effect of topical steroid treatment upon topical administration of aqueous methyl nicotinate, a powerful vasodilator. Here again topical steroid treatment had no effect. This group concluded that laser Doppler velocimetry did not provide a clear-cut system with which the vasoconstrictor effects of the topical steroids could be investigated. However, we have further developed and adapted this technique to measure rat skin basal blood flow and stimulus-induced increases in blood flow to provide a system with which the effects of topical steroid upon skin blood flow may be investigated. Preliminary accounts of these results have been presented to the British Pharmacological Society (Ahluwalia & Flower, 1991; 1992).

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Methods

Male Wistar rats (250–350 g) were anaesthetized with a mixture of hypnorm and hypnovel in sterile water in a ratio of 1:1:2, 4 ml kg^{-1}, their backs shaved and a depilatory cream applied to remove all remaining hair. Rats were left for a minimum of 1 h, during which time recovery from anaesthesia was allowed, before any experimental procedure was carried out. Rats were anaesthetized and skin blood flow measured on the back by the LDV technique (Perimed, Periflux PF3). The effect of topical and systemic steroid and a variety of enzyme inhibitors and receptor antagonists upon two different types of stimuli were investigated: (i) heat-induced changes in blood flow and (ii), chemical vasodilator-induced changes in blood flow.

Local heating in rat skin

Prepared rats were anaesthetized and basal blood flow measured by LDV at a marked site. Heat, 44°C, was applied for 20 min to this site after which blood flow was again measured. The heat was supplied by a thermostatically controlled heated disc which was coupled to the LDV. This measurement was repeated in duplicate at sites of measured distances apart upon opposite sides on the back of the rat, approximately 5 cm from the base of the tail. To investigate the effect of steroid upon this heat response, 1 g betamethasone-17-valerate was rubbed into one side of the back of the rat for approximately 2–3 min. After 18 h the effect of heat upon skin blood flow on the steroid-treated site was again measured and compared to the response observed on the untreated side. Vehicle controls were also carried out. The effects upon heat-induced vasodilatation of systemically administered betamethasone (0.1–1 mg kg^{-1}, s.c.) with 1 and 3 h pretreatment periods and a variety of enzyme inhibitors and receptor antagonists, with varying pretreatment periods, were also investigated. Saline controls were performed. All doses of blocking agents used in these experiments have previously been shown to be effective in several studies.

Intradermal vasodilators in rat skin

Prepared rats were anaesthetized and basal blood flow measured at a marked site. Once a stable basal flow had been obtained, vasodilators at different concentrations were injected intradermally (50 µl) 2–3 mm from the site at which flow was being measured and the vasodilator effect observed. The following agents were studied: bradykinin (BK) 50.0 nmol per site), des-Arg^9-BK (5.0 nmol per site), substance P (SP) (0.1 nmol per site) and capsaicin (1 µmol per site) and saline control. There were 6 available sites on the back of the rat, 3 on each side. For the series of experiments investigating the effect of topical betamethasone-17-valerate upon the dilator effects of the aforenamed agonists, 3 of the stimuli could be tested on any one rat. Control responses were measured on both sides prior to topical betamethasone application. After 18 h the responses to the vasodilators on the treated side were compared to the responses to these agonists on the untreated side of the rat back.

We then investigated the effect of the angiotensin converting enzyme (ACE) inhibitor, captopril (5 mg kg^{-1}, i.v. with a 30 min pretreatment) upon the dilator responses to each of the intradermal stimuli. Control responses were carried out on one side of the back of the rat, captopril administered and then the responses repeated on the opposite side of the rat back.

Intradermal captopril in rat skin

Prepared rats were anaesthetized and basal blood flow measured at a marked site. The heat stimulus was then applied and the peak flow response measured. This was then repeated on the contralateral side of the back of the rat. Once both controls had been established captopril (10–100 µg) was injected, in 50 µl of saline, intradermally approximately 2–3 mm from the site at which flow was measured. Basal flow was measured for the following 5 min, after which the heat stimulus was reapplied and the response measured. This was repeated on the opposite side, but with an intradermal injection of saline alone.

Materials

Betamethasone sodium phosphate was produced by Glaxo. Topical betamethasone-17-valerate (Betnovate) and vehicle cream were generous gifts from Glaxo, UK. Indomethacin, atropine, pentolinium, captopril, enalapril, aprotinin, bradykinin, des-Arg^9-bradykinin, substance P and capsaicin were purchased from Sigma, UK. Methysergide maleate was purchased from Sandoz, UK, mepyramine maleate from May and Baker Ltd, UK.

All peptides were dissolved in sterile water and kept at −20°C until used. Indomethacin was dissolved in 1% sodium bicarbonate in saline. Methysergide, mepyramine, captopril, enalapril, aprotinin, atropine, pentolinium, N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) were all dissolved in saline and made up freshly each day.

Data and statistical analysis

All the results are expressed as mean ± s.e.mean with n representing the number of animals. Flow is measured by LDV in an arbitrary unit called the Perfusion Unit (PU), where:

Blood flow (PU) =
no. of red blood cells × speed at which the cells are moving

The response to heat or intradermal vasodilator was measured as the maximal change in flow from basal flow and this response after drug treatment was expressed as a percentage of the response before drug treatment. Student's paired and un-paired t tests were used to determine the levels of significance with a $^*P < 0.05$ being considered as significant.

Results

Local heating

Basal blood flow in the rat skin measured 19.7 ± 1.03 PU ($n = 32$). Local heating of rat skin gave a peak flow of 60.5 ± 5.2 PU ($n = 32$), therefore causing approximately a 40 PU increase in blood flow. Topical treatment of the skin with betamethasone-17-valerate significantly attenuated the responses to the heat stimulus, whereas the vehicle cream had no significant inhibitory effect (Figure 1). Systemic betamethasone at both 0.1 mg kg^{-1} with a 3 h pretreatment and 1 mg kg^{-1} with a 1 h pretreatment had no significant inhibitory effects upon the heat-induced vasodilatation, the response after steroid treatment being 117.9 ± 35.9%, $n = 3$, and 95.1 ± 15.5%, $n = 5$, of the response before treatment, respectively. However a 3 h pretreatment of betamethasone of 1 mg kg^{-1} inhibited the dilator response to the heat stimulus, the response being 44.9 ± 17.6%, $n = 4$ of the response before betamethasone. This was significant at the 5% level when compared to the response after a 3 h pretreatment with saline (114.3 ± 7.6%, $n = 3$).

Table 1 shows the effects of a range of antagonists and enzyme inhibitors upon the heat-induced vasodilatation. Only the ACE inhibitors, captopril and enalapril, significantly inhibited the heat-induced increase in flow and aprotinin, a serine protease inhibitor, significantly potentiated the heat response.

Intradermal vasodilatation

All of the dilators used caused increases in flow within the first 5 min after injection. The changes in flow induced by each of the stimuli were approximately the same and the particular doses used here were chosen from dose-response curves to each of the stimuli. The change in flow in response to BK (50 nmol) was 24.3 ± 3.3 PU ($n = 6$), des-Arg^9-BK (5 nmol) 18.7 ± 4.6 PU ($n = 4$), SP (0.1 nmol) 19.5 ± 2.2 PU ($n = 6$) and capsaicin (1 μmol) 23.6 ± 6.2 PU ($n = 8$). We found that after topical betamethasone treatment only the dilator response to des-Arg^9-BK was significantly inhibited at the 5% level by the paired Student's t test as compared to the response on the untreated side, see Figure 2. An interesting observation was that although the response to capsaicin after steroid treatment remained unaffected, the response on the untreated side was reduced, although the two were not significantly different.

Unlike topical steroid treatment, systemic administration of captopril resulted in significant inhibition of the flow responses to all the intradermal stimuli (see Figure 3). Systemically administered saline had no effect upon the dilator responses.

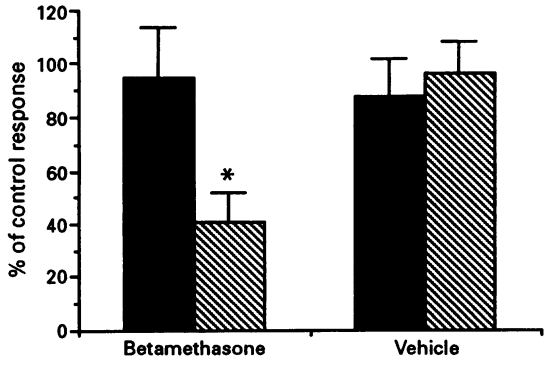

Figure 1 Inhibition of the response to heat-induced vasodilatation by topical application of betamethasone-17-valerate cream ($n = 7$) or vehicle cream ($n = 3$) (1 g rubbed in to one side of the back of the rat), with an 18 h pretreatment represented by the hatched columns with the response on the untreated control side represented by the solid columns. The results are expressed as mean (the response after drug treatment is expressed as a % of the response before drug treatment) with s.e.mean shown by vertical bars and statistical significance (comparison to untreated side of rat skin) is represented by $*P < 0.05$ (Student's paired t test). The control changes in blood flow in response to heat were 44.1 ± 9.7 PU and 40.1 ± 9.5 PU on the steroid-treated and untreated sides respectively and 33.2 ± 14.5 PU on the vehicle-treated and 32.8 ± 11.6 PU on the untreated side prior to topical treatment measured by laser Doppler velocimetry.

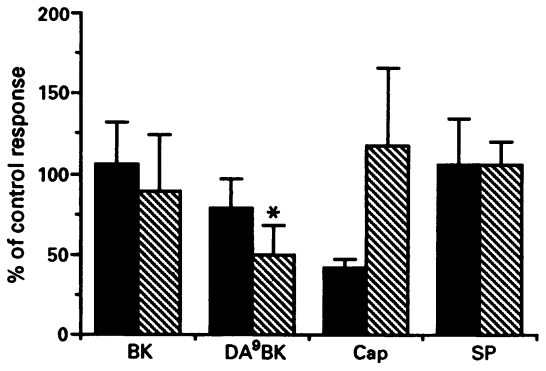

Figure 2 Effect of topical application of betamethasone-17-valerate upon the response to bradykinin (BK, 50 nmol per site, $n = 8$), des-Arg^9-bradykinin (DA^{9}BK, 5 nmol per site, $n = 6$), capsaicin (Cap, 1 μmol per site, $n = 8$) and substance P (SP, 0.1 nmol per site, $n = 6$) shown by the hatched columns. All of the doses of vasodilators used gave similar control peak changes in blood flow of 18.7–24.3 PU, as measured by laser Doppler velocimetry. Values are means (response after drug treatment is expressed as a % of the response before drug treatment) with s.e.mean shown by vertical bars and statistical significance (compared to responses upon untreated side of rat skin shown by the filled columns) represented by $*P < 0.05$ (Student's paired t test).

Intradermal captopril

Captopril (10–100 μg) injected intradermally caused a dose-dependent inhibition of the heat-induced vasodilatation as shown in Figure 4. Captopril alone had no effect upon basal blood flow in the skin when injected intradermally as was the case with systemic captopril administration.

Discussion

The aim of this study was to investigate the mechanisms involved in topical steroid-induced blanching or vasoconstriction. It is well known that steroid when applied topically will induce a blanching of the skin after several hours (McKenzie & Stoughton, 1962). In accordance with others (Amantea *et al.*, 1983) no measurable change in basal skin blood flow

Table 1 Effect of a range of different drug types upon the increase in blood flow shown in response to local heating (44°C) of the rat skin

Drug treatment	*% of control heat-induced change in blood flow*	
Saline (i.v. 30 min)	99.6 ± 6.4	($n = 4$)
Indomethacin (5 mg kg^{-1}, i.v. 30 min)	104.2 ± 21.4	($n = 5$)
Atropine (1 mg kg^{-1}, i.v. 30 min)	90.7 ± 21.4	($n = 3$)
L-NMMA (100 mg kg^{-1}, i.v. 30 min)	107.8 ± 13.3	($n = 5$)
L-NAME (10 mg kg^{-1}, i.v. 15 min)	136.8 ± 22.1	($n = 5$)
Methysergide (6 mg kg^{-1}, i.p. 15 min)	159.8 ± 26.1	($n = 4$)
Mepyramine (6 mg kg^{-1}, i.p. 15 min)	98.5 ± 15.3	($n = 4$)
Pentolinium (5 mg kg^{-1}, i.v. 30 min)	84.7 ± 18.9	($n = 7$)
Captopril (5 mg kg^{-1}, i.v. 30 min)	32.3 ± 8.6***	($n = 8$)
Enalapril (1 mg kg^{-1}, i.v. 30 min)	24.4 ± 5.3***	($n = 5$)
Aprotinin (100,000 KIU kg^{-1}, i.v. 5 min)	240.5 ± 51.5*	($n = 5$)

Results are expressed as mean (the response after drug treatment being expressed as a % of the response before drug treatment) ± s.e.mean.
n = number of animals.
Statistical significance (Student's t test): $*P < 0.05$; $***P < 0.001$, compared to saline control.

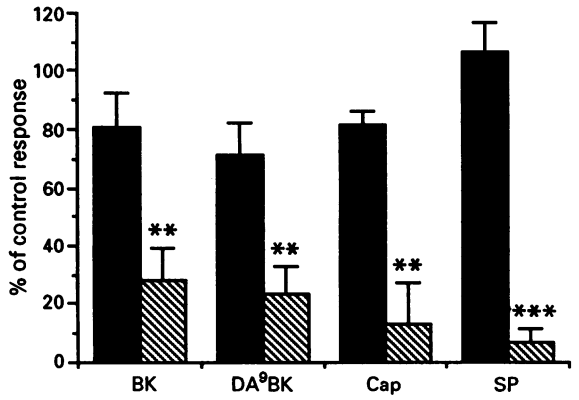

Figure 3 Inhibitory effects of systemic captopril (5 mg kg^{-1}, 30 min pretreatment) with saline controls upon the dilator responses to bradykinin (BK, 50 nmol per site) ($n = 7$, i.v. saline $n = 7$), des-Arg^9-bradykinin (DA^9BK, 5 nmol per site) ($n = 7$, i.v. saline $n = 5$), substance P (SP, 0.1 nmol per site) ($n = 9$, saline i.v. $n = 8$) and capsaicin (Cap 1 μmol per site) ($n = 3$, saline i.v $n = 3$) in the rat skin. Values are means (response after drug treatment is expressed as a % of the response before drug treatment) with s.e.mean shown by vertical bars and statistical significance (comparison to the responses in saline-treated animals shown by solid columns) represented by $^{**}P < 0.01$ and $^{***}P < 0.001$.

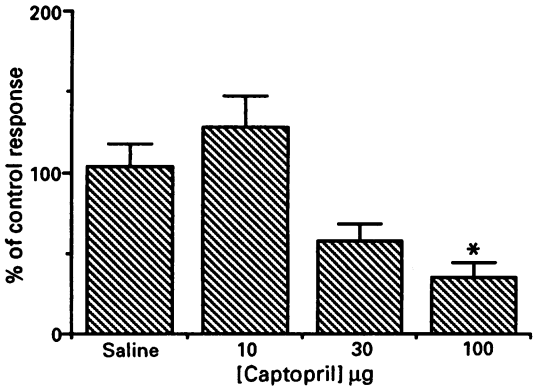

Figure 4 Inhibitory effects of intradermally administered captopril of doses of 10, 30 and 100 μg per site ($n = 5$ animals in each case) upon local heat-induced (44°C) vasodilatation in the rat skin. Values are means (responses after drug treatment are expressed as a % of the response before drug treatment) with s.e.mean shown by vertical bars and statistical significance (comparison to saline control, $n = 15$ animals) represented by $^{*}P < 0.05$ (Student's unpaired t test).

after steroid treatment was found. We next investigated the effect of steroid application upon a stimulus-induced increase in flow. The stimulus chosen was one of local skin heating. The reasons for this choice were manifold. Firstly, heat at 44°C provided a non-pathological response causing no injury, the threshold for thermal injury being 45–46°C (Rocha e Silva, 1964; Sevitt, 1964; Saria, 1984). Also the response to this heat stimulus is both reproducible and completely reversible by cooling. Thirdly, the technique is non-invasive, an ideal setting for investigating skin blood flow. Finally, a mild heat stimulus of vasodilatation seemed an appropriate stimulus to use as it resembled that seen in an inflammatory response, where the vasodilatation is insufficient to produce oedema unless there is accompanying permeability changes. Topical steroid administration will reduce the flow response after induced reactive hyperaemia (Bisgaard *et al.*, 1986), however, this does not provide a system in which extrapolation to the inflammatory situation can be made, as is we believe, the case in our model.

The topical betamethasone treatment used for these studies has been shown to be sufficient to inhibit oedema formation by a range of different inflammatory mediators in the rat (Ahluwalia & Flower, 1992) and is comparable to the doses used in the vasoconstriction assay in man of Marks & Sawyer (1986). We found that although basal flow in the rat skin appeared to be unaffected by topical betamethasone treatment, the increase in flow produced in response to heat was significantly attenuated. This suggests that the vasoconstrictor effects of the steroid, in this case, are probably not brought about through the production of a vasoconstrictor substance but, rather, that the steroid is affecting the processing of a substance(s) responsible for the vasodilator response to heat and consequently only becomes apparent when applying the stimulus. It seems also that this inhibition of induced flow is a general feature of steroids, as systemic administration of steroid also inhibited the response to heat.

Table 1 shows the effects of a variety of enzyme inhibitors and receptor antagonists upon basal flow and heat-induced vasodilatation. The reason for looking at the effects of these antagonists and inhibitors was to find an agent which produced a similar pattern of inhibition of the increase in flow as the topical steroid. This could provide some indication of the mediator involved in the heat response and the system in which the steroids are exerting their inhibitory effects. Many of the mediators known to play various roles in the inflammatory response are not involved in this system; from the data we can exclude the prostanoids, nitric oxide (endothelium-derived relaxing factor), the cholinergic system, histamine and 5-hydroxytryptamine (5-HT). The ACE inhibitors were the only drugs that inhibited the heat response. The classical action of these inhibitors is to inhibit ACE resulting in a decrease in the conversion of angiotensin I to the active mediator of the renin-angiotensin-system, the vasoconstrictor, angiotensin II. Also ACE inhibition will induce an increase in the presence of the vasodilator bradykinin, by inhibiting its breakdown. These actions however do not explain the effects seen in this model and, in fact, are exactly the contrary to that observed i.e. the resultant effect of ACE inhibition would normally itself be vasodilatation whereas here it appears that ACE inhibitors result in an inhibition of vasodilatation. These findings suggest that the ACE inhibitors are acting on a peptide(s) or protein(s) other than BK or angiotensin II. Indeed these inhibitors can influence the activity of other proteolytic enzymes such as tripeptide aminopeptidases (Sach & Marks, 1982). The only other agent that we tested that had any significant effect upon the test response was aprotinin, the serine protease inhibitor. Aprotinin caused a significant potentiation of heat-induced vasodilatation. This when combined with the ACE inhibitor effects suggest that the heat-induced increase in flow may be brought about by a released peptide or protein, the processing of which may be altered by both serine protease and ACE inhibitors.

In situations of thermal injury it has been shown that a variety of vasodilatators are released which contribute to the pathology of the injury. These include bradykinin (Rocha e Silva & Antonio, 1960; Rocha e Silva & Rosenthal, 1961) and substance P (Saria, 1984). Although our model is not one of thermal injury it is conceivable that these mediators may also be involved in this heat response. Thus, we investigated the effect of steroid and captopril treatment upon the vasodilator effects of both BK, SP, des-arg^9-BK and capsaicin. Des-Arg^9-BK is an active metabolite of BK, which is active at B_1 receptors. B_1 receptors have been found to be present only in situations of stress such as shock or inflammation. Capsaicin produces a vasodilatation by inducing the release of the contents of the sensory neurone, one of which is SP.

We found that topical steroid inhibited only the response to des-Arg^9-BK; however, systemic captopril significantly inhibited the vasodilator responses to all of the stimuli, suggesting some non-specificity in the inhibitory effects of this

drug. B_1 receptors as indicated earlier are not normally constitutively present but are induced in the presence of inflammatory stimuli by a process which depends upon new protein synthesis (Bouthillier *et al.*, 1987). It has been shown that the induction of B_1 receptors, by various cytokines, in the rabbit isolated aorta is prevented by exposure to the steroid, dexamethasone (Deblois *et al.*, 1988). Possibly the topical steroid, here, is acting at the level of the B_1 receptor to produce its inhibitory effect upon the response to des-Arg^9-BK. Captopril introduced intradermally at the site of the heat stimulus also inhibited the response, while saline administration on the contralateral side had no effect, implying that there was no systemic component to the observed captopril effects here. This indicates that the inhibitory effect of captopril in this system is not merely an artifact due to peripheral vasodilatation and hence recruitment of flow away from the skin, but rather a local action within the skin microcirculation.

Thus it appears that the heat-induced vasodilatation may be brought about by the activation of a peptide(s) or protein(s), the processing of which can be manipulated by both ACE inhibitors and serine protease inhibition. Since, both the ACE inhibitors and topical betamethasone appear to have similar resultant effects, it is conceivable that the skin blood flow effects of topical betamethasone are also brought about via the processing of this same unknown substance.

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Cibenzoline inhibits diazoxide- and 2,4-dinitrophenol-activated ATP-sensitive K^+ channels in guinea-pig ventricular cells

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1 We have investigated the effects of diazoxide (a sulphonamide derivative) and cibenzoline (a class I antiarrhythmic drug) on ATP-sensitive K^+ currents in guinea-pig ventricular cells, using whole-cell clamp techniques.

2 Diazoxide (50 μM) produced a marked shortening of action potential duration which was antagonized by 1 μM glibenclamide, an ATP-sensitive K^+ channel blocker.

3 Diazoxide (50 μM) increased the quasi-steady state outward current elicited by a ramp voltage protocol (− 20 mV s^{-1}) at potentials positive to about − 70 mV. This effect was completely prevented in the presence of glibenclamide (1 μM), thereby suggesting that diazoxide opens ATP-sensitive K^+ channels.

4 Cibenzoline (5 μM) depressed the diazoxide-induced increases in the outward current and the pretreatment with this agent prevented the development of the diazoxide-induced outward current.

5 Cibenzoline (10 μM) reversed the 2,4-dinitrophenol (50 μM)-induced shortening of the action potential duration partially but significantly.

6 These results suggest that diazoxide activates ATP-sensitive K^+ channels of guinea-pig ventricular cells and that cibenzoline, at therapeutic concentrations, inhibits this channel.

Keywords: Cibenzoline; diazoxide; ATP-sensitive K^+ channel; glibenclamide; guinea-pig ventricular cell

Introduction

Adenosine triphosphate (ATP)-sensitive K^+ channels are present in cardiac cells (Noma, 1983; Trube & Hescheler, 1984), in pancreatic β-cells (Cook & Hales, 1984; Ashcroft *et al.*, 1984), in skeletal muscle cells (Spruce *et al.*, 1985) and in neuronal cells (Ashford *et al.*, 1988). In cardiac cells, activation of the ATP-sensitive K^+ channel plays a major role in shortening the action potential induced by ischaemia or by metabolic inhibitors (Trube & Hescheler, 1984; Noma & Shibasaki, 1985). K^+ channel openers, such as pinacidil, nicorandil and cromakalim, are suggested to be specific in opening the ATP-sensitive K^+ channels of cardiac cells (Escande *et al.*, 1988; Arena & Kass, 1989; Hiraoka & Fan, 1989). Little information is available with regard to the effect of diazoxide (a sulphonamide derivative) on the ATP-sensitive K^+ channels of cardiac cells, although Faivre & Findlay (1989) reported that diazoxide did not activate the ATP-sensitive K^+ channels in rat ventricular myocytes. In pancreatic β-cells, however, application of diazoxide led to opening of the ATP-sensitive K^+ channels (Trube *et al.*, 1986; Dunne *et al.*, 1987; Zünkler *et al.*, 1988; Kozlowski *et al.*, 1989). The ATP-sensitive K^+ channel of pancreatic β-cells plays a pivotal role in eliciting insulin secretion (Petersen & Dunne, 1989). Sulphonylureas, such as glibenclamide and tolbutamide, are reported to inhibit the ATP-sensitive K^+ channel not only in pancreatic β-cells (Sturgess *et al.*, 1985; Trube *et al.*, 1986; Zünkler *et al.*, 1988) but also in cardiac cells (Belles *et al.*, 1987; Fosset *et al.*, 1988). In mouse pancreatic β-cells, tolbutamide reversed the diazoxide-induced hyperpolarization (Henquin & Meissner, 1982).

It has been reported that cibenzoline, a novel class I antiarrhythmic drug, induced hypoglycaemia in some patients (Hilleman *et al.*, 1987; Gachot *et al.*, 1988). However, details of the mechanism of this effect are not available. It seems reasonable to speculate that cibenzoline, like sulphonylureas, might block the ATP-sensitive K^+ channels in pancreatic β-cells, and produce hypoglycaemia by enhancing the release of insulin secretion. Such speculation prompted us to examine whether or not cibenzoline inhibits the ATP-sensitive K^+ channels in cardiac cells, using whole-cell current- and voltage-clamp methods.

Our results demonstrate that diazoxide activates the ATP-sensitive K^+ channel and cibenzoline inhibits this channel.

Methods

Cell isolation

Single ventricular cells from guinea-pigs were prepared as described by Taniguchi *et al.* (1981). In brief, guinea-pigs weighing 250–300 g were stunned by a blow on the neck and the heart was quickly dissected and perfused through the coronary artery with modified Tyrode solution for 3–5 min. The composition of modified Tyrode solution was (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.16, $NaHCO_3$ 3.0, glucose 5.5 and HEPES 5.0 (pH = 7.4 adjusted with NaOH). The perfusing solution was changed to nominally calcium-free modified Tyrode solution containing 0.004% collagenase (Type I Yakult, Tokyo, Japan). After 2–3 min perfusion, the heart was immersed in high-[K^+], low-[Cl^-] solution of the following composition (in mM): KCl 5, glutamic acid 70, taurine 10, oxalic acid 10, KH_2PO_4 5, HEPES 5, glucose 11 and EGTA 0.5 (pH = 7.4 adjusted with KOH, making final K^+ concentrations of ~136 mM). The temperature of these perfusates was kept at 35–36°C. A small piece of the tissue was detached and cells were dispersed by stirring in the recording chamber (0.8 ml in volume) and was placed on an inverted microscope (TMD Nikon, Tokyo, Japan). The chamber was continuously superfused with the modified Tyrode solution at a rate of 2–3 ml min^{-1} and the temperature of the solution was kept at 33–34°C. Only rod-shaped cells with a clear margin and striation were used for the experiments.

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Electrophysiological measurements

Current- and voltage-clamp experiments were performed using the tight-seal whole-cell recording method (Hamill *et al.*, 1981). The heat-polished glass pipettes were filled with (in mM) KCl 140, $MgCl_2$ 2.0, $CaCl_2$ 1.0, EGTA 11, HEPES 10 (pH = 7.2 adjusted with KOH). The resistance of the electrodes ranged from 2.5–5 megaohms. The 'giga-ohm seal' was accomplished between the pipette tip and the cell surface via gentle negative pressure applied to the pipette interior. Thereafter the patch membrane was disrupted by suction with a much greater negative pressure of -40 to $-100\ cmH_2O$. The membrane potentials and currents were recorded with a patch clamp amplifier (EPC-7 List, Darmstadt, Germany).

In current-clamp recording mode, action potentials were elicited at a rate of 0.2 Hz by an intracellular current injection with supra-threshold pulses of 5–10 ms duration. In whole-cell voltage clamp mode, we used a rectangular or ramp pulse protocol. In rectangular pulse protocol, the membrane potential was held at -40 mV to block the sodium current, and 500 ms rectangular voltage steps in 10 mV increments and decrements were applied from -100 mV to $+60$ mV at a rate of 0.2 Hz. In ramp pulse protocol, the membrane potential was held at -40 mV and depolarized first to $+60$ mV at a rate of $20\ mV\ s^{-1}$. It was then repolarized or hyperpolarized to -140 mV with a slope of $-20\ mV\ s^{-1}$, during which time the change in current was automatically plotted against the membrane potential. The voltage protocol was repeated once every 30 s.

The potential and current signals were filtered at 3 kHz and stored on magnetic tapes using a PCM data recording system (RP-880 NF Corp, Tokyo, Japan). The data were replayed and processed by a computer (PC-98XA NEC, Tokyo, Japan) equipped with an analogue-to-digital converter (ADX-98 Canopus Corp., Kobe, Japan), using the analysis porogramme DSS-98 (Canopus Corp., Kobe, Japan). The current signals were digitalized with a sampling interval of 1 kHz.

Drugs

Diazoxide (Sigma Chemical, St. Louis, U.S.A.) was prepared as 5 mM stock solution in 10% dimethyl sulphoxide (DMSO, Sigma) and glibenclamide (Hoechst, Japan, Tokyo, Japan) was dissolved as 0.4 mM stock solution in 0.05 N NaOH. Each stock solution was added to the Tyrode solution immediately before use to produce the final concentration given in the text and pH was readjusted to 7.4 if necessary. After dilution, the maximal DMSO concentration was 0.1% which had no effect on recordings. Cibenzoline succinate (a kind gift from Fujisawa Pharmaceutical Co., Osaka, Japan) and 2,4-dinitrophenol (Wako Pure Chemicals, Osaka, Japan) were prepared as stock solutions in distilled water and diluted in the Tyrode solution to produce the final concentrations indicated in the text.

Statistical analysis

Data in the text and figures are expressed as mean ± s.d. Statistical analyses were performed by Student's *t* test. A *P* value of less than 0.05 was considered significant.

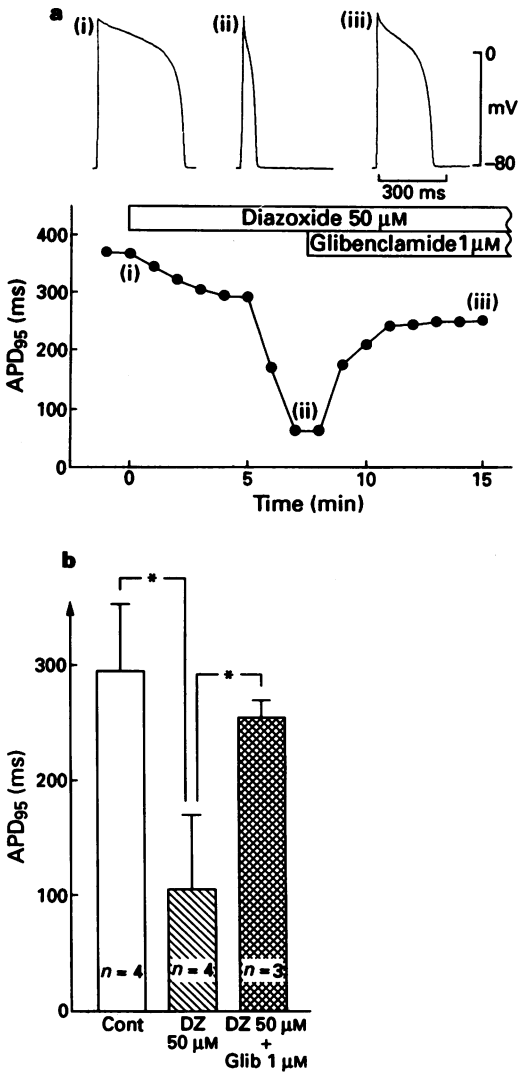

Figure 1 Reversal of diazoxide-induced action potential shortening by glibenclamide in a guinea-pig ventricular cell, stimulated at a rate of 0.2 Hz. (a) Top panels: action potentials during control (i), in the presence of 50 μM diazoxide (ii) and after application of 1 μM glibenclamide in the presence of diazoxide (iii). Bottom panel: the time course of changes in action potential duration measured at 95% repolarization levels (APD_{95}), with the experimental protocol shown above. The points designated by (i), (ii) and (iii) correspond to the action potentials (i), (ii) and (iii) shown in the top. The recording of action poential was started at 2 min after disruption of the patch. (b) Summarized effects of diazoxide and glibenclamide on the APD_{95}. Cont, control conditions; DZ 50 μM, effects of 50 μM diazoxide; DZ 50 μM + Glib 1 μM, effects of 1 μM glibenclamide in the presence of 50 μM diazoxide. Each column with a vertical bar indicates the mean and s.d. respectively. The number within each column denotes the number of cells tested. $*P < 0.05$.

Results

Glibenclamide reverses diazoxide-induced action potential shortening

We first examined the effect of diazoxide on guinea-pig ventricular cell action potentials evoked at a stimulation rate of 0.2 Hz. Figure 1a shows the typical effects of diazoxide on the action potential. After a period of equilibration in normal Tyrode solution, the myocyte was exposed to the Tyrode solution containing 50 μM diazoxide. The drug shortened the action potential duration (APD) slowly for the initial 4–5 min and then very rapidly for the later periods (5–8 min) with the resting membrane potential unchanged. After 7 min exposure to 50 μM diazoxide, the APD at the level of 95% repolarization (APD_{95}) was decreased from the control value of 360 ms (i) to 62 ms (ii). The subsequent application of 1 μM glibenclamide restored the APD_{95} up to 248 ms (iii), even in the continued presence of diazoxide. The results obtained from similar experiments are summarized in Figure 1b. Diazoxide at a concentration of 50 μM significantly ($P < 0.05$) shortened the APD_{95} from 292.8 ± 61.7 ms

($n = 4$) to 105.5 ± 64.9 ms ($n = 4$) within 5–12 min. In one of the four cells tested, the action potential could not be evoked after the application of diazoxide and the APD_{95} was measured immediately before disappearance of the action potential, even though the subsequent application of glibenclamide restored the action potential. Glibenclamide at a concentration of 1 μM significantly ($P < 0.05$) reversed the APD_{95} to 256.0 ± 15.6 ms ($n = 3$) within 3–7 min after application. Glibenclamide (1 μM) *per se* (in the absence of diazoxide) changed the APD_{95} from 318.0 ± 86.5 ms (before application) to 297.0 ± 82.0 ms (after 3–5 min application) but the change was not significant ($n = 4$). Therefore, glibenclamide, a potent and specific blocker of the ATP-sensitive K^+ channel (Fosset *et al.*, 1988), could restore the action potential shortening produced by diazoxide.

However, there remains the possibility that the diazoxide-induced shortening of APD_{95} simply reflects the activation of ATP-sensitive K^+ channels caused by spontaneous decline of the endogenous ATP since the pipette solution did not contain ATP. To exclude this possibility, we examined the effect of glibenclamide on time-dependent changes of APD_{95}. A typical experiment is shown in Figure 2, where the recording was started within 2–3 min after disruption of the membrane patch to make whole-cell recording. The APD_{95} decreased at a rate of about 20 ms min^{-1} with the elapse of time and reached steady state in about 20 min. Similar time-dependent shortenings of APD_{95} were seen in two other experiments, regardless of the presence or absence of glibenclamide. Furthermore, the steady state outward current tested in one cell remained unchanged during the 20 min observation period that was started 2–3 min after disruption of the membrane patch. Application of glibenclamide (1 μM and 4 μM) on the decay phase of the APD_{95} could not prolong the APD_{95}. Furthermore, there occurred no shortening of APD_{95} when glibenclamide was removed from the perfusate. It must be pointed out, however that, when the recording of action potentials was started well over 20 min after disruption of the membrane patch, almost no time-dependent change could be recognized, because the APD shortening reached a steady state already after 20 min (cf. Figure 2) in most cells. These results suggest that the decrease of APD_{95} recorded after application of diazoxide is not produced by the activation of ATP-sensitive K^+ channels secondary to a spontaneous decline of the endogenous ATP. Thus, the time-dependent decline of APD_{95} in control conditions may be, for the most part, ascribable to the so-called 'run-down' of the calcium current (I_{Ca}), which is reported to lead to APD shortening in the same myocytes (Belles *et al.*, 1988). Therefore intial slow

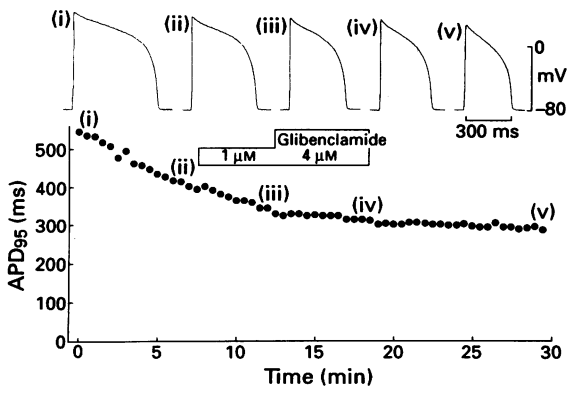

Figure 2 Time-dependent changes of action potential duration (APD) in the presence and absence of glibenclamide. Action potentials were elicited at a rate of 0.2 Hz. Top panels: action potentials taken at the points indicated below in the bottom panel (i), (ii), (iii), (iv) and (v). Bottom panel: time course of changes in the APD measured at 95% repolarization levels (APD_{95}). Application of glibenclamide (1 μM and 4 μM) did not affect the overall time-dependent changes of APD_{95}.

decay of the APD_{95} seen during application of diazoxide (Figure 1a) should be, at least in part, attributed to the run-down of I_{Ca}, while the second rapid decay reflects an authentic diazoxide effect, i.e., opening of the ATP-sensitive K^+ channels (cf. Figures 4a and 5a).

Ionic current underlying the changes in action potential duration induced by diazoxide and glibenclamide

The membrane ionic currents underyling the action potential changes produced by diazoxide and glibenclamide were studied by whole-cell voltage clamp techniques. We first examined the effect of diazoxide on the ionic currents using a rectangular pulse protocol. Figure 3a shows a typical series of currents before application (left), during application (middle) and after washout (right) of diazoxide (1 mM). Figure 3b illustrates the current-voltage relationships that were plotted immediately (left) and 500 ms (right) after step changes in the membrane potential. We observed that diazoxide shifted the holding current levels outward and markedly increased the time-independent outward current. In contrast to these prominent increase in the outward current, inward rectifier K^+ current (I_{K1}) remained unchanged at potentials more negative to − 60 mV. There were also no consistent changes in delayed rectifier K^+ current (I_K) measured from the amplitude of the tail current. However, as diazoxide markedly increased the time-dependent outward current, it was difficult to evaluate the effect of diazoxide on the I_{Ca} *per se*. To circumvent this problem, in Figure 3c, we plotted the amplitude of the initial inward current as the difference between the initial inward peak current and the current at 100 ms from the beginning of the pulse (left) or the current at the end of the pulse (right). Diazoxide (up to 1 mM) did not affect the I_{Ca} in either measurement. Since diazoxide increased only the time-independent outward current, we used the ramp pulse protocol in subsequent experiments to obtain a handy measure of the current-voltage relationship.

In Figure 4a, the current changes are examined by use of the ramp clamp method. The experimental protocol is shown on the top. After obtaining a control record (i), we superfused the cell with the solution containing diazoxide at a concentration of 50 μM; diazoxide began to increase the steady-state outward current at potentials positive to about − 70 mV and reached its peak after 11 min (ii), essentially the same findings as shown in Figure 3b (right). The increased outward current was completely suppressed within 5 min (iii) after subsequent application of 1 μM glibenclamide. Qualitatively similar results were obtained in three other experiments. The data show that glibenclamide inhibits the diazoxide-induced outward current.

We also studied the effect of diazoxide in the presence of glibenclamide. As illustrated in Figure 4b, 1 μM glibenclamide alone produced no change in the outward current after 5 min of exposure (ii) in comparison with the control record (i). The subsequent application of diazoxide (50 μM) failed to increase the outward current (iii), under these conditions. Similar findings were obtained in all three cells tested. All these findings, taken together, suggest that diazoxide activates the ATP-sensitive K^+ channels in guinea-pig ventricular cells which results in the shortening of the action potential duration, as shown in Figure 1.

Inhibitory effects of cibenzoline on diazoxide-induced changes of membrane currents

We then examined whether or not cibenzoline, a class I antiarrhythmic drug, could depress the diazoxide-induced outward current. Figure 5a shows representative traces of the effects of cibenzoline on the diazoxide-induced outward current. Application of diazoxide (50 μM) produced a marked increase in the steady-state outward current at potentials positive to about − 70 mV within 5 min (ii), the same findings as in Figure 4a. The subsequent application of ciben-

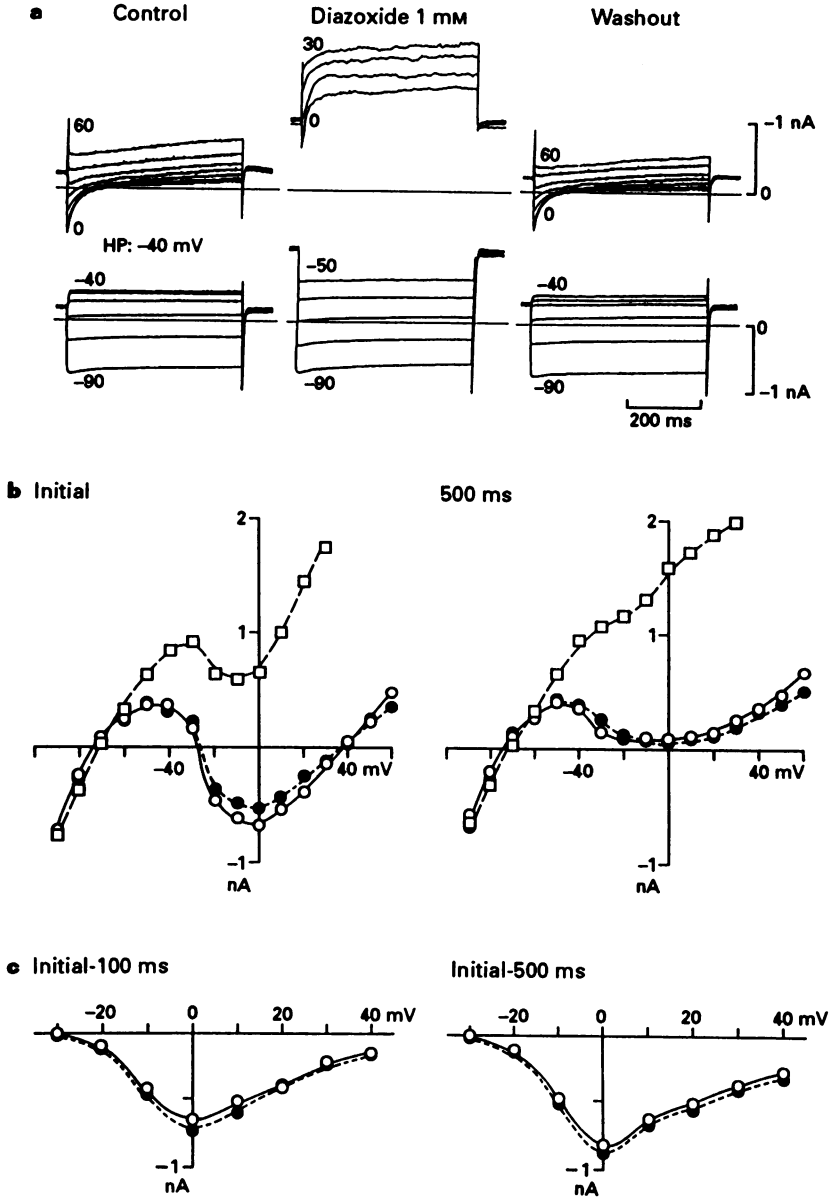

Figure 3 Effect of diazoxide on membrane currents and current-voltage relationships in whole-cell voltage clamp experiments. The holding potential (HP) was − 40 mV and 500 ms rectangular voltage steps were applied at rate of 0.2 Hz. (a) Family of current traces induced by the voltage steps between 0 and + 60 mV (or + 30 mV) (upper traces) and by negative voltage steps between − 40 mV (or − 50 mV) and − 90 mV (lower traces). Left, control; middle, 5 min after application of 1 mM diazoxide; right, washout of the drug. Each horizontal line indicates zero current level. (b) Current-voltage relationships of initial (left) and steady state (right) obtained from the same cell in (a). The current (ordinate scale) is plotted as net current, i.e., as difference from the zero current levels: (○), control; (□), in the presence of diazoxide; (●), after washout of the drug. (c) Effects of diazoxide on the amplitude of calcium current. Calcium current was measured as the difference between the initial peak inward current and the current 100 ms after the beginning of the pulse (left), and as the difference between the initial peak inward current and the terminal current at the end of the 500 ms depolarization (right): (○), control; (●), in the presence of diazoxide.

zoline (5 μM) depressed the diazoxide-induced outward current to a level close to that of control within 5 min (iii), as was observed for glibenclamide in Figure 4a. Identical results were obtained in three other cells.

Since activation of the ATP-sensitive K^+ channels by diazoxide was inhibited in the presence of glibenclamide, we also determined whether the pretreatment with cibenzoline would prevent the effect of diazoxide on the ATP-sensitive K^+ channels. As shown in Figure 5b, application of cibenzoline (5 μM) *per se* slightly decreased the outward current [(i)→(ii)], a finding not seen with glibenclamide (Figure 4b). In three cells tested, cibenzoline (5 μM) decreased the outward current at + 60 mV from 1.10 ± 0.16 nA to 0.93 ± 0.15 nA ($n = 3$, $P < 0.05$) significantly. Subsequent application of diazoxide (50 μM) failed to increase the outward current (iii). Similar findings were obtained with all three cells tested. Thus, cibenzoline seems to inhibit the diazoxide-activated ATP-sensitive K^+ channels in guinea-pig ventricular cells.

Cibenzoline reverses the 2,4-dinitrophenol-induced action potential shortening

It seems likely that cibenzoline directly inhibited the ATP-sensitive K^+ current activated by diazoxide (i.e., not mediated by decreases in intracellular concentrations of ATP, $[ATP]_i$). We therefore examined the effect of cibenzoline on the ATP-sensitive K^+ channel activated by a metabolic in-

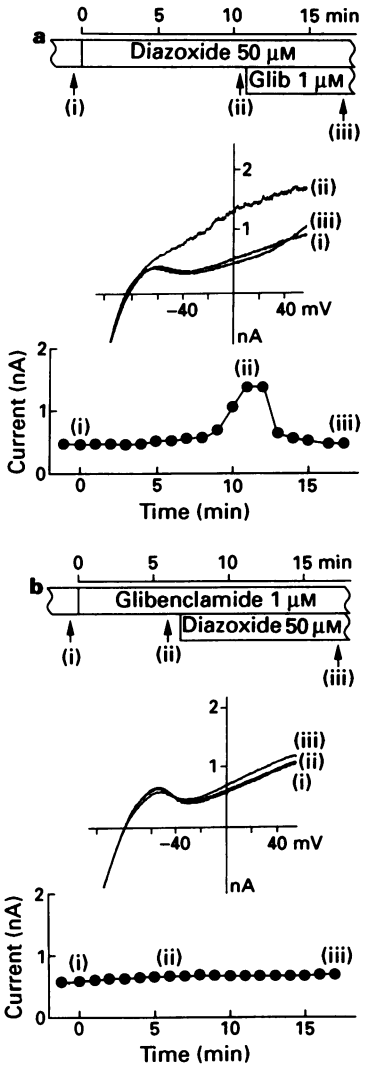

Figure 4 Inhibitory effects of glibenclamide (Glib) on diazoxide-induced increase of outward current (a) and protective effects of glibenclamide on diazoxide-induced increase of the outward current (b). Top: protocol of experiment. Middle: superimposed current-voltage relationships taken at the points designated by arrows (i), (ii) and (iii) in top panel. Bottom: time course of changes in the outward current measured at the membrane potential of 0 mV (or y intercept of the current-voltage curves). Points designated by (i), (ii) and (iii) correspond to those noted in top and middle panels.

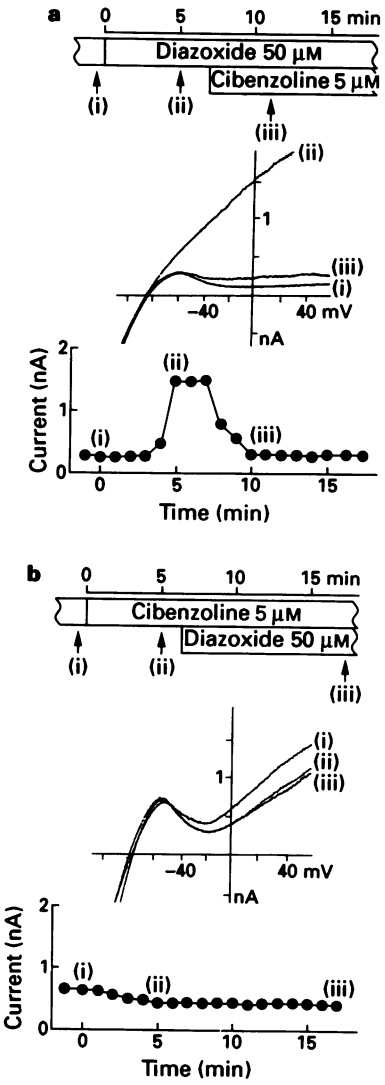

Figure 5 Inhibitory effect of cibenzoline on diazoxide-induced outward current (a) and protective effects of cibenzoline on diazoxide-induced increase of outward current (b). Top, protocol of experiment. Middle: superimposed current-voltage relationships obtained at points indicated by arrows (i), (ii) and (iii) in top panel. Bottom: time course of outward current changes measured at 0 mV. The points designated by (i), (ii) and (iii) correspond to those in above panels.

hibitor (i.e., presumably by a decrease in $[ATP]_i$). We tested the effect of cibenzoline on ventricular cell action potentials that has been shortened by a 5–10 min exposure to 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation. There is convincing evidence that the shortening of guinea-pig ventricular cell action potentials caused by 2,4-dinitrophenol is, for the most part, due to an increase in the outward K^+ current through activated ATP-sensitive K^+ channels (Isenberg *et al.*, 1983; Nakamura *et al.*, 1989).

As we found earlier (Nakamura *et al.*, 1989), 2,4-dinitrophenol (50 μM) increased the quasi-steady state outward current at potentials positive to about − 70 mV, as shown in Figure 4a and 5a. This current was completely inhibited by subsequent application of 1 μM glibenclamide. Cibenzoline at a concentration of 5 μM inhibited the 2,4-dinitrophenol (50 μM)-activated outward current only partially but 10 μM cibenzoline inhibited it completely (not illustrated). Therefore, in the later series of experiments, we used 10 μM cibenzoline. Cibenzoline (10 μM) *per se* shortened the ventricular cell APD_{95} significantly ($P < 0.01$) from 150.7 ± 18.8 ms to 121.9 ± 17.3 ms or to 80.5 ± 4.2% of the control APD_{95} ($n = 4$). This was tested when the APD_{95} reached a steady state (more than about 20 min, cf. Figure 2) after disruption of the membrane patch.

As shown in Figure 6a, 2,4-dinitrophenol (50 μM) produced a remarkable shortening of the APD by activating the ATP-sensitive K^+ channel (Nakamura *et al.*, 1989). In 6 min, the APD_{95} was shortened from 221 ms (i) to 83 ms (ii). Subsequent application of cibenzoline (10 μM) restored the APD_{95} to 121 ms (iii) in 4 min. Figure 6b summarizes the results of similar experiments on six cells. 2,4-Dinitrophenol at a concentration of 50 μM significantly ($P < 0.01$) shortened the APD_{95} from 230.2 ± 45.4 ms ($n = 6$) to 59.8 ± 22.3 ms ($n = 6$) in 4–7 min. In two of six cells tested, after superfusion with 2,4-dinitrophenol, the action potential discharge could not be triggered by a limited intensity of intracellular current injection. In such cases, the APD_{95} was measured immediately before abolition of action potential discharge. Subsequent application of cibenzoline (10 μM) significantly restored the shortened APD_{95} (59.8 ± 22.3 ms) to 101.8 ± 33.7 ms ($n = 4$, $P < 0.05$) in 3–5 min. These findings, taken together, indicate that cibenzoline inhibits the

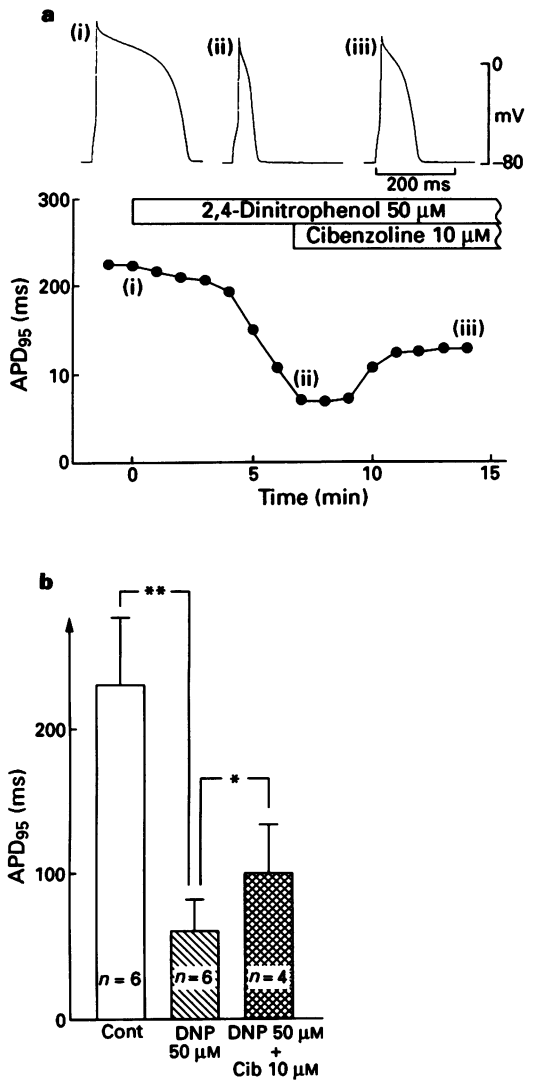

Figure 6 Reversing effects of cibenzoline on 2,4-dinitrophenol-induced shortening of action potential duration (APD). Action potentials were elicited at a rate of 0.2 Hz. (a) Top: action potentials in control (i), in the presence of 50 μM 2,4-dinitrophenol (ii), and subsequent application of 10 μM cibenzoline (iii). Recording of action potentials was started 3 min after disruption of the patch. Bottom: the time course of changes in APD measured at 95% repolarization levels (APD_{95}) with the experimental protocol to the top. The points designated by each letter correspond to the action potentials (i), (ii) and (iii) in the top. (b) Summarized effects of cibenzoline on the 2,4-dinitrophenol-induced shortening of APD_{95}. Cont, control condition; DNP 50 μM, effects of 2,4-dinitrophenol (50 μM); DNP 50 μM + Cib 10 μM, effects of 10 μM cibenzoline in the presence of 50 μM 2,4-dinitrophenol. Columns and vertical bars indicate mean and s.d. The number within each column denotes the number of cells tested. $*P < 0.05$; $**P < 0.01$.

2,4-dinitrophenol-activated ATP-sensitive K^+ channels in guinea-pig ventricular cells, as does glibenclamide (Fosset *et al.*, 1988) and restores action potential duration.

Discussion

Diazoxide activates the ATP-sensitive K^+ channel in guinea-pig ventricular cells

We found that diazoxide significantly shortened the action potential duration without changing the resting membrane potential. This shortening was similar to that induced by 2,4-dinitrophenol, a drug which activates the ATP-sensitive K^+ channels by decreasing $[ATP]_i$. A relatively high concentration of diazoxide (1 mM) increased only the time-independent outward current but did not change the other ionic current, i.e., I_{Ca}, I_K and I_{K1} (Figure 3). In keeping with this observation, diazoxide increased the quasi-steady state outward current at potentials positive to about −70 mV (Figures 4a and 5a), a finding identical to that of 2,4-dinitrophenol (Nakamura *et al.*, 1989). These effects were antagonized by glibenclamide, a specific blocker of the ATP-sensitive K^+ channel. Therefore, it is reasonable to consider that diazoxide directly opens the ATP-sensitive K^+ channels in guinea-pig ventricular cells, contrary to the observation reported for rat ventricular myocytes (Faivre & Findlay, 1989).

It has been reported that, the concentrations of diazoxide causing 50% inhibition of insulin secretion in rat isolated islets ranged between 10 and 40 μM (Henquin *et al.*, 1982). In mouse pancreatic β-cells, half maximum effective concentrations of diazoxide on ATP-sensitive K^+ currents were 20 μM with 0.3 mM $[ATP]_i$ and 102 μM with 1 mM $[ATP]_i$ (Zünkler *et al.*, 1988). In severe hypoglycaemic patients treated successfully with diazoxide, its free plasma level was found to be 6–22 μM (Pruitt *et al.*, 1973). Since 50 μM diazoxide used in the present study was close to these concentrations, the efficacy of diazoxide for the ATP-sensitive K^+ channels of guinea-pig ventricular cells seemed comparable to that seen in the pancreatic β-cells.

Diazoxide leads to activation of ATP-sensitive K^+ channels in pancreatic β-cells by increasing the open-state probability of the single channels and by increasing the number of functional channels, provided that Mg-ATP was present on the cytoplasmic side (Kozlowski *et al.*, 1989). Thus, phosphorylation of some part of the channel may be a prerequisite for diazoxide-induced activation of ATP-sensitive K^+ channels. Further investigations are required to determine the precise mechanisms of diazoxide interaction with ATP-sensitive K^+ channels of cardiac cells.

Cibenzoline inhibits the diazoxide-induced opening of the ATP-sensitive K^+ channel

It has been reported that some class Ia antiarrhythmic drugs, e.g., disopyramide and quinidine produced hypoglycaemia in some patients (Goldberg *et al.*, 1980; Phillips *et al.*, 1986). Cibenzoline belongs to this category and is alleged to cause the same side-effects, in some patients (Hilleman *et al.*, 1987; Gachot *et al.*, 1988). Several hypotheses have been proposed to account for this cibenzoline-induced hypoglycaemia, included are an impaired glycogenolysis, an increased glucose utilization by peripheral tissues and even an endogenous insulin-like effect. However, all of these hypotheses failed to account thoroughly for the drug-induced hypoglycaemia.

Blocking of ATP-sensitive K^+ channel by sulphonylureas, such as glibenclamide or tolbutamide, results in membrane depolarization, enhanced Ca^{2+} influx and insulin secretion in pancreatic β-cells (Sturgess *et al.*, 1985; Dunne *et al.*, 1987). We have found that cibenzoline at a concentration close to that used clinically (1–5 μM, Canal *et al.*, 1983) inhibited the diazoxide-activated ATP-sensitive K^+ channels in guinea-pig ventricular cells. Diazoxide is an ATP-sensitive K^+ channel opener in pancreatic β-cells (Trube *et al.*, 1986; Dunne *et al.*, 1987; Zünkler *et al.*, 1988; Kozlowski *et al.*, 1989) and also in ventricular myocytes (this study). Although there is no direct evidence that cibenzoline inhibits ATP-sensitive K^+ channels in pancreatic β-cells, our present data suggest that such may be the case. We propose the cibenzoline may stimulate insulin secretion by blocking the ATP-sensitive K^+ channels in pancreatic β-cells. In fact, most patients revealed fairly high levels of serum insulin during episodes of cibenzoline-induced hypoglycaemia (Gachot *et al.*, 1988).

Cibenzoline restores the 2,4-dinitrophenol-induced shortening of action potential duration

Cibenzoline decreased the maximum rate of rise of the action potential ($\dot{V}_{max}$) and lengthened the action potential duration in isolated cardiac tissues, characteristics shared by class Ia antiarrhythmic drugs (Millar & Vaughan Williams, 1982; Dangman, 1984). Cibenzaline also decreased the slow inward Ca^{2+} current of frog atrial muscle (Masse *et al.*, 1984) and guinea-pig ventricular myocytes (Holck & Osterrieder, 1986).

In the present study, cibenzoline by itself significantly decreased the steady-state outward current (Figure 5b). At least two possibilities may underlie this phenomenon: (1) blockade of ATP-sensitive K^+ channels that happened to be open under control conditions; (2) inhibition of the time- and voltage-dependent K^+ current (I_K). The former possibility was excluded when the application of glibenclamide had no effect on the steady-state outward current (Figure 4b). Cibenzoline decreased the I_K in guinea-pig ventricular cells (our unpublished observation), and in rabbit sinoatrial node (Kotake *et al.*, 1987). Therefore, the inhibiton of the steady-state outward current by cibenzoline (found in the absence of diazoxide) may be attributed to a reduction of I_K.

Cibenzoline (10 μM) significantly reversed the 2,4-dinitrophenol-induced shortening of APD_{95} (Figure 6) by inhibiting ATP-sensitive K^+ channel (Figure 5). Millar & Vaughan Williams (1982) have also reported that cibenzoline prevented shortening of the APD induced by hypoxia in rabbit isolated atria. The latter finding can be readily explained by a blockade of ATP-sensitive K^+ channel by this agent. Likewise, glibenclamide prevented the hypoxia-induced shortening of APD in guinea-pig papillary muscles (Wilde *et al.*, 1990; Deutsch *et al.*, 1991; Nakaya *et al.*, 1991).

ATP-sensitive K^+ channels in cardiac cells have been extensively studied and effects of openers of this channel, such as nicorandil, pinacidil and cromakalim have been documented (Escande *et al.*, 1988; Arena & Kass, 1989; Hiraoka & Fan, 1989). In contrast, surprisingly few blockers of this channel have so far been reported, except for the sulphonylureas (Belles *et al.*, 1987; Fosset *et al.*, 1988). In this respect, Undrovinas *et al.* (1990) found that quinidine, a classic antiarrhythmic drug, blocked the ATP-sensitive K^+ channels in rat ventricular cells. In the present study, we found cibenzoline to be a novel and potent blocker of ATP-sensitive K^+ channels, although it does also have blocking effects on I_{Na} and I_{Ca} (Millar & Vaughan Williams, 1982; Dangman, 1984; Holck & Osterrieder, 1986). For the latter reason, the recovery of 2,4-dinitrophenol-induced shortening of APD_{95} by this agent was relatively small (Figure 6), as compared to the recovery caused by glibenclamide (Figure 1).

Faivre & Findlay (1989) found that diazoxide 'inhibited' the ATP-sensitive K^+ channels in rat ventricular myocytes, whereas our present study reveals that diazoxide 'opened' this channel in guinea-pig ventricular cells. The reason for this discrepancy cannot be explained at this time.

Implication for the use of cibenzoline in the management of ventricular arrhythmias

The clinical efficacy of cibenzoline as a potent antiarrhythmic drug may depend on its inhibitory effects on I_{Na} and more or less on I_{Ca} in the ventricular muscle. In addition, we found that cibenzoline also inhibited the ATP-sensitive K^+ channels of ventricular cells. The concentrations of cibenzoline (5 and 10 μM) which inhibited the ATP-sensitive K^+ channels of the ventricular cells were fairly close to the concentrations used clinically (1–5 μM) (Canal *et al.*, 1983).

The role of the ATP-sensitive K^+ channel in arrhythmogenesis is not clearly understood. Opening of the ATP-sensitive K^+ channels contributes to the extracellular accumulation of potassium ions during early ischaemia (Wilde *et al.*, 1990) and this accumulation is responsible for the slowing of conduction and development of non-uniform refractoriness, leading to re-entrant arrhythmias. Glibenclamide decreases K^+ loss from the ischaemic myocardium and prevents arrhythmias emerging during ischaemia in rat heart (Kantor *et al.*, 1990). On the other hand, Noma (1983) suggested that activation of the ATP-sensitive K^+ channel and the consequent shortening of action potential duration may be important for preservation of cellular ATP. In fact, glibenclamide enhanced the myocardial damage caused by reperfusion (Cole *et al.*, 1991). From these points of view, advantages and disadvantages with regard to the use of antiarrhythmic drugs with or without effects on the ATP-sensitive K^+ channels should be carefully evaluated in basic as well as clinical settings.

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Incomplete inhibition of the pressor effects of endothelin-1 and related peptides in the anaesthetized rat with BQ-123 provides evidence for more than one vasoconstrictor receptor

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1 The effects of the ET_A receptor antagonist, BQ-123 on blood pressure changes induced by various members of the endothelin (ET)/sarafotoxin (SX) peptide superfamily were investigated in the anaesthetized rat.

2 ET-1 (1 nmol kg^{-1}, i.v. bolus) induced a sustained increase in mean arterial pressure (MAP, maximum increase 44 ± 3 mmHg). Intravenous injection of BQ-123 at 0.2, 1.0 or 5.0 mg kg^{-1} 5 min before ET-1 inhibited the pressor response by 18, 50 and 61%, respectively. The ET-1 pressor response was inhibited by 75% when the peptide was given 60 min after the start of a 120 min i.v. infusion of BQ-123 (0.2 mg kg^{-1} min^{-1}).

3 In addition to ET-1, BQ-123 (1 mg kg^{-1}, i.v. bolus) attenuated the pressor responses to big ET-1 (1 nmol kg^{-1}, i.v., bolus, maximum increase in MAP: 68 ± 7 mmHg), ET-3 (3 nmol kg^{-1}, i.v., bolus, maximum response: 30 ± 3 mmHg), SX6b (1 nmol kg^{-1}, i.v., bolus, maximum response: 41 ± 5 mmHg) and SX6c (1 nmol kg^{-1}, i.v., bolus, maximum response: 24 ± 4 mmHg) by 65, 60, 88 and 50%, respectively.

4 With the exception of big ET-1, all the peptides used in this study induced an initial transient depressor response (−32 ± 3 mmHg, $n = 18$). Although BQ-123 (1 mg kg^{-1}, i.v., bolus) did not affect the absolute magnitude of the fall in MAP, the ET_A receptor antagonist significantly prolonged the depressor responses induced by ET-3 and SX6b.

5 Thus, BQ-123 attenuates the pressor, but not the depressor effects of ET-1, big ET-1, ET-3, SX6b and SX6c. Complete inhibition of the pressor responses could not be achieved, suggesting that a component of the pressor response is not mediated via the ET_A receptor.

Keywords: Endothelin; sarafotoxin; ET_A receptor; ET_B receptor; BQ-123

Introduction

The endothelins (ETs) and sarafotoxins (SXs) constitute a family of structurally homologous peptides composed of 21 amino acids, of which there are now considered to be at least 8 members. ET-1, ET-2 and ET-3 are found in mammalian tissues (see Simonson & Dunn, 1990), and the sarafotoxins SX6a, SX6b, SX6c, and SX6d are constituents of the venom of the Israelia burrowing asp, *Atractaspis engaddensis* (Kloog *et al.*, 1988; Bdolah *et al.*, 1989). Murine vasoactive intestinal contractor (VIC) is an additional member of this peptide family (Saida *et al.*, 1989). A number of studies have demonstrated a widespread tissue distribution of specific ET receptors (see Randall, 1991).

At present the cDNAs encoding two ET receptors, classified as ET_A and ET_B have been cloned and expressed. Each receptor contains 7 trans-membrane domains and shows remarkable similarity to the rhodopsin receptor and other G protein coupled receptors (see Webb, 1991). The ET_A receptor is highly selective for ET-1 and is characterized by the rank order of binding affinities: ET-1 > SX6b > ET-3 (Arai *et al.*, 1990), while the ET_B receptor is non-isopeptide selective (ET-1 = ET-3) (Sakurai *et al.*, 1990). The existence of a third ET receptor has been proposed, present on bovine endothelial cells (Emori *et al.*, 1990) and probably mediating the release of nitric oxide (NO) (Emori *et al.*, 1991). It may be similar to the receptor present in porcine isolated pulmonary and coronary vessels (Fukuroda *et al.*, 1992).

Elevated levels of circulating ET-1 have been reported in a variety of cardiovascular diseases, including hypertension, renal failure, myocardial ischaemia and subarachnoid haemorrhage (see Thiemermann, 1991). However, it is not yet clear whether ET-1 is involved in the pathogenesis of these disorders. Clarification will only be obtained with the use of potent and selective ET receptor antagonists for assessing the contribution of the ETs to physiology and pathophysiology. BQ-123 (cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp]) antagonizes ET-1-induced constriction of porcine isolated coronary artery strips and inhibits ET-1 binding to porcine aortic smooth muscle cells (Ihara *et al.*, 1992) and is therefore a putative ET_A receptor antagonist.

Here, we have investigated the effects of BQ-123 on blood pressure changes induced by ET-1, big ET-1, ET-3, SX6b and SX6c in the anaesthetized rat.

Methods

Surgical procedure

Male Wistar rats (250–400 g) were anaesthetized with Trapanal (120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration and body temperature was maintained at 37°C by means of a rectal probe connected to a homeothermic blanket (Harvard, Edenbridge, Kent). The right carotid artery was cannulated and connected to a pressure transducer (Transamerica type 4-422-0001) for the measurement of systemic blood pressure, from which mean arterial blood pressure (MAP) and heart rate were derived and recorded on a Grass 7D polygraph (Grass Instruments, Quincy, Mass., U.S.A.). The left jugular and right femoral veins were cannulated for the administration of drugs.

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Experimental design

After surgery, all animals were allowed to stabilize for 20 min before receiving a 5 min infusion of hexamethonium (10 mg kg^{-1}, i.v.). Twenty min later, animals were given either BQ-123 (0.2, 1.0 or 5.0 mg kg^{-1}, i.v., bolus; approximately 0.28, 1.4 and 7 μmol kg^{-1}) or vehicle (0.9% w/v saline containing 10 mM sodium bicarbonate, 1 ml kg^{-1}). Five minutes later, animals ($n = 4-6$ for each group) were given either ET-1 (1 nmol kg^{-1}), ET-3 (3 nmol kg^{-1}), big ET-1 (1 nmol kg^{-1}), SX6b (1 nmol kg^{-1}) or SX6c (1 nmol kg^{-1}), all as i.v. bolus injections. In a separate series of experiments BQ-123 was administered as an infusion of 0.2 mg kg^{-1} min^{-1} (preceded by a loading dose of 1 mg kg^{-1}) for 60 min prior to and continued for a further 60 min following an i.v. bolus injection of ET-1 (1 nmol kg^{-1}; $n = 6$).

Calculations of changes in mean arterial pressure (MAP)

Changes in MAP were calculated as the peak increase or decrease of MAP (mmHg) from control levels. In some experiments, the changes in MAP were calculated as the area under the curve, expressed in mm^2, to permit comparisons between the durations of depressor responses induced by the various peptides.

Materials

Sodium-thiopentone (Trapanal) was obtained from Byk Gulden (Konstanz, Germany). Hexamethonium bromide, phenylephrine and angiotensin II were purchased from Sigma Chemical Co. (Poole, Dorset) and were dissolved in 0.9% w/v saline. ET-1, ET-3, big ET-1 and SX6b were purchased from the Peptide Institute (Osaka, Japan), and SX6c from Peninsula Laboratories Inc. (Belmont, U.S.A.). The peptides were reconstituted in 0.1% acetic acid and then diluted in 0.9% w/v saline containing 1% w/v bovine serum albumin and 0.06% sodium bicarbonate. BQ-123 was synthesized by the Parke-Davis Pharmaceutical Research Division of Warner-Lambert Co. (Ann Arbor, U.S.A.) and was dissolved in 0.9% w/v saline containing 10 mM sodium bicarbonate. Aliquots of the peptides and BQ-123 were stored frozen (−20°C) until use.

Statistical comparisons

All values in the figures and text are expressed as mean ± s.e.mean of n observations. Statistical evaluation of the data was by Student's t test for unpaired determinations or by ANOVA. A P value of <0.05 was considered significant.

Results

Mean resting values for mean arterial blood pressure (MAP) were 109 ± 2 mmHg ($n = 74$) and for heart rate were 377 ± 5 beats min^{-1} ($n = 74$). Treatment with hexamethonium (2 mg kg^{-1} min^{-1} for 5 min) caused MAP to fall to 83 ± 1 mmHg and heart rate to 330 ± 4 beats min^{-1}. These values were unaffected by vehicle or BQ-123 at any of the doses used in this study.

Duration of action of BQ-123

BQ-123 (1 mg kg^{-1}) was given as an i.v. bolus injection 1, 5, 15 or 30 min before ET-1 (1 nmol kg^{-1}, i.v., bolus; $n = 4-6$) or as an infusion 60 min before and continuing for 60 min after ET-1 ($n = 6$). BQ-123 was ineffective when given 30 or 15 min prior to ET-1. Furthermore, BQ-123 was no more effective when given 1 min rather than 5 min before ET-1 (data not shown). A pretreatment time of 5 min (for BQ-123 bolus injections) was, therefore, used in this study.

BQ-123 inhibits ET/SX induced pressor responses

ET-1 (1 nmol kg^{-1}) produced a sustained increase in arterial blood pressure which reached a maximum (44 ± 3 mmHg) within 5 min and returned to control levels within 60 min (Figure 1a). The peak effect was decreased significantly to 22 ± 3 mmHg ($n = 6$) and 17 ± 3 mmHg ($n = 6$) by 1 and 5 mg kg^{-1} BQ-123 (i.v., bolus), respectively (Figure 2b). BQ-123 at 5 mg kg^{-1} did not cause a significantly greater reduction in either the maximum response or the duration of the ET-1 induced pressor effect than 1 mg kg^{-1} (Figure 2a and 2b). In comparison to BQ-123 at 1 and 5 mg kg^{-1}, 0.2 mg kg^{-1} was clearly a threshold dose for inhibition of the ET-1 pressor response (Figure 2a and 2b). BQ-123 at 10 mg kg^{-1} (i.v., bolus) was not more effective than at lower doses ($n = 2$, data not shown). BQ-123 (1 mg kg^{-1}, i.v., bolus) was, therefore, selected for use in subsequent experiments with the other ET/SX agonists.

SX6c or SX6b (1 nmol kg^{-1}) or big ET-1 (1 nmol kg^{-1}) caused peak increases in MAP of 24 ± 4 mmHg ($n = 4$), 41 ± 5 mmHg ($n = 4$) and 67 ± 7 mmHg ($n = 6$), respectively. ET-3 at 3 nmol kg^{-1} increased MAP by 30 ± 3 mmHg ($n = 4$). SX6c at 3 nmol kg^{-1} caused rapid death (2 out of 2 experiments). The maximum elevations of MAP were seen at 5–10 min after peptide injection except with SX6c where the peak occurred at 20 min (Figure 3a–3d). The peak pressor effects of SX6b, big ET-1, ET-3, SX6c and ET-1 were decreased in the presence of BQ-123 to 12, 35, 40, 50 and 50% of control, respectively. The ET-1-induced increase in MAP was reduced by 75% when the peptide was given at 60 min after the start of a 120 min infusion of BQ-123 (0.2 mg kg^{-1} min^{-1}) (Figure 1c).

BQ-123 did not inhibit the pressor actions of phenylephrine or angiotensin II. Phenylephrine (0.1 μmol kg^{-1}, i.v., bolus; $n = 4$) increased MAP by 62 ± 6 mmHg in the absence and 66 ± 5 mmHg in the presence of BQ-123. Angiotensin II (1 nmol kg^{-1}, i.v., bolus; $n = 4$) increased MAP by 76 ± 4

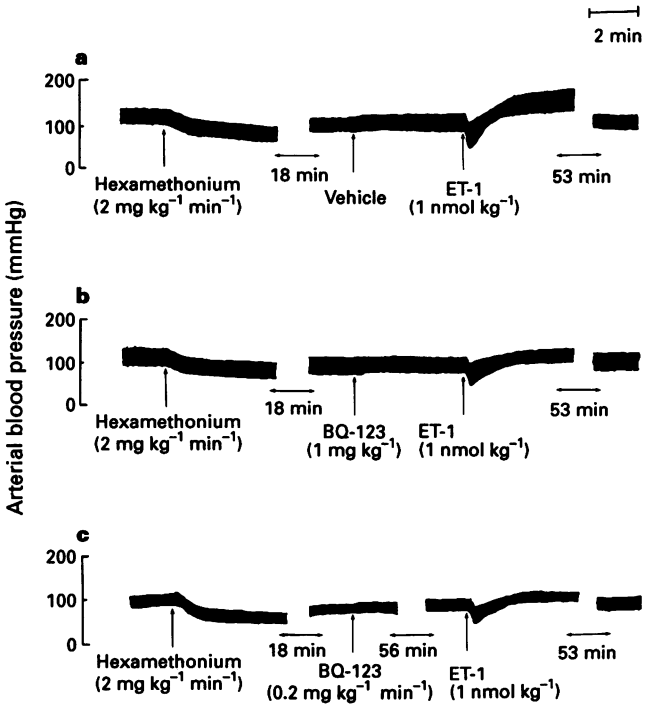

Figure 1 The figure shows three representative, original traces comparing endothelin-1 (ET-1, 1 nmol kg^{-1}, i.v., bolus)-induced pressor responses in (a) vehicle-treated rats; (b) rats given BQ-123 (1 mg kg^{-1}, i.v., bolus) 5 min prior to ET-1 injection and (c) rats given ET-1 at 60 min after the start of an infusion of BQ-123 (loading dose of 1.0 mg kg^{-1} then 0.2 mg kg^{-1} min^{-1} for 120 min, i.v.).

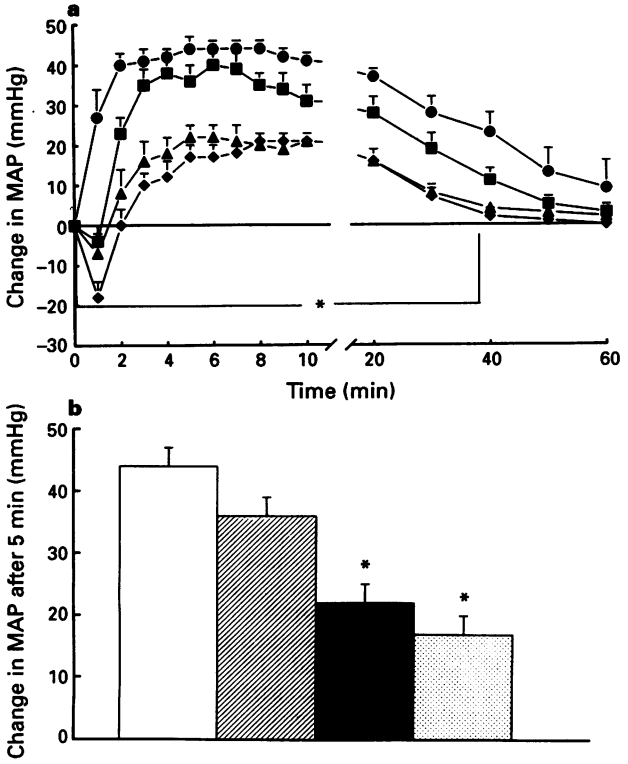

Figure 2 BQ-123 inhibits endothelin-1 (ET-1) pressor responses in a dose-dependent manner. (a) Time course of the effect of ET-1 (1 nmol kg^{-1}, i.v., bolus given at time = 0 min) on mean arterial pressure (MAP). Five minutes prior to ET-1 injection, different animals received either vehicle (●, $n = 6$), BQ-123 at 0.2 mg kg^{-1} (■, $n = 5$), BQ-123 at 1.0 mg kg^{-1} (▲, $n = 6$) or BQ-123 at 5.0 mg kg^{-1} (◆, $n = 6$), all as i.v. bolus injections. (b) BQ-123 inhibits the ET-1-induced rise in MAP (at 5 min) in a dose-dependent fashion. For example, 0.2 mg kg^{-1} (hatched column; $n = 6$) caused 18% inhibition and 1.0 mg kg^{-1} (solid column; $n = 6$) caused 50% inhibition. While, 5.0 mg kg^{-1} (stippled column; $n = 6$) produced 61% inhibition. Data are expressed as mean ± s.e. mean (vertical bars) of n observations. *$P < 0.05$ when compared to vehicle control.

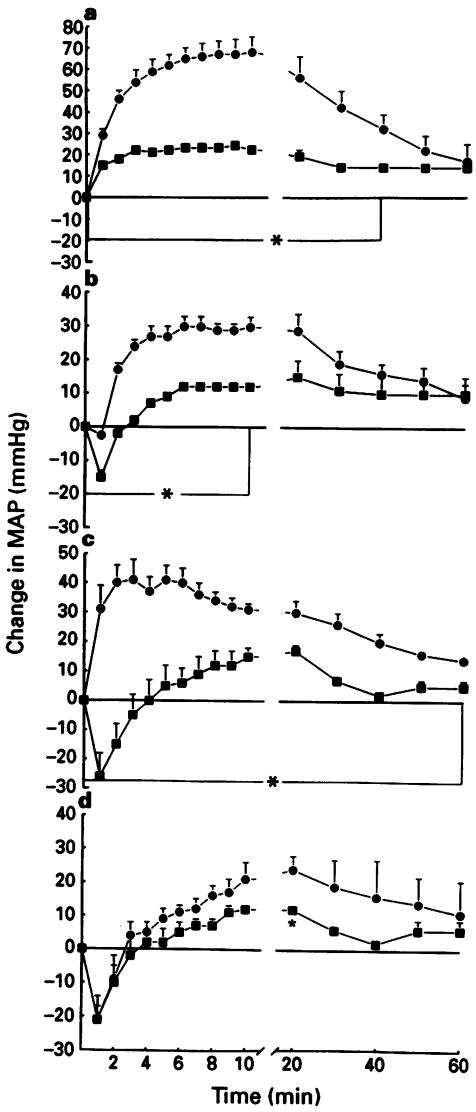

Figure 3 BQ-123 inhibits the pressor responses elicited by big endothelin 1 (ET-1), ET-3, sarafotoxin 6b (SX6b) or SX6c in the anaesthetized rat. Different groups of animals received (a) big ET-1 (1 nmol kg^{-1}; $n = 4$); (b) ET-3 (3 nmol kg^{-1}; $n = 4$); (c) SX6b (1 nmol kg^{-1}; $n = 4$) or (d) SX6c (1 nmol kg^{-1}; $n = 4$), all as i.v. bolus injections at time 0 min in the absence (●, vehicle-treatment) or presence (■) of BQ-123 (1 mg kg^{-1}, i.v. bolus). Data are mean ± s.e. mean (vertical bars) of n observations.
*$P < 0.05$ when compared to vehicle control.

mmHg in the absence and 79 ± 4 mmHg in the presence of BQ-123.

BQ-123 prolongs the duration of the ET/SX induced depressor responses

Injection of all peptides except big ET-1 produced an initial depressor response. ET-1 (1 nmol kg^{-1}), ET-3 (3 nmol kg^{-1}), SX6b (1 nmol kg^{-1}) and SX6c (1 nmol kg^{-1}) caused similar falls in MAP (ranging from −25 ± 5 mmHg to −36 ± 3 mmHg; data not shown). However, the SX6c-induced depressor response was longer than the other peptides (Figure 3) as confirmed by measurement of area under the curve (Table 1).

Although, BQ-123 (1 mg kg^{-1}) had no effect on the fall in MAP induced by any of the peptides (range: −30 ± 3 mmHg to −40 ± 5 mmHg), it prolonged the depressor actions of ET-1, ET-3 and SX6b. The duration of the SX6c-induced fall in MAP was unaffected by BQ-123 (Table 1).

Discussion

Our results show that the ET_A receptor antagonist, BQ-123, attenuates the pressor but not the depressor responses to ET-1, big ET-1, ET-3, SX6b and SX6c in the anaesthetized rat. BQ-123 was developed from another ET_A receptor antagonist (BE-18257B) which was isolated from the fermentation products of *Streptomyces misakiensis* and found to antagonize ET-1 pressor responses in conscious rats (Ihara *et al.*, 1991). Moreover, BQ-123 is similar to the ET_A receptor antagonist, BQ-153, which also antagonizes ET-1 pressor effects *in vivo* (Ihara *et al.*, 1992).

When ET-1 is injected into an anaesthetized rat there is an initial transient depressor response followed by a sustained pressor response. These changes in arterial blood pressure are mediated via at least two different ET receptors. The receptor responsible for the initial fall in blood pressure appears to be non-isopeptide selective (Inoue *et al.*, 1989). This ET_B receptor may be located on the endothelium and mediate vasodilatation via the release of prostacyclin (Lidbury *et al.*, 1990) or NO (Warner *et al.*, 1989; Whittle *et al.*, 1989). The 'vasoconstrictor' receptor (ET_A) has greater selectivity for ET-1 than ET-3 and is located on the vascular smooth muscle (see Sakurai *et al.*, 1992). Therefore, BQ-123 would be expected to inhibit the ET_A-mediated pressor, but not the

Table 1 Depressor effects of endothelin-1 (ET-1), endothelin-3 (ET-3), sarafotoxin S6b (SX6b) and sarafotoxin S6c (SX6c) in the presence or absence of BQ-123

	Area under the curve (mm^2)	
Peptide	*Control*	*+BQ-123*
ET-1	1.9 ± 0.3	3.4 ± 0.9
ET-3	2.0 ± 0.2	3.4 ± 0.4*
SX6b	1.1 ± 0.2	8.3 ± 2*
SX6c	4.3 ± 0.7	5.0 ± 2

This table shows the depressor responses induced by ET-1 (1 nmol kg^{-1}; $n = 6$), ET-3 (3 nmol kg^{-1}; $n = 4$), SX6b (1 nmol kg^{-1}; $n = 4$) or SX6c (1 nmol kg^{-1}; $n = 4$), all as i.v. bolus injections, in vehicle or BQ-123 (1 mg kg^{-1}, i.v., bolus)-treated rats. Data are mean ± s.e. mean of n observations. *$P < 0.05$ when compared to control.

ET_B-induced depressor responses.

On injection into the anaesthetized rat, the rank order of pressor potency of the peptides we used was: big ET-1 > ET-1 = SX6b > ET-3 ≥ SX6c. These data show that big ET-1 was significantly more potent than ET-1 at the same dose. The reason for this is unclear. The ET-1 pressor response was not completely abolished by any of the doses of BQ-123 used in this study. Even an infusion of BQ-123 (0.2 mg kg^{-1} min^{-1} for 60 min) only reduced the pressor response to 25% of control. Thus, there is a component of the ET-1-induced vasoconstrictor response which cannot be inhibited by BQ-123 and, therefore, may not be mediated via the ET_A receptor. The hypothesis that the ET-1-induced rise in blood pressure is not entirely due to activation of the ET_A receptor is supported by the finding that BQ-123 at concentrations up to 10 mg kg^{-1} does not completely inhibit the pressor action of ET-1 in conscious rats (Ihara *et al.*, 1992). BQ-123 failed to abolish the pressor responses to the other members of the ET/SX superfamily of vasoconstrictor peptides. Although SX6b was equipotent to ET-1 in this study, BQ-123 inhibited the SX6b pressor activity by 88% compared to a reduction of only 50% for ET-1. It is possible that the pressor activity produced by SX6b is mediated almost entirely via the ET_A receptor, while ET-1 may also be acting on another receptor or activating a mechanism which is not blocked by BQ-123. Alternatively, SX6b may have a greater intrinsic activity than ET-1 on the ET_B receptor. This would result in a greater vasodilator response which, when unmasked after inhibition of the ET_A receptor, would contribute to a more pronounced attenuation of the pressor response in the presence of BQ-123.

With the exception of big ET-1 all of the peptides used induced similar vasodepressor effects. Significantly, SX6c produced a longer lasting depressor response than the other peptides and this was unaffected by BQ-123. BQ-123 did not affect the depth of the fall in MAP, but significantly prolonged the depressor action probably by suppressing agonist-induced vasoconstrictor activity. This was best seen in the SX6b-treated animals in which BQ-123 was most effective at inhibiting the pressor response, allowing the depressor response to persist unchallenged by vasoconstriction. This prolonged fall in blood pressure in the presence of BQ-123 may indicate that the ET/SX peptides cause sustained activation of endothelial cells producing NO or prostacyclin. This would give rise to a tonic release of these vasodilators, thereby ameliorating the pressor effects of the peptides. The development of specific ET_B receptor antagonists will help further to characterize this depressor response.

It has been reported that SX6c (in doses up to 0.3 nmol kg^{-1}) is equipotent to SX6b as a pressor agent in the pithed rat and that this rise in blood pressure is an ET_B-mediated effect (Williams *et al.*, 1991). In contrast, in the present study in the ganglion-blocked rat, SX6c caused a smaller and more slowly developing increase in blood pressure when compared to SX6b or any of the other peptides. It has been suggested that there are two distinct phases of ET pressor response: an early phase immediately following the depressor response (3–10 min), followed by a later pressor effect which develops 10–20 min after peptide injection and lasts for more than an hour (Inoue *et al.*, 1989). These phases may be mediated via different receptors or mechanisms and SX6c may elicit only the latter of the phases. In our study the SX6c induced maximum increase in MAP was inhibited (by 50%) by BQ-123, indicating that the SX6c-induced pressor response is not wholly mediated via the ET_B receptor as suggested previously (Williams *et al.*, 1991). However, in non-ganglion blocked rats Clozel and co-authors (1992) have demonstrated that SX6c (0.8 nmol kg^{-1}) produces an increase in MAP which is not affected by BQ-123 (3 mg kg^{-1}). Although the different effects of BQ-123 reported in our study and by Clozel *et al.* may be due to the different preparations used, this seems unlikely for Cristol *et al.* (1992) have recently demonstrated that the dose-dependent increases in MAP obtained with 0.1, 0.25 and 0.5 nmol kg^{-1} SX6c are significantly attenuated by BQ-123 (1 mg kg^{-1}) in the anaesthetized rat. In our study, SX6c at 3 nmol kg^{-1} was lethal. The SXs probably cause death by inducing coronary constriction accompanied by ST-segment elevation, a slow positive inotropic effect and atrio-ventricular block (Wollberg *et al.*, 1987).

This work extends a preliminary report on the ET_A receptor antagonist, BQ-123 (Pollock *et al.*, 1992). We have shown that complete inhibition of the ET- or SX-induced pressor responses could not be achieved using BQ-123. It could be that ET/SX peptides induce the release of other agents which will increase blood pressure such as renin, aldosterone or catecholamines (Goetz *et al.*, 1988; Miller *et al.*, 1989). Contrary to this suggestion, the pressor effects of SX6c do not appear to be mediated via the central nervous system, by catecholamines from the adrenal medulla, by prostanoids or by ET-1 (Clozel *et al.*, 1992). Alternatively, the ET_B receptor may mediate a pressor action, as already indicated (Williams *et al.*, 1991). There is increasing evidence pointing to the existence of multiple ET receptor subtypes (Emori *et al.*, 1990; Samson *et al.*, 1990; Fukuroda *et al.*, 1992; Harrison *et al.*, 1992). Supporting the contention that ET/SX-induced smooth muscle contractions are not all mediated via the ET_A receptor is the finding that BQ-123 (10^{-5} M) did not affect the EC_{50} threshold concentration or maximum response to ET-1 in rings of guinea-pig bronchi, rabbit pulmonary artery or rat stomach strip. In contrast, this dose of BQ-123 shifted the EC_{50} for ET-1 in the rat thoracic aorta from 3×10^{-10} M to 1×10^{-7} M and the threshold concentration for contraction from 10^{-10} M to 3×10^{-8} M (Warner *et al.*, 1992). In a similar study Hay (1992) demonstrated that BQ-123 antagonizes ET-1-induced contractions of guinea-pig aorta but is without effect on ET-1-induced contractions of guinea-pig bronchus. Furthermore, BQ-123 does not inhibit ET- or SX-induced renal vasoconstriction *in vivo* (Cristol *et al.*, 1992).

In summary, the present study supports the hypothesis that the pressor response to members of the ET/SX family of vasoconstrictor peptides is not mediated entirely via activation of the ET_A receptor.

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Investigation into the role of phosphodiesterase IV in bronchorelaxation, including studies with human bronchus

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1 We have investigated the role of cyclic nucleotide phosphodiesterase IV (PDE IV) in the relaxation of human bronchus and guinea-pig trachea *in vitro* and in guinea-pigs *in vivo*.

2 Functional studies showed that the selective PDE IV inhibitors, rolipram and denbufylline, relaxed human and guinea-pig preparations *in vitro*.

3 Two clinically used xanthine non-selective PDE inhibitors, theophylline and pentoxifylline, were also effective in these preparations, but were much less potent than the selective agents used.

4 The rank order of potency for the four PDE inhibitors in both species was similar.

5 Biochemical studies indicated that PDE IV was the major PDE isoform present in the human bronchial tissue. PDEs I, II and V were also identified.

6 Theophylline and pentoxifylline were, as expected, non-selective inhibitors of the human enzymes, but there was a good correlation between PDE IV inhibitory and bronchorelaxation potencies, suggesting that PDE IV inhibition is important for the clinical bronchodilator activities of the two xanthine compounds.

7 We have confirmed the ability of selective PDE IV inhibitors to cause bronchodilatation in guinea-pigs *in vivo*.

8 We conclude that our study has provided further evidence that selective PDE IV inhibitors could act as bronchodilators in the clinic.

Keywords: Phosphodiesterase isoenzymes; PDE IV; human bronchus; theophylline; pentoxifylline; rolipram; denbufylline; bronchorelaxation

Introduction

There is currently interest in the possible use of selective inhibitors of cyclic nucleotide phosphodiesterases (PDEs) in the treatment of asthma (see e.g. Torphy & Undem, 1991; Giembycz & Dent, 1992). There are known to be at least five distinct families of PDE isoenzymes, PDEs I to V, which differ in their substrate specificity and affinity as well as in their regulatory properties and tissue distribution (see Beavo & Houslay, 1990). Of these, PDE IV inhibitors are receiving special interest as anti-asthmatic agents due to evidence that these can act as both anti-inflammatory agents and bronchodilators in animal species (see Nicholson *et al.*, 1991; Giembycz & Dent, 1992). There are, however, few clinical data generally available concerning selective PDE IV inhibitors in asthma.

Theophylline, currently widely used clinically in asthma, is a weak non-selective PDE inhibitor, but it is believed that PDE inhibitory activity (including PDE IV inhibition) may contribute to both its bronchodilator and anti-inflammatory activities in man (see e.g. Torphy & Undem, 1991; Gristwood *et al.*, 1991). Theophylline, therefore, may be an important link compound between *in vitro* human and animal pharmacological studies and the potential clinical efficacy of PDE IV inhibitors.

The purpose of the present investigation was to compare, using human *in vitro* studies, the bronchorelaxant and PDE inhibitory activities of two selective PDE IV inhibitors, rolipram (Reeves *et al.*, 1987) and denbufylline (Nicholson *et al.*, 1989), and two clinically used xanthine non-selective PDE inhibitors, theophylline (Persson, 1986) and pentoxifylline (Ward & Clissold, 1987). Isoprenaline was included as a standard bronchodilator agent.

A further important aim of the study was to compare the human *in vitro* bronchodilator activities of these agents with their *in vitro* and *in vivo* bronchodilator activities in the guinea-pig, a commonly used animal for models of human asthma.

Methods

Relaxant activity in human isolated bronchus

Lung tissue was obtained from 19 patients (18 males and 1 female, 35 to 78 years old, mean age 61.7 ± 2.8 years) who were undergoing surgery (Hospital de La Fe, Valencia, Spain) for lung carcinoma. None of the patients had a history of asthma. After the resection of one or more lung lobes, a piece of macroscopically normal tissue was cut free and submerged in Krebs solution (for composition see drugs and solutions) at 4°C for transport to the laboratory.

Once in the laboratory (Valencia) parts of the bronchus were dissected free from parenchymal lung tissue and preparations cut (3–4 mm length × 2–3 mm internal diameter) as previously described (Cortijo *et al.*, 1992). Preparations were stored in Krebs solution, equilibrated with 5% CO_2 in O_2 at 4°C until used. Experiments were routinely completed within 24 h of initiating storage.

For experiments, the bronchial rings were suspended on tissue hooks in 10 ml organ baths containing Krebs solution, gassed with 5% CO_2 in O_2 at 37°C (pH 7.4). Each preparation was connected to a force displacement transducer (Grass FTO3) and isometric tension changes recorded on a Grass polygraph (model 7P).

The preparations were equilibrated for 60–90 min with changes in bath Krebs solution every 20 min before adding drugs.

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A load of 2 g was maintained throughout the equilibrium period and a stable resting level of tone was present immediately prior to drug administration. The effects of test drugs were investigated by adding cumulative concentrations of these to the baths. Responses were allowed time to stabilize prior to increasing the bath drug concentration (usually within 15 min of addition).

Only one concentration-effect curve was constructed with each preparation. Experiments were terminated by the addition of theophylline, 1×10^{-3} M, the effect of which was taken to represent the maximum relaxation possible in the tissue.

Changes in force were measured from isometric recordings and expressed in g. The maximum response induced with a relaxant agent (E_{max}) and the molar concentration required to produce 25% (EC_{25}) and 50% (EC_{50}) of maximal relaxation was calculated by linear regression from the individual concentration-effect curves.

Relaxant activity in guinea-pig isolated trachea

Guinea-pigs (male Dunkin-Hartley) of weight range 400–600 g were maintained without food but with free access to water for 16 h before experimentation. Animals were then killed by cranial percussion, the trachea dissected free and placed into Krebs solution at 4°C. The trachea was cut into preparations consisting of 2 cartilaginous rings which were then cut through the cartilaginous zones.

The preparations were suspended in 30 ml organ baths containing Krebs solution at 37°C gassed with 5% CO_2 in O_2. These were allowed at least 1 h to equilibrate during which time the resting tension was maintained at 1 g. A control response to isoprenaline (1×10^{-7} M) was then obtained, to indicate the maximum relaxation possible in this tissue. The tissues were then washed and allowed 30 min to re-equilibrate before one of the drugs under study was added by a cumulative concentration procedure. Responses were allowed to stabilize (usually within 15 min of drug addition).

Tension was recorded with isometric transducers (Letica TR1 010) onto a Letica polygraph (model 4000). Changes in tension were measured, and EC_{25} and EC_{50} values calculated, as described for human preparations.

Bronchodilator activity in anaesthetized guinea-pigs

Animals of weight range 400–500 g were anaesthetized with sodium pentobarbitone 60 mg kg^{-1}, i.p. A tracheotomy was performed and a polyethylene tube inserted into the cut trachea, connected to a respirator (Ugo Basile 7025) and a side arm connected to a pressure transducer (Letica TRA021). The left carotid artery was cannulated for blood pressure and heart rate recordings and the right jugular vein for drug administration. After a 15 min stabilization period, a continual infusion (Braun infusion pump) of histamine (100 μg ml^{-1}, 2–6 ml h^{-1}) sufficient to elevate insufflation pressure by 150–200% was administered via the left jugular vein and maintained throughout the experiment. The histamine infusion was continued for 45 min, by which time a steady state bronchoconstrictor response had been observed, before one of the test drugs was given by i.v. bolus injections using an ascending dose scheme.

The maximum bronchodilatation produced by each drug was measured and expressed as the % inhibition of the histamine-induced bronchoconstrictor response.

Extraction, separation and characterization of PDE isoenzymes from human bronchus

Individual human bronchi, weighing 0.9–2.7 g were homogenized for 60 s, with an Ultraturrax at 9000 r.p.m. in 5 volumes of ice-cold buffer A (20 mM Bis Tris, pH 6.5, containing 50 mM sodium acetate, 2 mM benzamidine, 2 mM EDTA, 5 mM β-mercaptoethanol and 50 mM phenylmethylsulphonylfluoride (PMSF)). The homogenate was centrifuged at 15000 *g* for 10 min and the clear supernatant was filtered through 0.22 μm Millex filters. The sample was injected into a MONO-Q HR 5/5 column (1 ml of gel bed, Pharmacia) attached to an FPLC chromatography system and equilibrated in the same buffer. After washing with 15 ml of buffer A, the PDEs were eluted by developing a 20 ml linear sodium acetate gradient from 50 to 1000 mM in buffer A. Flow rate was 1 ml min^{-1} throughout. Fractions of 0.5 ml were collected, analyzed and stored as previously described (Gristwood *et al.*, 1992).

Molecular weights of purified PDEs were determined by gel filtration on a Superose 12 HR 10/30 column (Pharmacia) attached to an FPLC system. The column was equilibrated at room temperature in 20 mM Bis Tris, 150 mM sodium acetate, 2 mM EDTA, 5 mM β-mercaptoethanol, 2 mM benzamidine pH 6.5 buffer. The column was calibrated with proteins of known molecular weight by running 0.1 ml samples at a flow rate of 0.4 ml min^{-1}. Elution volumes were determined by following the absorbance at 280 nm on line, and the obtained K_{av} values were correlated with the logarithm of their molecular weights. Column void volume was determined with Blue Dextran 2000 under the same conditions as the standards. Phosphodiesterase samples obtained from the ion exchange chromatography were also run in a similar manner and 0.20 ml fractions were collected. Elution volumes for the different enzymes were determined by assaying the enzyme activity in the fractions. Molecular weights were calculated by interpolation of the K_{av} values on the regression line obtained for the standards.

Cyclic nucleotide PDEs were assayed following the procedure of Thompson & Strada (1984). Inhibition assays were run in duplicate at 30°C for 20 min at a substrate concentration of 0.25 μM. The substrate was adenosine 3′:5′-cyclic monophosphate (cyclic AMP) unless otherwise stated. PDE II was assayed in the presence of 5 μM unlabelled guanosine 3′:5′-cyclic monophosphate (cyclic GMP). For each of the assayed drugs, 6 to 8 different concentrations were tested in at least two different enzyme preparations. IC_{50} values were obtained by non-linear regression using InPLot, GraphPad Software on an IBM computer.

Drugs, reagents and solutions

The following drugs were used: zaprinast (a gift from Rhône-Poulenc Rorer U.K.), SK&F 94120 (a gift from SK&B Ltd, Welwyn, U.K.), isoprenaline sulphate, obtained from Boehringer Ingelheim (Germany) and theophylline, purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). Denbufylline, rolipram and pentoxifylline were synthesized in the Department of Chemical Synthesis, Almirall.

Calmodulin was obtained from Boehringer Mannheim (Barcelona, Spain). Benzamidine, histamine, PMSF, cyclic GMP and cyclic AMP were from Sigma-Aldrich Química, S.A. (Madrid, Spain). The low and high molecular weight markers and Blue Dextran 2000 were from Pharmacia Iberica (Barcelona, Spain). [8-^{3}H]-adenosine 3′:5′-cyclic monophosphate and [8-^{3}H]-guanosine 3′:5′-cyclic monophosphate were from Amersham International (U.K.).

The drugs used for the biochemical studies were dissolved in dilute NaOH, dimethylsulphoxide (DMSO) or water. Drug vehicles at concentrations employed did not affect enzyme activities.

For the pharmacological studies, isoprenaline was dissolved in distilled water containing 0.57 mM ascorbic acid. Stock solutions of denbufylline were prepared in 40% polyethyleneglycol 300 and rolipram in 20% polyethyleneglycol 300. Theophylline and pentoxifylline were dissolved in water. Subsequent dilutions of drugs were made in the Krebs solution which had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.6, $NaHPO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.

Statistics

Values are given as mean ± s.e. mean. Statistical analysis of results was carried out by analysis of variance (ANOVA) followed by Duncan's test or by Student's t test for paired or unpaired data as appropriate.

Results

Bronchorelaxant activity in human bronchus

All drugs tested caused concentration-dependent inhibition of the spontaneous tone of human bronchi as shown in Figure 1. All produced full relaxation, i.e. the maximal relaxation was not significantly different from that obtained with theophylline, 1×10^{-3} M, see Table 1.

The threshold concentrations for isoprenaline, rolipram, pentoxifylline and denbufylline were similar at between 10^{-9} M and 10^{-8} M, whereas the threshold for theophylline was higher, as shown in Figure 1. Considering both EC_{25} and EC_{50} values, the potency order of drugs for relaxation was isoprenaline > rolipram = denbufylline ≫ pentoxifylline > theophylline, as indicated in Table 1.

One further important observation was that in preparations with spontaneous tone, rolipram, denbufylline, and pentoxifylline all produced clear biphasic concentration-response curves (see Figure 1).

Bronchorelaxant activity in guinea-pigs in vitro

All drugs produced concentration-dependent relaxation of the guinea-pig tracheal preparations, as shown in Figure 2. The maximum observed relaxations were as follows; isoprenaline 0.67 ± 0.03 g, rolipram 0.44 ± 0.04 g, denbufylline 0.40 ± 0.04 g, theophylline 0.66 ± 0.03 g and pentoxifylline 0.34 ± 0.07 g, $n = 5-12$.

In terms of threshold concentrations there was a clear order of isoprenaline ≪ denbufylline = rolipram ≪ pentoxifylline = theophylline.

EC_{25} and EC_{50} values for the compounds on guinea-pig trachea are shown in Table 2. For EC_{25} values the potency order was isoprenaline ≫ rolipram = denbufylline ≫ pentoxifylline > theophylline. For EC_{50} values the order was now isoprenaline ≫ denbufylline = rolipram > pentoxifylline > theophylline.

Of all agents tested only rolipram produced a clear biphasic concentration-response curve (Figure 2).

Bronchodilator activity in anaesthetized guinea-pigs

All drugs produced dose-dependent inhibitions of the histamine-induced bronchospasm, as shown in Figure 3. In terms of threshold doses the order was isoprenaline > rolipram > denbufylline ≫ pentoxifylline = theophylline. In order to compare drug potencies, doses causing a mean decrease of 50% of the histamine-induced bronchoconstriction were cal-

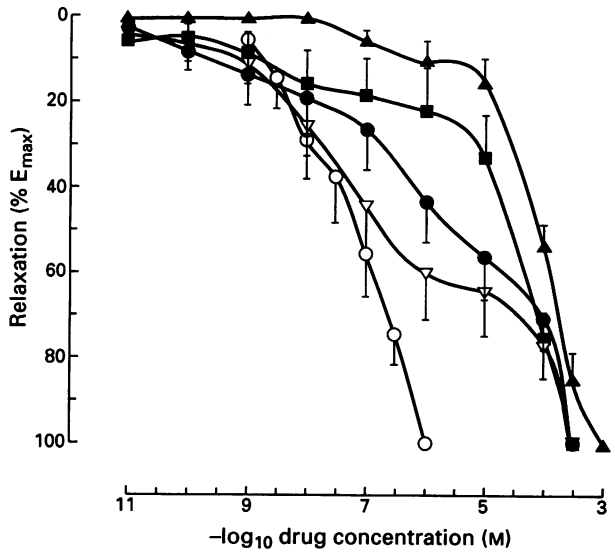

Figure 1 Relaxant effects of theophylline (▲), pentoxifylline (■), denbufylline (●), rolipram (▽) and isoprenaline (○) in human bronchi with spontaneous tone. Points are means ± s.e.mean (vertical bars). n values are indicated in Table 1.

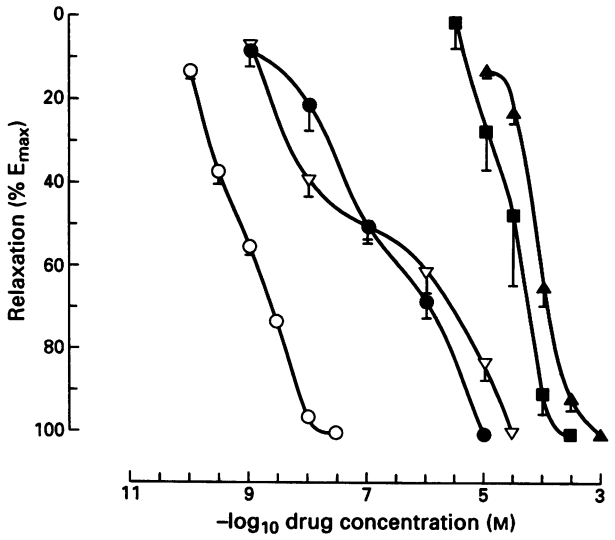

Figure 2 Relaxant effects of theophylline (▲), pentoxifylline (■), denbufylline (●), rolipram (▽) and isoprenaline (○) in guinea-pig tracheal ring preparations. Points are means ± s.e.mean (vertical bars). n values are indicated in Table 2.

Table 1 Relaxant effects of cyclic nucleotide phosphodiesterase (PDE) inhibitors on spontaneous tone of human bronchial preparations

	p/n	$-log\ EC_{50}$ (M)	$-log\ EC_{25}$ (M)	E_{max} (g)	*Maximal relaxation* (g) *induced by theophylline* (1×10^{-3} M)
Theophylline	10/6	4.11 ± 0.13*	4.98 ± 0.30‡	1.53 ± 0.25	-
Pentoxifylline	11/6	5.30 ± 0.53	6.10 ± 0.70	1.31 ± 0.17	1.52 ± 0.19
Denbufylline	9/5	5.94 ± 0.62	7.21 ± 0.74	1.23 ± 0.09	1.74 ± 0.13
Rolipram	9/6	6.23 ± 0.54	7.75 ± 0.59	1.34 ± 0.14	1.56 ± 0.16
Isoprenaline	8/6	7.35 ± 0.30†	7.80 ± 0.20†	1.35 ± 0.22	1.72 ± 0.17

Values are means ± s.e.mean. p represents the number of preparations and n the number of patients.
*$P < 0.05$ from the other agents; †$P < 0.05$ from theophylline and pentoxifylline; ‡$P < 0.05$ from the other agents except pentoxifylline.

Table 2 Relaxant effects of cyclic nucleotide phosphodiesterase (PDE) inhibitors on spontaneous tone of guinea-pig tracheal preparations

	n	$-log\ EC_{50}$ (M)	$-log\ EC_{25}$ (M)
Theophylline	8	4.15 ± 0.15	4.51 ± 0.06
Pentoxifylline	6	4.71 ± 0.17*	5.03 ± 0.20*
Denbufylline	5	6.91 ± 0.13*	8.01 ± 0.24*
Rolipram	12	6.68 ± 0.23*	8.07 ± 0.17*
Isoprenaline	8	9.13 ± 0.04*	9.75 ± 0.05*

*$P < 0.05$ compared with theophylline.

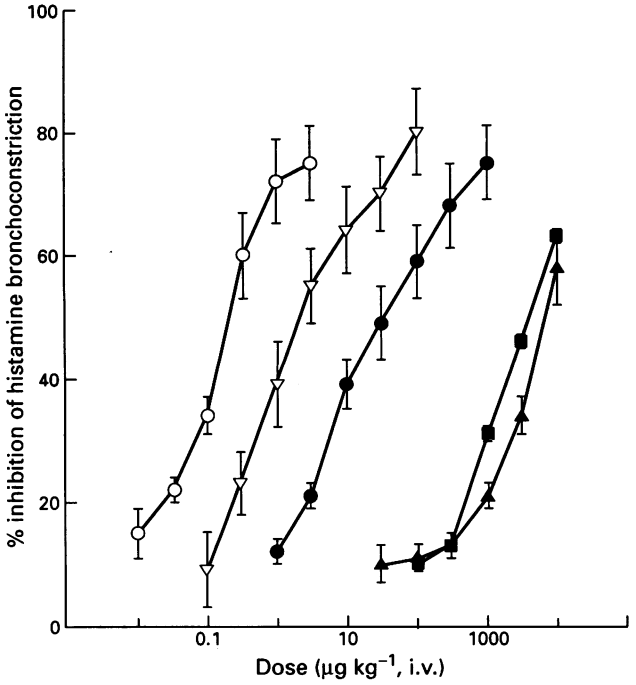

Figure 3 Inhibition of histamine-induced bronchoconstriction in anaesthetized guinea-pigs by isoprenaline (○), rolipram (▽), denbufylline (●), pentoxifylline (■) and theophylline (▲). Points are means ± s.e.mean (vertical bars). $n = 4-6$.

culated. These were as follows in μg kg^{-1} i.v.: isoprenaline 0.45 ± 0.1, rolipram, 4 ± 1, denbufylline 69 ± 20, pentoxifylline 4534 ± 860 and theophylline 5842 ± 911 ($n = 4-6$).

Isolation and characterization of the human bronchial phosphodiesterases

A representative chromatogram of the PDEs isolated from human bronchi by ion exchange is shown in Figure 4. In 5 out of 6 individual preparations, 4 peaks of PDE activity were observed.

The characterization of the peaks is shown in Table 3. When assayed with 1 μM cyclic AMP as substrate, peak A was the most variable in size, ranging from 8 to 21% of total PDE activity. When submitted to gel filtration, peak A was resolved into two distinct peaks. This, taken together with its differential sensitivity to zaprinast when cyclic GMP was used as substrate instead of cyclic AMP and its marginal activation by Ca^{2+}/calmodulin (Table 3) indicates that peak A is a mixture of PDE I and PDE V.

Peak B was always the smallest of all and could not be clearly identified in one of the samples. The characterization

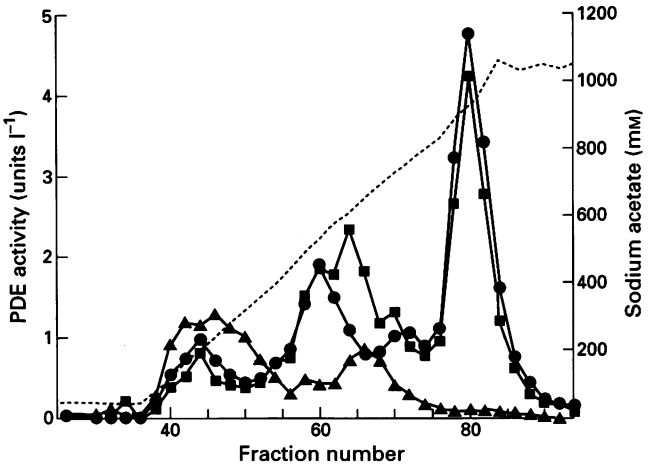

Figure 4 Representative elution profile of cyclic nucleotide phosphodiesterase (PDE) activities from human bronchus on a MONO Q ion exchange column. The low speed centrifugation supernatant (10 ml) from one individual sample was chromatographed as described in the text. Fractions (0.5 ml) were collected and assayed for PDE activity in the following conditions: 1 μM cyclic AMP (●), 1 μM cyclic AMP plus 1 μM cold cyclic GMP (■) and 1 μM cyclic GMP (▲). The 4 peaks collected were: fractions 37 to 53 (A), fractions 56 to 62 (B), fractions 64 to 68 (C) and fractions 76 to 85 (D). For the ordinate scale, 1 unit of enzyme activity is defined as the amount hydrolysing 1 μmol of substrate per min.

of its activity using kinetic and regulatory properties suggested that peak B corresponded to a form of PDE IV. IC_{50} values of 1.1 and 1.3 μM were obtained for this peak using denbufylline and rolipram respectively.

The low molecular weight obtained for peak B suggested that it could have been derived from native PDE IV by limited proteolysis. This, and the small amounts of peak B obtained, led us to exclude this enzyme from the biochemical studies with the rest of inhibitors.

Peak C represented 16.7 ± 3.4% of the total activity measured with 1 μM cyclic AMP as substrate and the data presented in Table 3 indicate that this enzyme is the cyclic GMP stimulated form (PDE II).

Peak D was the major peak in all samples (56 ± 1.7% of total). Its characterization, shown in Table 3, indicates that this peak is also PDE IV. Gel filtration studies indicated a molecular weight of 82300 ± 5600, as well as the homogeneity of the enzyme, with little contamination by other PDE isoenzymes (see Figure 5).

No clear evidence of cyclic GMP inhibited PDE (PDE III) was obtained either from the chromatograms or from the use of the selective PDE III inhibitor SK&F 94120 (Gristwood *et al.*, 1986) on selected PDE fractions.

Effects of the drugs on the isolated isoenzymes

The effects of the PDE inhibitors on the three major peaks of human bronchial PDE activities are shown in Table 4. As expected, rolipram and denbufylline displayed selectivity for Peak D, whereas theophylline and pentoxifylline inhibited all three peaks. The potency order of the inhibitors on peak D was denbufylline = rolipram ≫ pentoxifylline > theophylline.

Discussion

The results from this study have confirmed the relaxant activity of theophylline in human respiratory muscle *in vitro*. This is in agreement with previous findings (Cortijo *et al.*, 1992). Furthermore, we have shown that pentoxifylline, another clinically used xanthine, also has relaxant properties

Table 3 Characterization of the human bronchial phosphodiesterases separated by ion exchange chromatography

	Peak A	*Peak B*	*Peak C*	*Peak D*
K_m cyclic AMP	1.3	ND	77*	1.15
K_m cyclic GMP	0.8	>500	9.6**	>500
Ca^{2+}/Calmodulin (% activation)	31	NE	NE	NE
Cyclic GMP 5 μM (% activation)	NE	NE	87	NE
Mol. weight (KD)	ND	27.8	ND	82.3
Zaprinast[a] (IC_{50})	53	50	55	61
Zaprinast[b] (IC_{50})	0.32	ND	ND	ND
SK&F 94120 (IC_{50})	>500	>500	>500	>500

All concentrations are in μM. [a]Cyclic AMP as substrate; [b]Cyclic GMP as substrate. ND, not determined. NE, no effect. * indicates $S_{0.5}$ because of non hyperbolic kinetics. **K_m value for cyclic AMP in the presence of cyclic GMP. Dispersion values, lower than 10% in all cases, have been omitted for clarity.

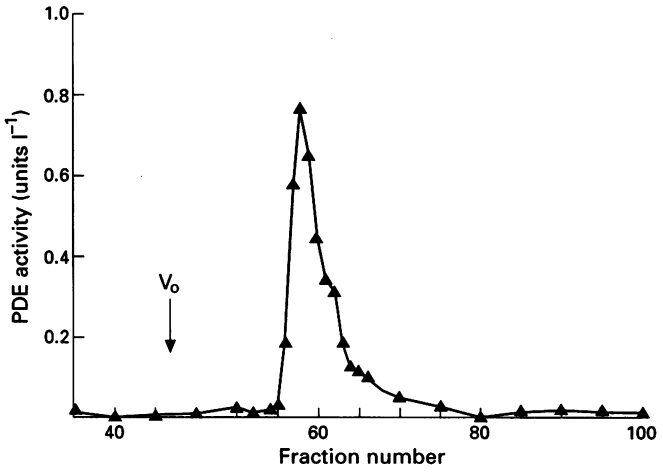

Figure 5 Gel filtration profile on Superose 12 of an aliquot of the 4th peak obtained from the ion exchange chromatography (peak D). The arrow indicates column void volume. See text for details. For the ordinate, 1 unit of enzyme activity is defined as the amount hydrolysing 1 μmol of substrate per min.

in human bronchial tissue *in vitro*. Although pentoxifylline is currently used clinically as a haemorheological agent (Ward & Clissold, 1987), clinical studies have shown that the drug has bronchodilator activity in man (Nolte, 1971).

We consider that the concentrations of both theophylline and pentoxifylline used in this study are relevant to the plasma concentrations of these drugs achieved clinically. Thus, in the case of theophylline, therapeutic plasma concentrations are between 50 and 100 μM, 60% of which is protein bound (Svedmyr, 1988). Therefore, free plasma concentrations would not be very different from the *in vitro* EC_{25} (≈10 μM) and EC_{50} (≈80 μM) values found in this study. For pentoxifylline, mean peak plasma concentrations in man (following a standard 400 mg dose) are approximately 5 μM (plasma binding is low, see Ward & Clissold, 1987), again a value close to the *in vitro* EC_{25} (≈1 μM) and EC_{50} (≈5 μM) found for pentoxifylline in this study.

Considering responses in human preparations at the EC_{50} level, both theophylline and pentoxifylline were relatively weakly active on the tissues, whereas the selective PDE IV inhibitors tested, rolipram and denbufylline, were much more potent relaxants. Indeed rolipram, at the response level of EC_{25}, was close to isoprenaline in its potency.

These data confirm the bronchodilator potential of PDE IV inhibitors in man, particularly in terms of potency and maximal effect.

Consistent with the bronchorelaxant activities of denbufylline and rolipram, our biochemical studies confirmed the presence of PDE IV in human bronchi and furthermore, indicated that it is the major PDE isoform present in this tissue. There were in fact two peaks of activity that could be characterized as PDE IV, (Peaks B and D) although we believe, based on their properties, that Peak B could be a proteolysed form of the enzyme. Thus, whereas peak D eluted in the position typical for PDE IV both from human (Reeves *et al.*, 1987) and animal sources (Gristwood *et al.*, 1992) and had a molecular weight close to that of cloned human PDE IV (Livi *et al.*, 1990) peak B was smaller and eluted before PDE II in the chromatogram.

A comparison of the inhibitory potencies of the four PDE inhibitors on PDE IV (Peak D activity) and relaxant activity in human bronchi (Tables 1 and 4) indicated a good correlation, and this suggests that PDE IV inhibitory activity for both theophylline and pentoxifylline is at least partly responsible for their *in vitro* bronchorelaxant activities in human tissue.

We cannot, of course, exclude the possibility that for the xanthine compounds, inhibition of the other PDE isoenzymes present, contributed to their relaxant activities, but no correlation could be found for the tested compounds between inhibition of the other peaks and their relaxant potencies, particularly in the case of rolipram.

Recently Shahid *et al.* (1992) have shown that up to six different peaks of PDE activity could be resolved by ion exchange chromatography in human bronchi. Although the chromatographic profile described by these authors appears to be more complex than that found in the present paper, no major discrepancies can be perceived. As with our samples, PDE IV was the main activity hydrolyzing cyclic AMP in the study of Shahid *et al.* (1992). PDE V and two slightly

Table 4 Effect of the compounds under study on the phosphodiesterase peaks isolated by ion exchange chromatography

	Peak A	*Peak C*	*Peak D*
Theophylline	3.75 ± 0.01	3.26 ± 0.15	3.82 ± 0.02
Pentoxifylline	4.19 ± 0.02	4.01 ± 0.04	4.35 ± 0.06
Denbufylline	4.57 ± 0.01	4.13 ± 0.06	6.33 ± 0.14
Rolipram	3.46 ± 0.05	3.59 ± 0.04	6.21 ± 0.10

For each drug 6–8 concentrations were tested in duplicate for are at least two different samples. Values are $-\log IC_{50}$ in molar concentration ± s.e.mean.

different forms of PDE I could be detected by the authors eluting at low salt concentrations. These match the enzymes that were identified in our peak A. PDE II was also detected. The main difference found was the presence of a minor form of PDE IV eluting at a different position from that of peak B in the study of Shahid *et al.* (1992).

It was interesting that another major PDE isoenzyme, PDE III, was not identified in our biochemical experiments. This is in agreement with recent data (Shahid *et al.*, 1992). Consistent with this, it has recently been shown that selective PDE III inhibitors, unlike selective PDE IV inhibitors, did not induce increases in intracellular cyclic AMP content in human cultured tracheal smooth muscle cells (Hall *et al.*, 1992). These data taken together suggest that PDE III inhibitors may not have important bronchodilator actions in man.

As in human preparations, all PDE inhibitors induced concentration-dependent relaxation of guinea-pig tracheal preparations with similar maximal effects. Furthermore, the rank order of potency of the drugs was similar for the two species. The only major quantitative difference between guinea-pig and human tissues was that isoprenaline was almost 2 orders of magnitude more potent on guinea-pig preparations. The potencies of the PDE inhibitors, however, appeared similar.

We did not investigate PDE enzymes in guinea-pig trachea in the present study. Biochemical studies have been previously reported in the literature and it is known that PDE IV is present in this tissue (Silver *et al.*, 1988; Takagi *et al.*, 1992). Unlike human bronchus, guinea-pig trachea also has PDE III present (Silver *et al.*, 1988), and PDE III inhibitors are effective bronchodilators in the guinea-pig both *in vitro* (Bryson & Roger, 1987) and *in vivo* (Gristwood & Sampford, 1987). It is therefore, possible that both PDE III and IV inhibition contributed to the bronchorelaxant activity of theophylline and pentoxifylline in the guinea-pig.

Rolipram and denbufylline both demonstrated potent dose-related bronchodilator activity in anaesthetized guinea-pigs. This confirms that selective PDE IV inhibitors can act as bronchodilators *in vivo*. In this *in vivo* preparation there was a difference in potency between rolipram and denbufylline that was not indicated by the guinea-pig *in vitro* studies. The reason for this is not known, but may have been due to pharmacokinetic considerations. Nevertheless, it is clear that both selective PDE IV inhibitors were much more potent than either pentoxifylline or theophylline *in vivo*.

The consistency of the bronchorelaxant efficacy of the PDE IV inhibitors and the xanthines in human bronchus and guinea-pig trachea *in vitro* coupled with their efficacy in the guinea-pig *in vivo*, and evidence that theophylline and pentoxifylline act as bronchodilators in man, all lead us to the conclusion that selective PDE IV inhibitors will have bronchodilator activity in man *in vivo*.

One additional finding of interest was that in human preparations, rolipram, denbufylline, pentoxifylline and theophylline all produced concentration-response curves which appeared somewhat biphasic in nature. This was particularly so for the rolipram curve which had evidence of a plateau between 10^{-6} M and 10^{-5} M.

A biphasic concentration-response curve for rolipram was also evident in guinea-pig tracheal preparations, where there was evidence of a plateau between 10^{-8} and 10^{-6} M.

Although a definite explanation for these findings cannot be given, the possibility of the involvement of another PDE isoenzyme or isoenzymes in the relaxation of human tissue cannot be excluded. If that were the case, the most selective compound of those tested (rolipram) might be expected to have the most evident plateau, as indeed is the case.

In conclusion, our study has indicated the importance of PDE IV in the relaxation of human bronchial smooth muscle *in vitro*. The data obtained indicate that the inhibition of this isoenzyme is important for the bronchorelaxation activities of rolipram, denbufylline as well as theophylline and pentoxifylline both in guinea-pig and man and, further, that selective PDE IV inhibitors could be clinically useful bronchodilators in man.

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