British Journal of Pharmacology

April 1991

Volume 102 Number 4 pages 783–1008

Br. J. Pharmacol. (1991), **102**, 785–787

SPECIAL REPORT

D-Arg[Hyp^3-Thi^5-D-Tic^7-Tic^8]-bradykinin, a potent antagonist of smooth muscle BK_2 receptors and BK_3 receptors

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D-Arg[Hyp^3-Thi^5-D-Tic^7-Tic^8]-bradykinin (NPC 16731) inhibited bradykinin (BK) binding and BK-induced contraction in guinea-pig ileum, being markedly more potent than D-Phe^7-BK analogues as a BK_2 receptor antagonist. In isolated trachea NPC 16731, unlike other BK_2 antagonists, inhibited BK binding and BK-induced contraction, and $^{45}Ca^{2+}$ efflux in tracheal smooth muscle cells. That NPC 16731 potently inhibits BK effects in trachea provides further evidence for the existence of the airway BK_3 receptor.

Keywords: Bradykinin; BK_2 receptor; BK_3 receptor; antagonist; trachea; ileum; smooth muscle; $^{45}Ca^{2+}$ efflux; contraction; bradykinin binding

Introduction Bradykinin (BK) receptors have been divided into BK_1 and BK_2 subtypes based on the relative potencies of BK analogues in isolated smooth muscle (Farmer & Burch, 1991). Many D-Phe^7-substituted BK analogues are BK_2 receptor antagonists (Vavrek & Stewart, 1985; Burch *et al.*, 1990), but exhibit pA_2 values of only 5.50–6.50 in ileum or uterus (Burch *et al.*, 1990). Also, these peptides are often spasmogenic at concentrations necessary to inhibit BK (Farmer *et al.*, 1989a,b).

BK_2 receptor antagonists, such as D-Arg[Hyp^3,D-Phe^7]-BK (NPC 567), do not displace [^{3}H]-BK binding from tracheal smooth muscle (Farmer *et al.*, 1989a). In addition, BK_2 antagonists exhibit minimal inhibition of BK-induced contraction of guinea-pig airways, and NPC 567 does not inhibit BK-induced $^{45}Ca^{2+}$ efflux from tracheal smooth muscle cells (Farmer *et al.*, 1991). These data led to the proposal that a novel receptor, designated BK_3, mediates BK-induced tracheal contraction (Farmer *et al.*, 1989a). Its existence, however, can be established only with the discovery of a BK_3 receptor antagonist. We have compared effects of D-Arg[Hyp^3-Thi^5-D-Tic^7-Tic^8]-BK (NPC 16731), a novel BK analogue, with those of NPC 567, a well-characterized BK_2 antagonist (Burch *et al.*, 1990; Farmer & Burch, 1990), on effects of BK in guinea-pig ileal and tracheal smooth muscle.

Methods *Isolated tissue studies* Ileal and tracheal strips, from male Dunkin-Hartley guinea-pigs (300–400 g), were prepared (Farmer *et al.*, 1986; 1989b). Smooth muscle preparations were suspended in organ baths (resting tension, 1.5 g) for 60 min with washes every 15 min. At the end of this period, BK (0.1 nM–0.1 mM) was added non-cumulatively. At the end of the experiment a maximum, reference response to methacholine (MCh, 1 mM) was obtained.

NPC 16731 (0.1, 1.0 or 10 μM) was added 15 min before each application of BK. One antagonist concentration was examined in each preparation, although different concentrations were tested in tissues from the same animal. pD_2 values for BK were obtained by regression analyses of concentration-response curves. pA_2 values for NPC 16731 were estimated by dividing the antagonist concentration by BK EC_{50} ratio-1 in the presence and absence of a given antagonist concentration (Tallarida *et al.*, 1979). We also examined the effect of NPC 16731 on tracheal sensitivity to MCh, histamine and substance P.

$^{45}Ca^{2+}$ efflux studies Culture of tracheal smooth muscle cells, and $^{45}Ca^{2+}$ efflux experiments therein are detailed elsewhere (Farmer *et al.*, 1991). Briefly, confluent cultures were incubated in Dulbecco's modified Eagle medium containing HEPES (10 mM, pH 7) and $^{45}Ca^{2+}$ (20 μCi), but no serum, for 2–4 h. Efflux of radioactivity was measured for 20 min by replacing media every 60 s. At the end of the experiment, radioactivity remaining in the cells was measured after incubation with trypsin (0.1 mg ml^{-1}). Radioactivity from supernatants and from solubilized cells was determined by liquid scintillation counting. Fractional $^{45}Ca^{2+}$ efflux at each interval was estimated by division of radioactivity lost during a given 60 s period by the amount of radioactivity associated with the cells. Where appropriate, NPC 567 and NPC 16731 were added 5 min prior to and at the same time as BK.

Bradykinin binding Displacement of [^{3}H]-BK binding in ileal and tracheal smooth muscle membranes by NPC 567 or NPC 16731, and subsequent determination of K_d and K_i values, were performed exactly as described previously (Farmer *et al.*, 1989a,b). All data are expressed as the mean ± s.e.mean.

Materials D-Arg[Hyp^3-Thi^5-D-Tic^7-Tic^8]-BK was synthesized at Nova Pharmaceutical Corporation. NPC 567 was synthesized by Abbot Laboratories (North Chicago, U.S.A.), and BK and substance P were purchased from Bachem Inc. (Torrance). Acetyl-β-methylcholine chloride (MCh) and histamine dihydrochloride were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Drugs were dissolved as required in distilled water.

Results *Bradykinin binding* Trachealis and ileal membranes contain saturable BK binding sites with respective K_d values of 87 ± 10 pM and 115 ± 38 pM. Specific binding in trachea was 65 ± 3% and, in ileum, 96 ± 1%. In ileum NPC 567 and NPC 16731 inhibited BK binding with respective K_i values of 84.4 ± 6.2 nM and 0.98 ± 0.23 nM (Figure 1a). In contrast to

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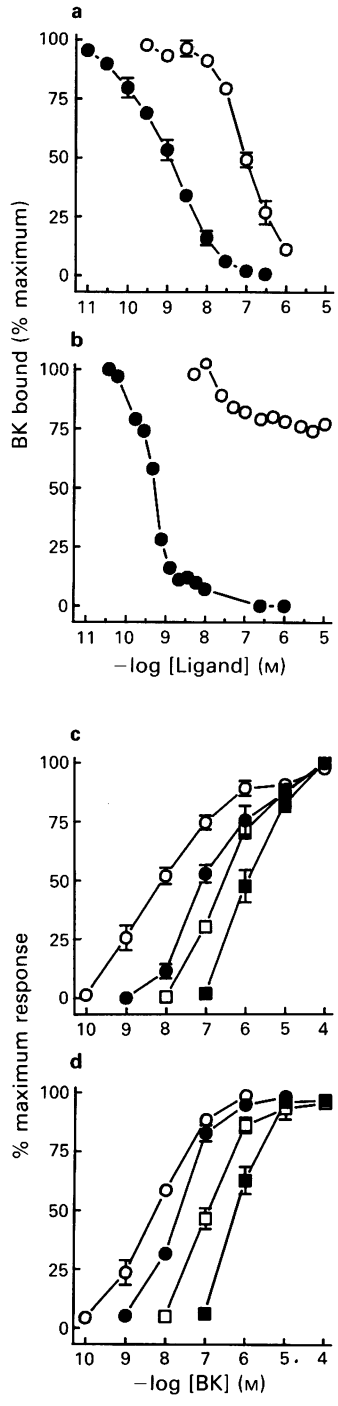

Figure 1 (a & b) [^{3}H]-bradykinin ([^{3}H]-BK) binding displacement curves for NPC 567 (○) and NPC 16731 (●) in (a) guinea-pig ileal and (b) tracheal membranes. [^{3}H]-BK was 0.2 nM, and EBDA corrected for BK when determining K_i values. In ileum, indirect Hill coefficients for NPC 567 and NPC 16731 were -1.14 ± 0.29 and -0.79 ± 0.03 respectively. In trachea, the Hill coefficient for NPC 16731 was 0.96 ± 0.11. (c & d) Effect of NPC 16731 on BK-induced contraction (c) of guinea-pig ileum and (d) trachea. (○) Control, (●) 0.1 μM NPC 16731, (□) 1 μM NPC 16731, (■) 10 μM NPC 16731. Each point is the mean of at least 7 experiments; vertical bars show s.e.mean.

ileum, only NPC 16731 caused 100% displacement of [^{3}H]-BK binding in tracheal smooth muscle membranes, exhibiting a K_i value of 0.14 ± 0.03 nM. NPC 567 (10 μM) displaced BK binding by only 23% (Figure 1b).

Isolated smooth muscle NPC 16731 displaced BK concentration-response curves to the right in trachea and ileum (Figure 1c and d), with respective pA_2 values of 7.63 ± 0.10 ($n = 8$) and 7.28 ± 0.09 ($n = 7$). In ileum the maximum response to BK (expressed as % maximum response to MCh) was unaffected by NPC 16731: control, $92.0 \pm 2.3\%$; in presence of NPC 16731, $86.3 \pm 4.9\%$. In trachea the maximum response to BK, $34.3 \pm 2.3\%$, was comparable to $45.5 \pm 3.3\%$ in the presence of NPC 16731. NPC 16731 (10 μM) did not alter tracheal sensitivity to MCh (control pD_2 6.48 ± 0.15; NPC 16731 6.43 ± 0.03), histamine (control pD_2 5.31 ± 0.06; NPC 16731 5.47 ± 0.13), or substance P (control pD_2 7.13 ± 0.06; NPC 16731 7.18 ± 0.10). Spasmogenic activity of NPC 16731 was never observed in either tissue.

$^{45}Ca^{2+}$ efflux BK (0.1 nM–10 μM) stimulated efflux of $^{45}Ca^{2+}$ with a pD_2 value of 7.60 ± 0.20 ($n = 3$). NPC 16731 inhibited BK-induced $^{45}Ca^{2+}$ efflux ($-\log IC_{50}$ 8.48 ± 0.05; Figure 2), whereas at concentrations of up to 10 μM, NPC 567 was without effect on efflux (data not shown).

Discussion NPC 16731, a novel BK_2 receptor antagonist, is significantly more potent than the D-Phe^7-substituted BK analogues (Burch *et al.*, 1990). Thus, NPC 567 had a K_i value similar to that in earlier studies (Steranka *et al.*, 1988; Farmer *et al.*, 1989b), whereas NPC 16731 was approximately 120 fold more potent than NPC 567 in displacing [^{3}H]-BK binding. Moreover, NPC 16731 inhibited BK-induced ileal contractions with nearly 50 fold greater potency than NPC 567, the pA_2 of which is around 6.00 (Burch *et al.*, 1990).

Studies with D-Phe^7-BK analogues are complicated by their spasmogenic effects (Farmer *et al.*, 1989a,b). Whether this represents a true partial agonist action at BK receptors or is a non-specific effect is unknown. However, smooth muscle contractions to the D-Phe^7-analogues, unlike responses to BK, are phasic and exhibit tachyphylaxis. NPC 16731 exhibited no contractile activity in trachea or ileum.

Finally, the proposal for the BK_3 receptor was based on the inability of BK_1 or BK_2 antagonists to inhibit BK binding in airway smooth muscle (Farmer *et al.*, 1989a). Moreover, BK_1 and BK_2 antagonists are ineffective against BK-induced tracheal contraction or efflux of $^{45}Ca^{2+}$ in cultured tracheal smooth muscle cells (Farmer *et al.*, 1991). Although it did not influence responses to MCh, histamine or substance P, NPC 16731 was a potent inhibitor of BK-induced tracheal contraction and of $^{45}Ca^{2+}$ efflux. These data, coupled to the observation that NPC 16731 was a potent inhibitor of BK binding in tracheal membranes, substantiates the evidence for the BK_3 receptor in airway smooth muscle.

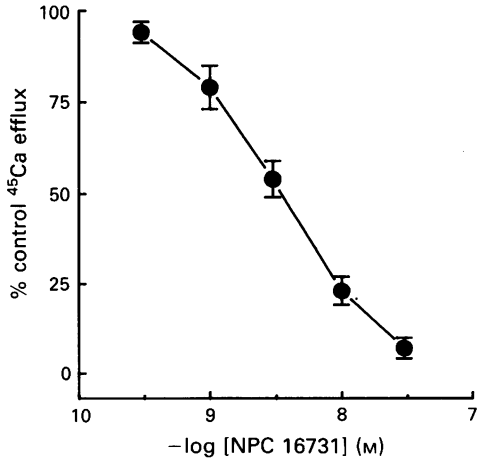

Figure 2 Inhibition by NPC 16731 of $^{45}Ca^{2+}$ efflux induced by bradykinin (10 nM) in cultured guinea-pig tracheal smooth muscle cells. Each point is the mean of 3 experiments; vertical bars show s.e.mean.

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(Received October 26, 1990
Accepted November 26, 1990)

Br. J. Pharmacol. (1991), **102**, 788–790

SPECIAL REPORT

Pinacidil inhibits the ryanodine-sensitive outward current and glibenclamide antagonizes its action in cells from the rabbit portal vein

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Pinacidil, a potassium-channel opener, inhibited the ryanodine-sensitive oscillatory outward potassium current induced by Ca released from an intracellular store. Glibenclamide, a blocker of the ATP-sensitive K-channel, prevented the action of pinacidil, suggesting the presence of an additional site (to K channels) for the vasodilator actions of pinacidil at which glibenclamide can act as an antagonist.

Keywords: Pinacidil; glibenclamide; Ca-dependent K current; oscillatory outward current; Ca release; intracellular Ca store sites; K-channel opener; ATP-sensitive K channel

Introduction Pinacidil is a known K-channel opener which activates ATP-sensitive K-channels in cardiac cells (Fan *et al.*, 1990). In smooth muscle cells, the vasodilator actions of pinacidil are thought to be mediated by the activation of ATP-sensitive K-channels (Standen *et al.*, 1989; Okabe *et al.*, 1990; Fan *et al.*, 1990; Kajioka *et al.*, 1990). These workers demonstrated that glibenclamide, a blocker of the ATP-sensitive K-channel, inhibited K-channel activities induced by various K-channel openers. However, it is not yet clear whether pinacidil and glibenclamide act solely on the K-channel or whether pinacidil activates additional vasodilator mechanisms (Cook *et al.*, 1989). In the present experiments, we describe an action of pinacidil which appears not to be related to K-channel opening.

Methods Single smooth muscle cells were prepared by collagenase treatment from rabbit portal vein. Cells were placed in a physiological salt solution (PSS) at room temperature and the patch-clamp technique was performed with a glass electrode (2–4 MΩ) filled with a high-K solution containing 0.1 mM EGTA. For single channel recording, outside-out membrane patches were obtained with electrodes of resistance 5–10 MΩ. The ionic composition of PSS and the high-K solution were as follows (mM): (PSS): NaCl 135, KCl 6, $CaCl_2$ 2.5, $MgCl_2$ 1.2 and glucose 12; (high-K solution): KCl 140, $MgCl_2$ 5, Na_2ATP 5, EGTA 0.1 and glucose 12. For single-channel current recording, 4 mM EGTA was added to the high-K solution and the concentration of free Ca was kept at 0.3 μM by addition of $CaCl_2$. The pH of the solutions was adjusted to 7.25 ± 0.05 with 10 mM HEPES titrated with Tris. Drugs used were pinacidil (Shionogi Pharmac. Ltd.), glibenclamide (Sigma Chem., St. Louis, MO) and ryanodine (Wako Pure Chem, Osaka).

Results Single smooth muscle cells of the rabbit portal vein at a holding potential of −40 mV produced an oscillatory outward current (Ioo; Ohya *et al.*, 1987; sometimes called a spontaneous transient outward current (STOC), Bolton & Lim, 1989). Superfusion of ryanodine (30 μM; Figure 1a) or caffeine (3 mM; data not shown), both of which are known releasers of Ca from its store site, inhibited Ioo within a few min, suggesting that, in the portal vein, the generation of Ioo is closely related to the release of Ca from an intracellular store (Ohya *et al.*, 1987; 1988; Sakai *et al.*, 1988). A high concentration of glibenclamide (30 μM) inhibited the activity of Ioo, but 1 μM glibenclamide had no action on Ioo (Figure 1b). Single channel current recording showed that 100 μM glibenclamide did not inhibit the large conductance Ca-dependent K-channel, which is reported to be closely related to generation of Ioo (Figure 1c(i); Sakai *et al.*, 1988). This idea is supported by the fact that charybdotoxin, a selective blocker of the large conductance Ca-dependent K channel, blocked Ioo (data not shown). Similarly, application of pinacidil (≤ 100 μM) did not change the activity of the large conductance Ca-dependent K-channel (Figure 1c(ii)). On the other hand, with the whole-cell voltage-clamp configuration, application of pinacidil (≥ 3 μM) rapidly produced an outward current and then inhibited Ioo after a delay of several min (Figure 1d(i)). A higher concentration of pinacidil (30 μM) further inhibited Ioo and after its removal, the outward current induced by pinacidil declined but it took over 10 min after washout for Ioo generation to recover (Figure 1d(ii)). Pinacidil (5 μM) neither produced an outward current nor inhibited Ioo, after pretreatment with 1 μM glibenclamide (Figure 1e(i)). However, 8 min after removal of both drugs, reapplication of pinacidil (5 μM) produced an outward current and inhibited the generation of Ioo. If pinacidil was applied first glibenclamide (1 μM) partly restored Ioo and inhibited the pinacidil-induced outward current (Figure 1e(ii)). Amplitude histograms showed that pinacidil reduced the number of Ioo (calculated from amplitudes larger than 100 pA) to 0.21 times of the control, and simultaneous application of glibenclamide partly restored Ioo generation (0.51 times of the control; Figure 1f). As shown in Figure 1g, the relative amount of current carried as Ioo was reduced by application of pinacidil (≥ 1 μM) dose-dependently, and glibenclamide (1 μM) significantly prevented the inhibitory actions of pinacidil.

Discussion The findings of the present experiments indicate (1) that pinacidil not only activates K-channels (Standen *et al.*, 1989; Kajioka *et al.*, 1990), but also inhibits the spontaneous K current induced by Ca release from intracellular stores and (2) that glibenclamide both prevents the pinacidil-induced inhibition of Ioo and blocks the pinacidil-activated outward current.

Inhibition of Ioo by pinacidil was not due to direct blockade of the Ca-dependent K-channel, since the activity of the K channel was not inhibited by pinacidil. Although the possibility that pinacidil might synthesize an endogenous channel-blocker for the Ca-dependent K-channels cannot be excluded, a more plausible explanation for the inhibition of Ioo is that pinacidil inhibits some step involved in the Ca release mechanism and/or the Ca refilling mechanism. Since both ryanodine-sensitive and IP_3-sensitive Ca release channels could modify the generation of Ioo (Ohya *et al.*, 1987; 1988; Sakai *et al.*, 1988), it is difficult to decide whether one or both

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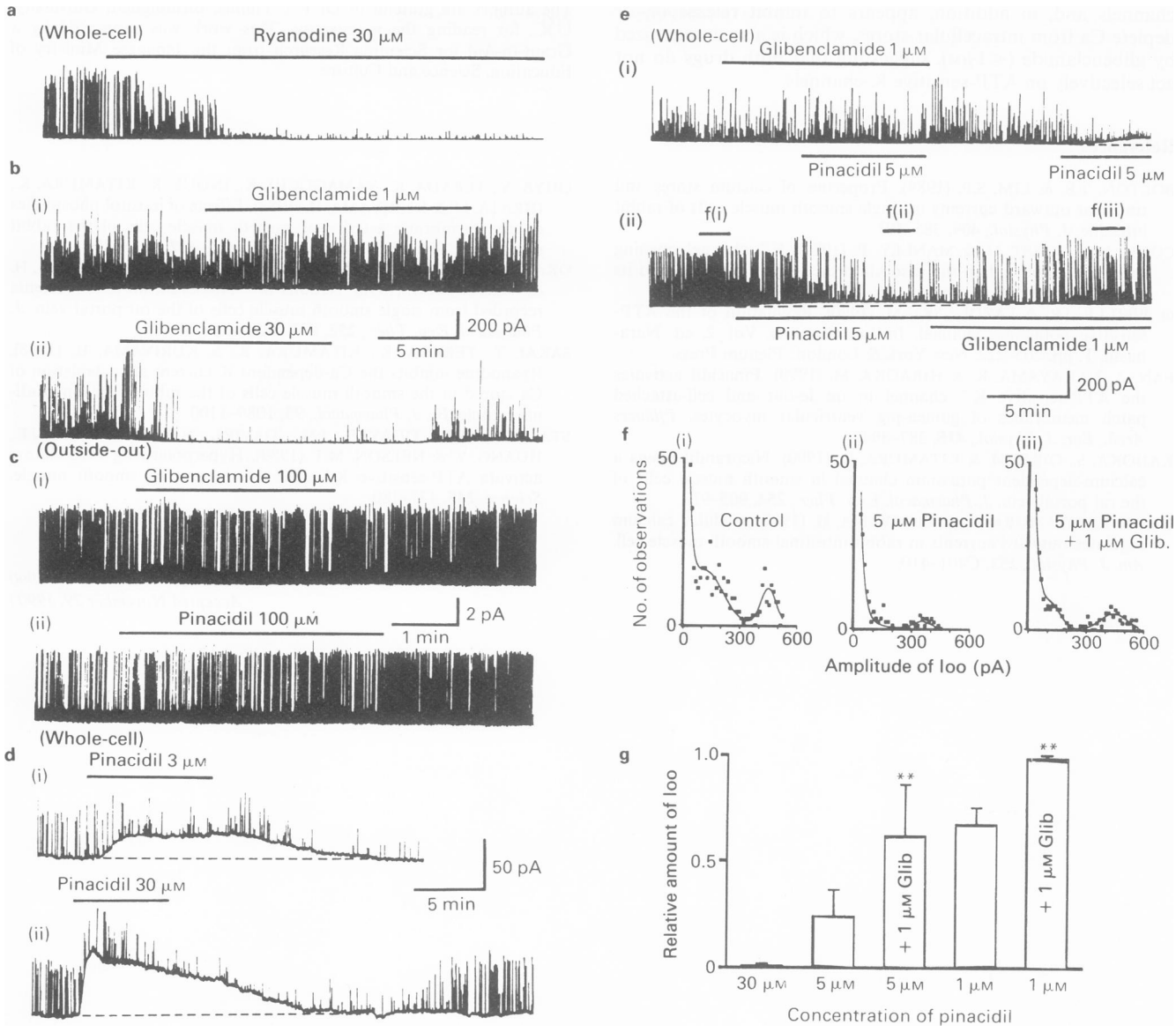

Figure 1 Effects of ryanodine, pinacidil and glibenclamide on the oscillatory outward current (Ioo) and single channel activity of the Ca-dependent K-channel with large conductance. (a) Effect of ryanodine (30 μM) on Ioo. (b) Effect of glibenclamide (1 μM and 30 μM) on Ioo. (c) Effects of glibenclamide (100 μM) and pinacidil (100 μM) on activity of the Ca-dependent K-channel. (d) Effects of pinacidil (3 μM or 30 μM) on Ioo. (e) Antagonism by glibenclamide (1 μM) of the pinacidil (5 μM)-induced inhibition of Ioo. The holding potentials were −30 mV (a, b and e), −40 mV (d) and 0 mV (c). Traces in (c) were recorded with the outside-out membrane patch configuration whereas the other traces were recorded with the whole-cell voltage-clamp configuration. All drugs, dissolved in deionized water and diluted with PSS, were superfused in the bath with a flow rate of 1 ml min^{-1}. (f) Amplitude histograms of Ioo measured in the absence (i) of and presence of 5 μM pinacidil (ii) or in the presence of 5 μM pinacidil and 1 μM glibenclamide (iii). Histograms were obtained from the result shown in Figure 1e(ii) (indicated as f(i), f(ii) and f(iii) above the trace) and the fluctuations of the current less than 30 pA were omitted from the measurement. Curves were fitted by an equation of:

$$Y = A_1 * \mathrm{Exp}(-X/M_1) + A_2 * \mathrm{Exp}(-(X - M_2)^2/S_2^2) + A_3 * \mathrm{Exp}(-(X - M_3)^2/S_3^2)$$

with following values; control, $A_1 = 150$, $M_1 = 20$ pA, $A_2 = 16$, $M_2 = 119$ pA, $S_2 = 130$, $A_3 = 11$, $M_3 = 448$ pA, $S_3 = 58$, correlation coefficient (r) = 0.92; 5 μM pinacidil, $A_1 = 150$, $M_1 = 23$ pA, $A_2 = 1$, $M_2 = 100$ pA, $S_2 = 96$, $A_3 = 2.2$, $M_3 = 353$ pA, $S_3 = 66$, r = 0.96; 5 μM pinacidil + 1 μM glibenclamide, $A_1 = 150$, $M_1 = 25$ pA, $A_2 = 7$, $M_2 = 119$ pA, $S_2 = 70$, $A_3 = 4.9$, $M_3 = 442$ pA, $S_3 = 98$, r = 0.98. (g) Inhibitory actions of pinacidil (1, 5 and 30 μM) on relative amount of current carried as Ioo observed in the absence and presence of 1 μM glibenclamide. Amount of Ioo was estimated by area of amplitude histogram and the histogram in the absence of drug was normalized as 1.0. Each column shows mean value with s.d. ($n = 3$). ** indicates $P < 0.01$ (P-values were calculated between the values in the presence and absence of 1 μM glibenclamide).

processes is responsible for the pinacidil-induced inhibitory action seen in the present experiments.

Glibenclamide is used to inhibit selectively ATP-sensitive K-channel in cardiac muscle and pancreatic β-cells (see review by De Weille & Lazdunski, 1990). In the present experiments, a low concentration of glibenclamide (1 μM) restored the generation of Ioo blocked by pinacidil without any action on Ioo by itself, while a high concentration of the drug (30 μM) had a similar action to pinacidil. This implies that glibenclamide (1 μM) itself, while not having the ability to activate Ioo, did antagonize the inhibitory actions of pinacidil. Although glibenclamide inhibited the pinacidil-induced outward current (ATP-sensitive K current), the antagonizing action of glibenclamide on Ioo was neither related to activation of ATP-sensitive K-channels by pinacidil, as lemakalim had no inhibitory action on Ioo in the same preparation (unpublished observations), nor was it due to direct activation of Ca-dependent K-channels, as glibenclamide had no excitatory action on these channels.

In summary, pinacidil activates glibenclamide-sensitive K-

channels and, in addition, appears to inhibit release of, or deplete Ca from intracellular stores, which is also antagonized by glibenclamide ($\leqslant 1\ \mu M$), suggesting that both drugs do not act selectively on ATP-sensitive K-channels.

The authors are grateful to Dr R.J. Timms, Birmingham University, U.K., for reading the manuscript. This work was supported by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science and Culture.

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(Received November 19, 1990
Accepted November 29, 1990)

Br. J. Pharmacol. (1991), **102**, 791–796

Comparison of the effects of intra-arterial and aerosol administration of endothelin-1 (ET-1) in the guinea-pig isolated lung

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1 Intra-arterial injection of endothelin-1 (ET-1, 400 pmol; 1 μg) in guinea-pig isolated perfused lungs, induced increases in pulmonary inflation pressure (PIP) and perfusion pressure (PPP), associated with oedema formation and thromboxane B_2 (TxB_2) release but not with the generation of sulphidopeptide leukotrienes or release of histamine. In contrast, aerosol administration of ET-1 (3, 6, 10 $\mu g\,ml^{-1}$, for 2 min) evoked a dose-dependent increase in PIP, without significant changes in PPP, oedema formation or TxB_2 release.

2 Addition of indomethacin (5 μM) or BW 755C (10 or 100 μM), but not nordihydroguaiaretic acid (NDGA, 50 μM) or FPL 55712 (10 μM), to the perfusion medium led to a significant inhibition of the increases in PIP and PPP, TxB_2 release and oedema formation evoked by intra-arterial injection of 400 pmol ET-1. In contrast, indomethacin (5 μM), BW 755C (100 μM) or FPL 55712 (10 μM), added to the perfusion medium 10 min prior to challenge, did not affect the increase in PIP induced by a 2-min aerosol of a solution of ET-1 10 $\mu g\,ml^{-1}$.

3 *In vivo* aerosol administration of indomethacin (100 $mg\,ml^{-1}$, for 20 min) to non-anaesthetized guinea-pigs, 15 min before lung removal, did not modify the bronchopulmonary response evoked in isolated perfused lungs by an aerosol of ET-1 10 $\mu g\,ml^{-1}$. However, under the same experimental conditions, indomethacin significantly inhibited TxB_2 release evoked by aerosolized arachidonic acid (2 $mg\,ml^{-1}$).

4 In conclusion, the present study shows that when injected by the intra-arterial route, ET-1 effects are mediated primarily via the generation of cyclo-oxygenase metabolites of arachidonic acid, whereas when the aerosol route is used, the peptide appears to act on airway smooth muscle cells, through an indomethacin-insensitive process which may involve some other, as yet unidentified, mediator(s).

Keywords: Endothelin-1; bronchoconstriction; indomethacin; BW 755C; FPL 55712; NDGA; guinea-pig lung; aerosol

Introduction

The spasmogenic effect of endothelin-1 (ET-1), a 21-amino acid peptide isolated from cultured porcine endothelial cells (Yanagisawa *et al.*, 1988), is not restricted to the vascular system. Indeed, this peptide induces the *in vitro* contraction of pulmonary tissue from various species (Uchida *et al.*, 1988; Maggi *et al.*, 1989; Turner *et al.*, 1989; Hay, 1989; Advenier *et al.*, 1990). Intravenous injection of ET-1 in the guinea-pig elicits a dose-dependent bronchoconstriction which is accompanied by a rapid and marked increase in mean arterial blood pressure (Payne & Whittle, 1988; Braquet *et al.*, 1989a; Macquin-Mavier *et al.*, 1989; Touvay *et al.*, 1990). However, it has been suggested that the *in vivo* effects of ET-1 on blood pressure and bronchopulmonary alterations in the guinea-pig are, at least in part, dissociated (Touvay *et al.*, 1990). This was strengthened by the finding that in the guinea-pig, aerosol administration of ET-1 also induces a potent bronchoconstriction without significant changes in blood pressure (Lagente *et al.*, 1989).

Pretreatment of guinea-pigs with the cyclo-oxygenase inhibitor, indomethacin, produced a marked but incomplete inhibition of the bronchoconstriction induced by both intravenous and aerosol administrations of ET-1 (Payne & Whittle, 1988; Braquet *et al.*, 1989b; Macquin-Mavier *et al.*, 1989; Lagente *et al.*, 1989). Furthermore, a marked release of cyclo-oxygenase metabolites, especially thromboxane (Tx) A_2, following injection of ET-1 into guinea-pig lung is observed (De Nucci *et al.*, 1988; Antunes *et al.*, 1989; Braquet *et al.*, 1989a; Touvay *et al.*, 1990). These results suggested that ET-1 exerts its bronchopulmonary activity mostly via the generation of cyclo-oxygenase metabolites such as TxA_2. However, the possible contribution of other mediators such as lipoxygenase-derived metabolites of arachidonic acid in the ET-1-induced bronchopulmonary effects have yet to be determined.

In the present study, we compared the effects of intra-arterial and aerosol administration of ET-1 in isolated and perfused guinea-pig lungs, in order to analyse the respective contribution of the direct and the indirect bronchopulmonary alterations of the peptide. Furthermore, the pharmacological modulation of the ET-1-induced bronchopulmonary effects, as well as the possible release of histamine, TxA_2 and of the bronchoconstrictor lipoxygenase metabolites of arachidonic acid upon challenge with the peptide were also investigated.

Methods

Lung perfusion experiments

Male Hartley guinea-pigs (400–500 g, Charles River, Saint-Aubin-les-Elbeuf, France) were anaesthetized with sodium pentobarbitone (60 $mg\,kg^{-1}$, i.p.) and the lungs were prepared as described by Pons *et al.* (1989). Briefly, after mid-thoracotomy the lungs were washed *in situ* through the pulmonary artery with 50 ml of Krebs solution containing 2% bovine serum albumin (Krebs-BSA) and heparin 20 units ml^{-1}. Then, the trachea was cannulated and the lungs rapidly excised and suspended in a warmed (37°C) glass chamber. The trachea was connected to a respiratory pump (Ugo Basile pump, Varese, Italy, 60 strokes min^{-1}, 1 ml $100\,g^{-1}$ body weight) and the pulmonary inflation pressure (PIP) was monitored with a Gould transducer (Cleveland, Ohio, USA) connected to the ventilation system and linked to a multichannel recorder (Gould). The lungs were perfused at a flow rate of 5 $ml\,min^{-1}$ through the pulmonary artery with thermostated (37°C) and oxygenated (95% O_2 : 5% CO_2) Krebs-BSA. The pulmonary perfusion pressure (PPP) was

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monitored via a transducer (Gould) connected to the perfusion system. The lungs were allowed to equilibrate for 10 min before intra-arterial or aerosol administration of ET-1 or the various agents tested. For the aerosol administrations, a Devilbiss 'Pulmosonic' ultrasonic Nebuliser (Sommerset, Pennsylvania, USA) was inserted in series with the afferent limb of the ventilator circuit (as modified from Payne & De Nucci, 1987). The various drugs tested, indomethacin (5 μM), BW 755C (10 and 100 μM), FPL 55712 (10 μM), nordihydroguaiaretic acid (NDGA; 50 μM), were added to the perfusion medium 10 min before the stimulation with ET-1 or the various agents. The lung effluent was collected for 1 min before and 5 min following intra-arterial or aerosol challenge for the measurement of histamine, inactive metabolite of TxA_2: thromboxane B_2 (TxB_2), and immunoreactive sulphidopeptide leukotrienes. In order to evaluate oedema formation, the wet weights of the lungs were measured prior to and at the end of the experiments. The increment of the lung weight is expressed as a percentage calculated over the value measured before the experiment.

Pretreatment of guinea-pigs by aerosol administration of indomethacin

In some experiments, non-anaesthetized guinea-pigs were placed in a plexiglass chamber (12 × 30 × 12 cm) and exposed to an aerosol of a solution of indomethacin 100 mg ml^{-1} for 20 min. The aerosol was generated by a Devilbiss 'Pulmosonic' ultrasonic nebuliser, directly connected to the plexiglass chamber. The guinea-pig lungs were prepared as described above, 15 min after the end of the aerosol of indomethacin.

Mediator assays

TxB_2 and sulphidopeptide leukotrienes were assayed according to the method described by Pradelles *et al.* (1985) and with commercial kits (Stallergenes, Fresnes, France). The minimal detectable concentration of TxB_2 was 10 pg ml^{-1} and within the range of 10–400 pg ml^{-1} the intra-assay variation was less than 10%. Cross-reactivities with TxB_1 and dinor TxB_2 were 17% and 11%, respectively. Cross-reactivity with other prostanoids was less than 1%. Background values of TxB_2 in the effluent, prior to ET-1 challenge never exceeded 1 ng ml^{-1}. The minimal detectable concentration of sulphidopeptide leukotriene in lung effluent was equivalent to 10 pg ml^{-1} leukotriene C_4 (LTC_4) and cross-reactivities with LTD_4, LTE_4 and LTB_4 were 56%, 2% and 0.1%, respectively.

For the assessment of histamine, 2 ml aliquots of the lung perfusate were mixed with 0.2 ml of a 4.8 N perchloric acid solution in water. After centrifugation for 20 min at 3500 r.p.m. and at 4°C, the supernatants were collected and stored at 4°C. The spectrofluorimetric assay for histamine was performed according to the method described by Shore (1959).

Drugs

The composition of the Krebs solution was (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$, $7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 5.6. The following drugs were used: sodium pentobarbitone (Nembutal, Clin-Midy, Saint-Jean-de-la-Ruelle, France), pancuronium bromide (Pavulon, Organon Technika, Fresnes, France), heparin (Roche, Neuilly-sur-Seine, France), BSA, TxB_2, histamine hydrochloride, NDGA (Sigma, Saint Louis, MO, U.S.A.). NDGA was solubilized in a 50% ethanol solution in water and then added to the Krebs-BSA. The inhibitor of cyclo-oxygenase and lipoxygenase, BW 755C (3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline, a gift from Drs M.D. Cooke and S. Moncada, Wellcome Research Laboratories, Beckenham, U.K.), and the antagonist of peptidoleukotrienes, FPL 55712 (7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid, a gift from Dr Shipman, Fisons Pharmaceuticals, Loughborough, U.K.), were dissolved in distilled water. The cyclo-oxygenase inhibitor, indomethacin, was solubilized either in 0.5 N NaOH in 0.15 M NaCl and the solution was then adjusted to pH 7.4–7.6 for aerosol administration, or dissolved in absolute ethanol and further diluted in Krebs-BSA. ET-1 (mol. wt. = 2492, Peptide Institut Inc., Osaka, Japan) was dissolved in distilled water and further diluted in a 0.15 M NaCl solution (saline).

Statistical analysis

The results are expressed as mean ± s.e.mean. Differences between means were analysed by Student's *t* test for unpaired data or by use of two-way analysis of variance for the time-course studies.

Results

Effect of intra-arterial and aerosol administration of endothelin-1 in isolated guinea-pig lungs

Aerosol administration of ET-1 (1, 3, 6 or 10 μg ml^{-1} solutions) for 2 min provoked a dose-dependent increase in PIP, developing slowly and reaching a maximal value by 2–3 min following the beginning of the aerosol (Figure 1). Aerosol administration of a solution containing 1 μg ml^{-1} ET-1 evoked no significant change in PIP, as compared to the effect of the solvent alone. When a solution containing ET-1, 3 μg ml^{-1}, was administered by aerosol an important increase in PIP was observed. This bronchoconstrictor response was not significantly different from the one induced by 6 μg ml^{-1} ET-1, but significantly lower than that evoked by aerosol administration of 10 μg ml^{-1} ET-1 (two way analysis of variance). These results are in keeping with the ones previously obtained when investigating the *in vivo* aerosol administration of the peptide in guinea-pigs (Lagente *et al.*, 1989).

The increase in PIP induced by aerosol administration of 10 μg ml^{-1} ET-1 was of lower intensity than the one induced by intra-arterial injection of 400 pmol (approx. 1 μg) of the peptide (Figure 2a). When administered by the intra-arterial route, the ET-1-induced bronchoconstriction was associated with an increase in PPP (Figure 2b), which lasted for at least 5 min following injection of the peptide. In contrast, the bronchoconstrictor effect induced by aerosolized ET-1 (10 μg ml^{-1}) was not associated with such a significant change in PPP (Figure 2b).

As presented in Figure 3, intra-arterial injection of 400 pmol ET-1 provoked a 10% oedema formation and generation of

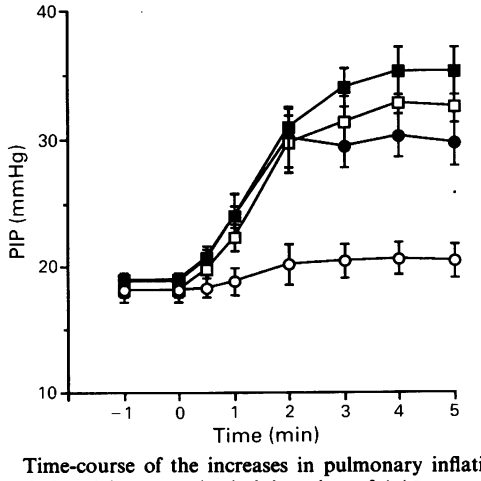

Figure 1 Time-course of the increases in pulmonary inflation pressure (PIP) induced by aerosol administration of 1 (○, $n = 7$), 3 (●, $n = 6$), 6 (□, $n = 7$) or 10 μg ml^{-1} (■, $n = 15$) for 2 min in isolated and perfused guinea-pig lungs. PIP is expressed in mmHg as means, with s.e.mean shown by vertical bars.

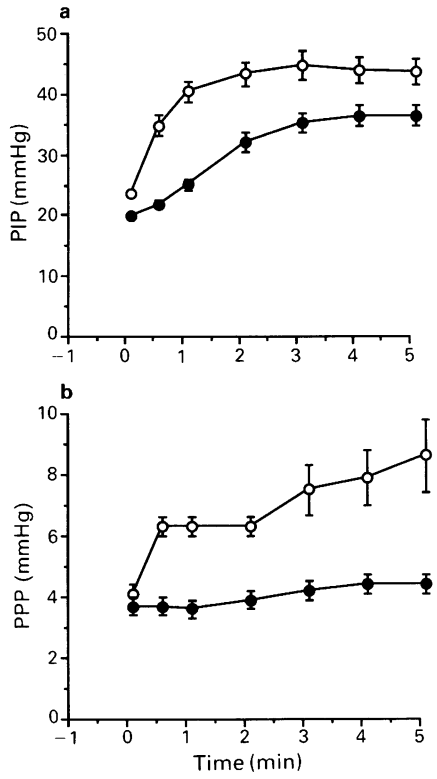

Figure 2 Time-course of the increases in pulmonary inflation pressure (PIP) (a) and pulmonary perfusion pressure (PPP) (b) induced by intra-arterial (400 pmol, ○, $n = 25$) or aerosol (10 μg ml^{-1} for 2 min, ●, $n = 15$) administration of endothelin-1 (ET-1) in isolated and perfused guinea-pig lungs. PIP and PPP are expressed in mmHg as means, with s.e.mean shown by vertical bars.

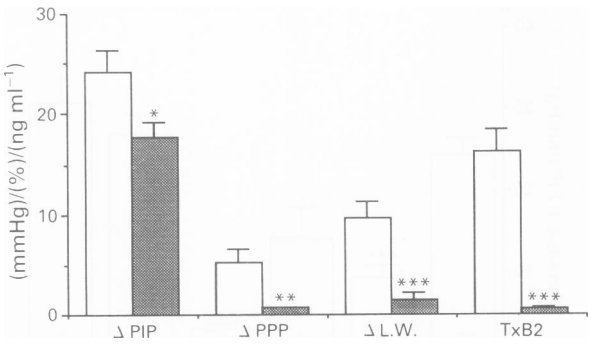

Figure 3 Comparison of the effects of intra-arterial (400 pmol, open columns, $n = 25$) or aerosol (10 μg ml^{-1} for 2 min, stippled columns, $n = 15$) administration of endothelin 1 (ET-1) on pulmonary inflation pressure (PIP), pulmonary perfusion pressure (PPP), oedema formation, thromboxane B_2 (TxB_2) release in isolated and perfused guinea-pig lungs. Increases in PIP (ΔPIP) and PPP (ΔPPP) are expressed in mmHg. Lung oedema (ΔL.W.) is expressed as percentage increase in the wet weight of the organ, as compared to the pre-experiment value. TxB_2 release is expressed in ng ml^{-1}. Results are presented as means with s.e.mean shown by vertical bars. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (t test).

TxB_2 in the lung effluent; however, there was no detectable release of histamine or sulphidopeptide leukotrienes (data not shown). In contrast, no generation of TxB_2 and little, if any, oedema formation was observed after aerosol administration of 10 μg ml^{-1} ET-1 (Figure 3).

Pharmacological modulation of the bronchopulmonary effects induced by intra-arterial injection of endothelin-1

The effects of indomethacin, BW 755C, FPL 55712 and NDGA on the bronchopulmonary alterations induced by intra-arterial injection of 400 pmol of ET-1 were investigated. Addition of indomethacin (5 μM) or BW 755C (10 or 100 μM) to the perfusion medium of isolated and perfused guinea-pig lungs led to a significant inhibition of the increase in PIP, TxB_2 release and oedema formation induced by ET-1 (Figure 4a, c and d). In the presence of indomethacin or BW 755C, the increase in PPP was also reduced, but this inhibition did not reach a significant level (Figure 4b). The effect of BW 755C was concentration-related and that of 100 μM abrogated the increase in PIP and in lung weight, as well as the TxB_2 release, induced by ET-1 (Figure 4a, c and d). In contrast, FPL 55712 (10 μM) or NDGA (50 μM), added to the perfusion medium, did not significantly affect the bronchopulmonary alterations evoked by ET-1 (Figure 4a, b, c and d). On the contrary, in the presence of NDGA (50 μM), the increases in PIP and PPP, TxB_2 release and oedema formation evoked by ET-1 were slightly enhanced (Figure 4a, b, c and d).

Pharmacological modulation of the bronchopulmonary effects induced by aerosol administration of endothelin-1

The effects of addition of indomethacin, BW 755C or FPL 55712 into the perfusion medium were investigated only on the increase in PIP, since no changes in PPP, TxB_2 release and lung wet weight were observed following ET-1 aerosol administration in guinea-pig isolated lungs (see above). The presence of indomethacin (5 μM) in the perfusion medium did not significantly modify the increase in PIP after aerosol administration of ET-1 (3, 6 or 10 μg ml^{-1}, for 2 min) (Table 1). In addition, *in vivo* aerosol administration of indomethacin (100 mg ml^{-1}, for 20 min) to non-anaesthetized guinea-pigs, 15 min before lung removal, did not modify the bronchopulmonary response induced by an aerosol of 10 μg ml^{-1} ET-1 to isolated perfused lungs (data not shown). Furthermore, BW 755C (100 μM) and FPL 55712 (10 μM), added to the perfusion medium, also failed to affect the increase in PIP induced by an aerosol of 10 μg ml^{-1} ET-1. Indeed, the ET-1-induced increases in PIP were 17.7 ± 1.5 mmHg, 18.2 ± 4.5 mmHg and 17.0 ± 2.9 mmHg in the absence and in the presence of BW 755C and FPL 55712, respectively.

Table 1 Effects of indomethacin on the increase in pulmonary inflation pressure (PIP) induced by aerosol administration of endothelin-1 (ET-1)

	Increase in PIP induced by aerosolized ET-1 (mmHg)			
ET-1 (μg ml^{-1})	n	*Control*	n	*Indomethacin*
3	6	13.3 ± 2.3	6	9.2 ± 2.6
6	7	15.4 ± 2.6	6	19.0 ± 3.9
10	15	17.7 ± 1.5	4	18.0 ± 1.9

Indomethacin (5 μM) was added to the perfusion medium 10 min before administration of ET-1. Increases in PIP following aerosol administration of ET-1 (3, 6 or 10 μg ml^{-1}, for 2 min) are expressed in mmHg as mean ± s.e.mean of the indicated number (n) of experiments. No significant differences were observed between control and indomethacin-treated lungs, regardless of the dose of ET-1 used.

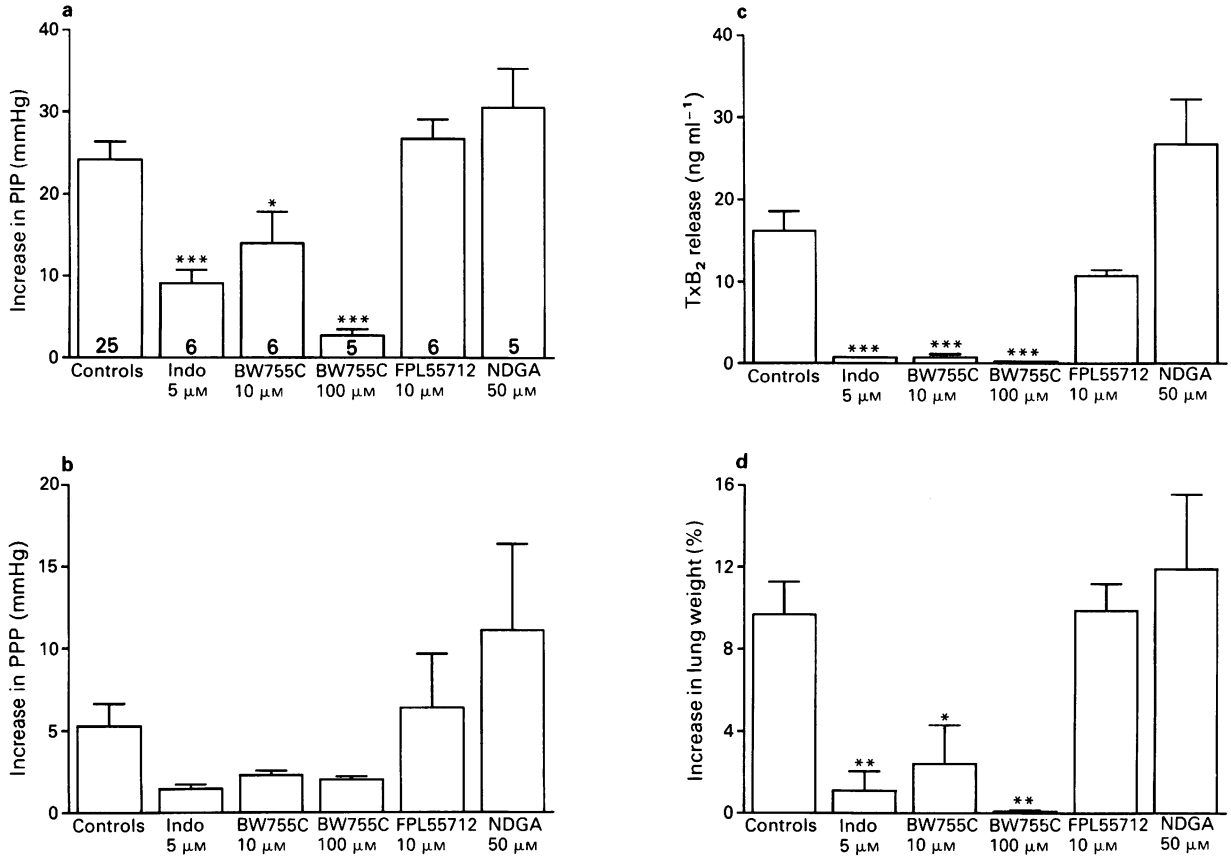

Figure 4 Effects of indomethacin (Indo, 5 μM), BW 755C (10 or 100 μM), FPL 55712 (10 μM) and nordihydroguaiaretic acid (NDGA, 50 μM) on the increases in pulmonary inflation pressure (PIP) (a) and pulmonary perfusion pressure (PPP) (b), thromboxane B_2 (TxB_2) release (c) and oedema formation (d) induced by intra-arterial injection of 400 pmol endothelin-1 (ET-1). The various drugs were added separately to the perfusion medium 10 min before the injection of ET-1. Increases in PIP and PPP are expressed in mmHg. TxB_2 release is expressed in ng ml^{-1}. Lung oedema is expressed as percentage increase in the wet weight of the organ, as compared to the pre-experimental value. Results are presented as mean with s.e.mean shown by vertical bars of the number of experiments indicated inside the bars of (a). * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$ (t test).

Bronchopulmonary effects induced by arachidonic acid administered by aerosol

The effect of indomethacin on the bronchopulmonary actions of aerosolized arachidonic acid was also studied, in order to ascertain that the dose of indomethacin chosen in this series of experiments was adequate. In isolated perfused guinea-pig lungs, aerosol administration of arachidonic acid (2 mg ml^{-1}) for 3 min provoked a slowly developing increase in PIP with a maximal value of 12.9 ± 1.5 mmHg by 3–4 min (Table 2). This peak increase in PIP was associated with no significant change in PPP and with only a minimal alteration (less than 3.9%) in the lung weight (data not shown). However, aerosol administration of arachidonic acid induced a marked generation of TxB_2 in the lung effluent (Table 2). Addition of indomethacin (5 μM) to the perfusion medium did not significantly affect the increase in PIP induced by arachidonic acid, although it reduced by 95% the generation of TxB_2 (Table 2). Also, *in vivo* aerosol administration of indomethacin to non-anaesthetized guinea-pigs, 15 min before lung removal, led to a 60% reduction ($P < 0.05$) of the arachidonic acid-induced generation of TxB_2 by isolated lung, without significant alteration of the bronchoconstrictor response (Table 2).

Discussion

Intra-arterial injection of ET-1 in isolated perfused lungs of the guinea-pig has been shown to result in a dose-dependent increase in PIP, associated with TxB_2 release (Antunes *et al.*, 1988; De Nucci *et al.*, 1988; Braquet *et al.*, 1989a; Touvay *et al.*, 1990). The present results indicate that ET-1 also induces an *in vitro* increase in PIP in isolated and perfused guinea-pig lungs when administered by aerosol. The threshold concentra-

Table 2 Effects of indomethacin on the increase in pulmonary inflation pressure (PIP) and thromboxane B_2 (TxB_2) release induced by aerosol administration of arachidonic acid

Treatment	n	*Increase in PIP* (mmHg)	*TxB_2 release* (ng ml^{-1})
Control	8	12.9 ± 1.5	15.3 ± 3.1
Indo (5 μM)	6	9.2 ± 1.7	0.8 ± 0.3***
Indo (100 mg ml^{-1})	6	20.0 ± 4.1	6.3 ± 1.7*

Indomethacin was added to the perfusion medium (5 μM) or administered by aerosol to non-anaesthetized guinea-pigs (100 mg ml^{-1}, for 20 min). Isolated and perfused guinea-pig lungs were challenged with arachidonic acid (2 mg ml^{-1}) for 3 min. Increases in PIP are expressed in mmHg and TxB_2 in ng ml^{-1}. Results are presented as mean ± s.e.mean of the indicated number (n) of experiments. * $P < 0.05$; *** $P < 0.001$ (t test).

tion of the peptide was $3\,\mu g\,ml^{-1}$, and in the range of 3–$10\,\mu g\,ml^{-1}$, the ET-1-induced effects were dose-dependent. These results are in agreement with those reported by Lagente *et al.* (1989), who initially described the *in vivo* bronchoconstrictor effects of aerosolized ET-1 in the guinea-pig. Administered by the aerosol route, however, ET-1 had no significant effect on PPP, oedema formation or generation of TxB_2 in the lung effluent. This contrasts with intra-arterial injection of the peptide.

The inhibition of the bronchopulmonary effects of intra-arterially administered ET-1 by indomethacin or BW 755C indicates that when this route is used, ET-1-induced effects are primarily mediated by cyclo-oxygenase metabolites of arachidonic acid, a result in agreement with previous reports (De Nucci *et al.*, 1988; Antunes *et al.*, 1988). In addition, no release of immunoreactive sulphidopeptide leukotrienes and no inhibitory effect of NDGA and FPL 55712 were observed, suggesting that lipoxygenase metabolites of arachidonic acid are not involved in the bronchopulmonary alterations induced by intra-arterial injection of ET-1.

The present results also indicate that, when administered by aerosol, ET-1 effects appear not to be mediated through the generation of cyclo-oxygenase metabolites. Indeed, no generation of TxB_2 was detected following aerosol administration of ET-1, under experimental conditions where aerosolized arachidonic acid evoked a marked release of this metabolite in the lung effluent. Furthermore, the bronchopulmonary response induced by aerosolized ET-1 was unaltered when indomethacin, BW 755C or NDGA were added to the perfusion medium. Previous work by Lagente *et al.* (1989) demonstrated that indomethacin, given intravenously to anaesthetized guinea-pigs, reduced by 60% the *in vivo* bronchopulmonary response evoked by aerosolized ET-1, suggesting that when the aerosol route is used, ET-1-induced effects are only partially mediated by the release of cyclo-oxygenase metabolites. Such a discrepancy between *in vivo* and *in vitro* results indicates that other components or cell types (like circulating blood cells) could be recruited during *in vivo* aerosol challenge with ET-1 and contribute to the indomethacin-sensitive part of the bronchopulmonary activity of the peptide. On the other hand, it has been recently reported (Battistini *et al.*, 1990) that the TxA_2 antagonist, BM 13177, markedly reduced the *in vitro* contraction of guinea-pig trachea induced by ET-1. However, in this study, the effect of indomethacin was not evaluated. In this regard, Maggi *et al.* (1989) and Advenier *et al.* (1990) have reported only a slight inhibition of the contraction of guinea-pig trachea and human bronchus induced by low concentrations of ET-1 in the presence of indomethacin. The lack of effect of this drug added to the perfusion medium under our experimental conditions is in keeping with these last results, but can also indicate that, in isolated and perfused lungs, large and small bronchi rather than trachea are the primary targets of ET-1.

In the present study, no inhibitory effect of indomethacin was observed when non-anaesthetized guinea-pigs were pretreated with this drug by aerosol. This pretreatment, however, significantly reduced the release of TxB_2 induced by aerosolized arachidonic acid, and has been reported to suppress the bronchoconstriction induced by aerosolized FMLP in the guinea-pig. Indeed, Boukili *et al.* (1989) have demonstrated that indomethacin administered by aerosol to anaesthetized guinea-pigs significantly reduced the bronchoconstriction induced by both intravenous and aerosol administration of FMLP and abrogated that evoked by intravenous injection of arachidonic acid, suggesting a predominant cyclo-oxygenase-dependent component in these experiments. Under our experimental conditions, aerosol administration of indomethacin ($100\,mg\,ml^{-1}$, for 20 min) suppressed the *in vivo* bronchoconstriction evoked by intravenous injection of arachidonic acid ($0.5\,mg\,kg^{-1}$; Pons *et al.*, unpublished results). Interestingly, although pretreatment of the animals or addition of indomethacin to the perfusion medium significantly decreased TxB_2 formation induced *in vitro* by arachidonic acid by more than 65%, minimal effect of this drug on the increase in PIP was noted. Nevertheless, Manzini *et al.* (1989) suggested that arachidonic acid-induced bronchoconstrictor activity is not exclusively mediated by the generation of cyclo-oxygenase metabolites but also through a local release of neuropeptides. The possibility that the bronchoconstriction evoked by aerosolized ET-1 may involve mediators other than the indomethacin-sensitive ones, for example platelet-activating factor or neuropeptides, deserves investigation.

In summary, these results demonstrate that in isolated and perfused guinea-pig lungs, ET-1 administered by the intra-arterial or aerosol route induces bronchopulmonary effects. When ET-1 is injected by the intra-arterial route, its effects are primarily mediated *via* the generation of cyclo-oxygenase metabolites of arachidonic acid, whereas when the aerosol route is used, the peptide appears to act on airway smooth muscle cells through an indomethacin-insensitive process which may involve some other, as yet unidentified, mediator(s).

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(Received March 23, 1990
Revised November 6, 1990
Accepted November 13, 1990)

Br. J. Pharmacol. (1991), **102**, 797–800

Morphine, but not sodium cromoglycate, modulates the release of substance P from capsaicin-sensitive neurones in the rat trachea *in vitro*

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1 Opioids have been shown to inhibit substance P (SP) release from primary afferent neurones (PAN). In addition, opioid receptors have been identified on PAN of the vagus nerves. Sodium cromoglycate (SCG) decreases the excitability of C-fibres in the lung of the dog *in vivo*. We have utilised a multi-superfusion system to investigate the effect of opioids and SCG on the release of SP from the rat trachea *in vitro*.

2 Pretreatment of newborn rats with capsaicin (50 mg kg^{-1} s.c. at day 1 and 2 of life) resulted in a 93.2 ± 6.3% reduction in tracheal substance P-like immunoreactivity (SP-LI) content when determined by radioimmunoassay in the adult.

3 Exposure to isotonically elevated potassium concentrations (37–90 mM), capsaicin (100 nM–10 μM), and bradykinin (BK; 10 nM–1 μM) but not des-Arg^9-BK (1 μM) stimulated SP-LI release by a calcium-dependent mechanism.

4 SCG (1 μM and 100 μM) did not affect spontaneous, potassium (60 mM)- or BK (1 μM)-stimulated SP-LI release.

5 Morphine (0.1–100 μM) caused dose-related inhibition of potassium (60 mM)-stimulated SP-LI release with the greatest inhibition of 60.4 ± 13.7% at 100 μM. The effect of morphine was not mimicked by the κ-opioid receptor agonist, U50,488H (10 μM) or the δ-opioid receptor agonist, Tyr-(D-Pen)-Gly-Phe-(D-Pen) (DPDPE).

6 The effect of morphine was totally abolished by prior and concomitant exposure to naloxone (100 nM) which had no effect on control release values.

7 We conclude that opioid receptors, predominantly of the μ-opioid receptor subtype, inhibit SP-LI release from PAN in the rat trachea and suggest that centrally inactive μ-opioid receptor agonists may have therapeutic potential in the treatment of asthma.

Keywords: Morphine; sodium cromoglycate; trachea substance P; primary afferent neurone

Introduction

In airways of mammals, including the rat, immunohistochemical and morphological studies have localised the undecapeptide substance P (SP), possibly coexisting with calcitonin gene-related peptide (CGRP), and the related tachykinins, neurokinin A (NKA) and neurokinin B (NKB), to capsaicin-sensitive vagal and non-vagal primary afferent neurones (PANs). These sensory fibres innervate both bronchial and vascular smooth muscle, submucosal glands and the respiratory epithelium (Lundberg *et al.*, 1984; Hua *et al.*, 1985; McDonald *et al.*, 1988).

Recent evidence suggests that airway irritation causes local defence reactions including bronchoconstriction, vasodilatation and an increase in vascular permeability (Lundberg & Saria, 1982a; 1983; Lundberg *et al.*, 1983a). Activation of local axon reflexes, with release of mediators including SP from peripheral endings of sensory fibres, contributes to these reactions (Lundberg *et al.*, 1985). Indeed it has been postulated that these axon reflexes may have relevance to the pathogenesis of asthma (Barnes, 1986).

Opioid receptors exist on small-diameter PANs in the spinal cord (LaMotte *et al.*, 1976). Autoradiographic studies have demonstrated opioid receptors on vagal PANs (Atweh *et al.*, 1978). Several studies have shown that opioids inhibit the release of SP from neurones *in vitro* (Jessell & Iversen, 1977; Lembeck & Donnerer, 1985) and *in vivo* (Yaksh *et al.*, 1980). Opioids have also been shown to modulate non-adrenergic, non-cholinergic (NANC) bronchoconstriction and plasma extravasation induced by antidromic stimulation of the vagus nerves in the guinea-pig *in vivo* (Belvisi *et al.*, 1988; 1989). These authors concluded that this effect may be mediated by presynaptic inhibition of SP release from the peripheral termini of vagal afferents.

Sodium cromoglycate (SCG) has been reported to depress the excitability of C-fibres in the canine lung *in vivo* (Dixon *et al.*, 1980). In addition, bradykinin, a known stimulant of vagal afferent C-fibres (Kaufman *et al.*, 1980), causes SCG-sensitive bronchoconstriction in man *in vivo* (Fuller *et al.*, 1987).

We have developed a multi-superfusion system to study the control of the release of SP-like immunoreactivity (LI) from rat trachea *in vitro* and have investigated the effect of morphine on potassium- and capsaicin-stimulated SP-LI release. In addition, the effect of SCG on potassium- and bradykinin-stimulated SP-LI release was studied.

Preliminary data have been presented to the British Pharmacological Society (Ray *et al.*, 1989).

Methods

Experimental protocol

Male albino rats (Wistar 350–400 g) were obtained from the departmental colony in Bristol. Animals were killed by a terminal injection of sodium pentobarbitone (100 mg kg^{-1}, i.p.). Eight or ten spirally cut tracheae (Constantine, 1965) were mounted in parallel surfasil (Pierce Chemical Co.)-coated glass chambers and simultaneously superfused (Watson-Marlow 202S/AA) at 37°C with oxygenated (95% O_2/5% CO_2) physiological salt solution (composition (mM): NaCl 120, KCl 4.7, $CaCl_2$ 2.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1) at 5 ml min^{-1} to allow equilibration. After at least 60 min

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bovine serum albumin (BSA; 1 mg ml^{-1}) and three peptidase inhibitors were added to the physiological salt solution and the flow rate adjusted to 1 ml min^{-1}. Peptidase inhibitors were phosphoramidon (1 μM), captopril (100 μM) and bacitracin (20 mg ml^{-1}) (Turner *et al.*, 1985). After a further 15 min four fractions of 5 min from each trachea were simultaneously collected in vials containing a final concentration of 0.1% trifluoroacetic acid (TFA), ethylenediaminetetracetic acid, disodium salt, dihydrate (EDTA, 1 mM), and dithiothreitol (DTT, 1 mM). Drugs were added to the superfusion fluid reservoir for 4 min during the third collection period. Where the KCl concentration was raised the NaCl concentration was correspondingly reduced to maintain the isotonicity of the superfusing medium. Some experiments were performed on trachea from rats which had been pretreated neonatally with capsaicin. Rats received capsaicin (50 mg kg^{-1}) subcutaneously 24 h and 48 h after birth. Control littermates received equal volumes of the vehicle which consisted of 10% ethanol: 10% Tween 80 (v/v) in 0.9% (w/v) saline.

Substance P-like immunoreactivity extraction and radioimmunoassay

Superfusates were applied to Sep-Pak C_{18} cartridges (Waters Associates) and washed with 0.1% TFA. Peptides were eluted with acetonitrile-0.1% TFA (80:20 v/v) and evaporated to 0.5 ml under nitrogen. The final SP-LI content of the trachea was extracted by plunging each trachea into a microcentrifuge tube containing 400 μl of boiling homogenizing medium (0.1% TFA, 1 mM EDTA, 1 mM DTT) for 10 min. The tubes were then transferred to ice and the samples homogenised in the tubes with a motor-driven Teflon pestle. A further 200 μl of homogenizing medium was added to wash the pestle. The samples were then centrifuged for 15 min at 8000 *g* and the supernatants decanted and retained for radioimmunoassay (RIA). Samples were then lyophilised and stored at −70°C. RIA of SP-LI was performed with an antiserum directed against the C-terminus of the peptide, as previously described (Harmar & Keen, 1986). None of the agents employed interfered with the RIA at the concentrations used.

Calculation of substance P-like immunoreactivity release

Changes in SP-LI release were calculated as the difference in fractional release constants (FRC) between the mean of fractions 1 and 2 and the mean of fractions 3 and 4 based on the recovery of exogenous SP (40 pg ml^{-1}) determined as 62.2 ± 3.2% (n = 12).

Statistical analysis

Results are expressed as means ± s.e.mean. Differences between means were analysed by Student's t test for unpaired data (two-tailed) or analysis of variance when appropriate. A P value of less than 0.05 was considered to be significant.

Drugs

Drugs and chemicals were obtained from the following sources: phosphoramidon, bacitracin, bovine serum albumin (BSA), DTT, capsaicin, SP, BK, des-Arg^9 BK, *trans*-3,4-dichloro-N-methyl-N(2-(1-pyrrolidinyl)cyclohexyl)-benzeneacetamine (U50,488H), Tyr-(D-Pen)-Gly-Phe-(D-Pen) (DPDPE), naloxone hydrochloride, cromolyn sodium, (Sigma Chemical Co., Poole, Dorset); EDTA, ethylene glycol *bis*-(2-aminoethyl) tetraacetic acid (EGTA; Fisons Ltd., Loughborough); captopril (E.R. Squibb & Sons, Inc., Princeton, U.S.A.), morphine hydrochloride (McFarlan Smith Ltd., Edinburgh) and sodium pentobarbitone (May & Baker Ltd., Dagenham).

Results

Effect of neonatal pretreatment with capsaisin

Neonatal administration of capsaicin caused a significant reduction in tracheal SP-LI content when extracted and assayed after the rats had attained 350 g body weight. SP-LI content of the trachea was reduced from 2125 ± 135 pg (n = 8) in vehicle-treated rats to 144 ± 17 pg (n = 8) in capsaicin-treated littermates representing a 93.2 ± 6.5% reduction.

Stimulation of SP-LI release

Capsaicin (100 nM–10 μM), KCl (37–90 mM), and BK (10 nM–1 μM), caused dose-related increases in SP-LI release above spontaneous values. Des-Arg^9 BK (1 μM) did not increase SP-LI release. Replacement of calcium ions by magnesium with addition of 1 mM EGTA did not affect spontaneous SP-LI release but caused a total abolition of the increase in release stimulated by 60 mM KCl, a 97.8 ± 21.0% reduction of that by 1 μM capsaicin and a 97.6 ± 23.7% reduction of that by 1 μM BK (see Table 1).

Effect of sodium cromoglycate on substance P-like immunoreactivity release

Tracheae were preincubated in SCG (1 μM and 100 μM) for 60 min prior to and during the equilibration period. SCG (1 μM and 100 μM) did not significantly affect spontaneous, 60 mM KCl or 1 μM BK stimulated SP-LI release when compared to control values (Table 2).

Effect of opioid agonists on KCl-stimulated substance P-like immunoreactivity release

Morphine (0.1–100 μM), when present 2 min prior to and during exposure to 60 mM KCl, produced dose-dependent inhibition of SP-LI release: at a dose of 3 μM the inhibition was 33.6 ± 11.2% (n = 10, $P < 0.01$), at 10 μM 49.3 ± 16.7% (n = 10, $P < 0.01$) and at 100 μM 60.4 ± 13.7% (n = 10, $P < 0.001$; Figure 1). Morphine (100 μM) did not inhibit SP-LI release by capsaicin (1 μM). If naloxone (100 nM) were present during the equilibration and stimulation periods then the inhibitory effect of morphine (30 μM) on KCl-stimulated SP-LI release was abolished (Figure 2). Naloxone (100 nM) alone did not affect SP-LI release. The κ-receptor agonist, U50,488H (10 μM) did not significantly alter 60 mM KCl-stimulated release. Results were: KCl-stimulated control (60 mM): 100.0 ± 9.0% (n = 14); U50,488H (10 μM): 111.2 ± 9.6% (n = 14; $P > 0.05$). In a series of experiments we examined the effect of the δ-receptor agonist DPDPE. The maximum dose which did not increase recovery of exogenous SP was 1 μM. This effect may reflect inhibition of neutral endopeptidase. At

Table 1 Effect of calcium-free medium on substance P-like immunoreactivity (SP-LI) release by (a) 60 mM KCl, (b) 1 μM capsaicin and (c) 1 μM bradykinin (BK)

	Calcium control	*Calcium-free*	*% inhibition*
(a) KCl 60 mM	100.0 ± 16.7 (8)	−3.9 ± 14.8 (8)	⩾100.0***
(b) Capsaicin 1 μM	100.0 ± 20.9 (10)	2.2 ± 1.6 (10)	97.8 ± 21.0***
(c) BK 1 μM	100.0 ± 22.3 (10)	2.4 ± 8.0 (10)	97.6 ± 23.7***

Release is expressed as a percentage of control for (n) preparations.
*** $P < 0.001$

Table 2 Effect of sodium cromoglycate (SCG) on (a) spontaneous, (b) 60 mM KCl and (c) 1 μM bradykinin (BK)-stimulated substance P-like immunoreactivity (SP-LI) release

	Spontaneous	*KCl*	*BK*
(1) Control	100.0 ± 6.6 (30)	100.0 ± 10.3 (15)	100.0 ± 12.0 (15)
SCG 1 μM	107.2 ± 7.5 (30)	97.5 ± 11.0 (15)	106.0 ± 16.2 (15)
(2) Control	100.0 ± 7.6 (30)	100.0 ± 10.9 (15)	100.0 ± 10.8 (15)
SCG 100 μM	104.8 ± 5.0 (30)	98.8 ± 14.6 (15)	95.9 ± 18.3 (15)

Release is expressed as a percentage of control.
Values are means ± s.e.mean of n animals.

this dose DPDPE did not affect SP-LI release by KCl. Results were: KCl-stimulated control (60 mM): 100.0 ± 10.3% (n = 10); DPDPE (1 μM): 99.3 ± 7.0% (n = 10; $P > 0.05$).

Discussion

We have investigated the release of SP-LI by a variety of neuronal stimulants in the superfused rat trachea. Treatment of newborn rats with capsaicin caused a greater than 90% reduction in tracheal SP-LI content. Capsaicin causes the selective destruction of chemosensitive C-fibre PANs under these conditions (Jancso *et al.*, 1977). This suggests that the great majority of tracheal SP-LI is localized to PANs, a finding that is consistent with those of Lundberg and associates who found that treatment of newborn rats with capsaicin abolishes vagally mediated neurogenic plasma extravasation in the trachea (Lundberg & Saria, 1982b) and causes an almost total loss of SP-immunoreactive fibres from the airways (Lundberg *et al.*, 1984).

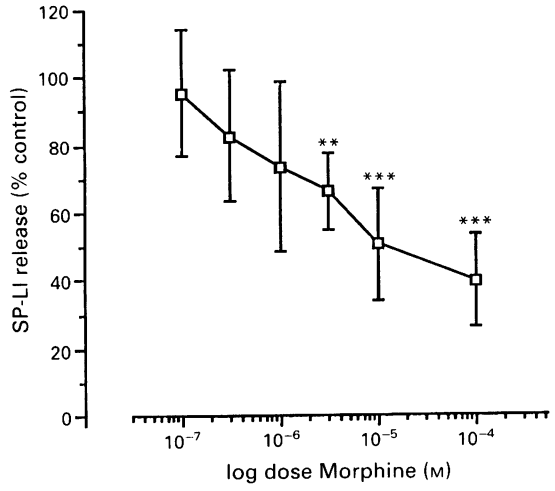

Figure 1 Effect of morphine on substance P-like immunoreactivity (SP-LI) release evoked by KCl 60 mM. Data are mean differences for 10 animals; vertical bars show s.e.mean. *** $P < 0.001$; ** $P < 0.01$.

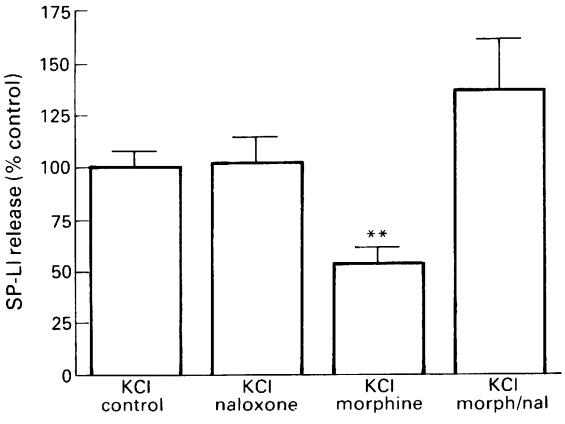

Figure 2 Effect of naloxone (100 nM) on morphine (30 μM)-sensitive substance P-like immunoreactivity (SP-LI) release evoked by KCl 60 mM. Data are means expressed as a percentage of 60 mM KCl-stimulated control; vertical bars show s.e.mean. ** $P < 0.01$ compared to control. n = 9 for control; n = 7 for naloxone; n = 8 for morphine; n = 6 for morphine (morph) plus naloxone (nal).

A markedly increased outflow of SP-LI was observed during exposure to KCl, capsaicin and BK. Removal of calcium ions from the superfusion medium abolished release to KCl and significantly reduced it to capsaicin and BK. The effect of BK was not mimicked by des-Arg^9-BK. BK receptors have two distinct populations, classified as B_1 and B_2 (Regoli & Barabé, 1980). BK is a potent activator of C-fibres (Kaufman *et al.*, 1980; Dray *et al.*, 1988) and stimulates SP-LI release from capsaicin-sensitive neurones in guinea-pig airways (Saria *et al.*, 1988). In our studies the BK B_1 receptor agonist, des-Arg^9-BK (Regoli & Barabé, 1980) was inactive suggesting that the BK receptor stimulating calcium-dependent SP-LI release is not of the B_1 subtype. This result agrees with the report that C-fibre activation by BK is mediated by the BK B_2 receptor (Dray *et al.*, 1988).

SCG has been reported to suppress C-fibre excitability in the canine lung *in vivo* (Dixon *et al.*, 1980) and to inhibit BK-stimulated bronchoconstriction in man (Fuller *et al.*, 1987). In both studies, subjects were exposed to SCG 10 min prior to challenge by capsaicin and BK respectively. Since BK is a known stimulant of vagal afferents (Kaufman *et al.*, 1980) we postulated that SCG might inhibit SP-LI release by BK from PANs in the rat trachea. However, we found no apparent changes in spontaneous, KCl- or BK-stimulated release of SP-LI after a prior exposure to SCG of 2 h. We conclude that it is possible that SCG can inhibit unmyelinated afferent pathways but that this effect does not manifest itself as a suppression of SP-LI release.

The neurotransmitter(s) mediating NANC neurogenic inflammatory reactions in the airways are currently unknown. Several neuropeptides, including SP, have been proposed as mediators of NANC neurogenic plasma extravasation, bronchoconstriction, vasodilatation and mucus secretion (Joos *et al.*, 1988). With regard to plasma extravasation, the most promising candidate is SP. SP is a powerful stimulant of plasma extravasation in rat airways *in vivo* (Lundberg & Saria, 1982b). Furthermore, vagally mediated NANC neurogenic plasma extravasation is abolished by capsaicin-pretreatment (Lundberg & Saria, 1982b) and inhibited by a SP receptor antagonist (Lundberg *et al.*, 1983b) although the latter observation is balanced by the finding that the SP receptor antagonist possesses potent local anaesthetic activity (Post *et al.*, 1985). In the present study, morphine inhibited 60 mM KCl-stimulated SP-LI release by a dose-related, naloxone-sensitive mechanism. Opioid receptors are known to be present on PANs in the spinal cord (LaMotte *et al.*, 1976) and vagus nerve (Atweh *et al.*, 1978). Opioids have been demonstrated to inhibit SP-LI release *in vitro* (Jessell & Iversen, 1977; Lembeck & Donnerer, 1985) and *in vivo* (Yaksh *et al.*, 1980). Furthermore, in guinea-pig airways, morphine inhibits NANC neurogenic plasma extravasation *in vivo* (Belvisi *et al.*, 1989). The receptor subtype mediating inhibition of SP-LI release is not known. Morphine is relatively selective for μ-opioid receptors but also stimulates δ-opioid

receptors at high doses (Paterson *et al.*, 1983). In our study, the effect of morphine was highly sensitive to antagonism by the selective μ-opioid receptor antagonist, naloxone (Paterson *et al.*, 1983). This finding, together with the fact that the κ-opioid receptor agonist, U50, 488H, and the δ-opioid receptor agonist, DPDPE, did not mimic the action of morphine suggests a predominant role for the μ-opioid receptor. Precise classification requires a highly selective full antagonist at the δ-opioid receptor. The usefulness of the best available, ICI 174,864, has been questioned because it exhibits agonist activity (Dray & Nunan, 1984).

Local or axon reflexes, with consequent release of inflammatory neuropeptides from sensory nerves have been implicated in the pathogenesis of asthma (Barnes, 1986). We suggest that opioid receptors, probably of the μ-opioid receptor subtype, inhibit SP-LI release from PANs in the rat trachea and that centrally inactive μ-opioid receptor agonists may have therapeutic potential in the treatment of asthma.

We are grateful to E.R. Squibb & Sons Inc. for the gift of captopril. N.J.R. is supported by the Medical Research Council.

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(Received February 22, 1990
Revised September 17, 1990
Accepted September 20, 1990)

Br. J. Pharmacol. (1991), **102**, 801–804

$GABA_B$ receptor modulation of the release of substance P from capsaicin-sensitive neurones in the rat trachea *in vitro*

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1 The role of γ-aminobutyric acid (GABA) as an inhibitory transmitter in the central nervous system is well documented. Recently, $GABA_A$ and $GABA_B$ receptors have been identified in the peripheral nervous system, notably on primary afferent neurones (PAN). We have utilised a multi-superfusion system to investigate the effect of selective GABA receptor agonists and antagonists on the release of substance P (SP) from the rat trachea *in vitro*.

2 GABA (1–100 μM) did not affect spontaneous release of SP-like immunoreactivity (LI) but caused dose-related inhibition of calcium-dependent potassium (60 mM)-stimulated SP-LI release. The greatest inhibition of 77.7 ± 18.8% was observed at 100 μM.

3 The inhibitory effect of GABA was mimicked by the $GABA_B$ receptor agonist, (±)-baclofen (1–100 μM), but not the $GABA_A$ receptor agonist, 3-amino-1-propane-sulphonic acid (3-APS, 1–100 μM). Baclofen (100 μM) had no effect on SP-LI release stimulated by capsaicin (1 μM).

4 The inhibitory effect of baclofen (30 μM) was significantly reduced by prior and concomitant exposure to the $GABA_B$ receptor antagonist, phacolofen (100 μM) but not the $GABA_A$ receptor antagonist, bicuculline (10 μM). Neither antagonist, alone, affected spontaneous or potassium-stimulated SP-LI release.

5 We conclude that activation of pre-synaptic $GABA_B$ receptors on the peripheral termini of PANs in the rat trachea inhibits SP-LI release and suggest that $GABA_B$ receptor agonists may be of value in the therapeutic treatment of asthma.

Keywords: GABA; $GABA_B$ receptor; trachea; substance P; primary afferent neurone

Introduction

The airways receive a dense sensory innervation (Lundberg *et al.*, 1984; Hua *et al.*, 1985; McDonald, 1988). Irritation of the airways causes local defence reactions, including brochoconstriction, vasodilatation and an increase in vascular permeability (Lundberg & Saria, 1982a; Lundberg & Saria, 1983). Activation of local axon reflexes, with release of mediators including substance P (SP) from peripheral endings of sensory fibres, contributes to these reactions (Lundberg *et al.*, 1983; 1985). Indeed it has been postulated that these axon reflexes may have relevance to the pathogenesis of asthma (Barnes, 1986). The role of γ-aminobutyric acid (GABA) as an inhibitory transmitter in the central nervous system (Fagg & Foster, 1983; Curtis & Johnston, 1974); has recently been complemented by the identification of GABA receptors on primary afferent neurones (PANs) of the peripheral nervous system (Dunlap, 1981; Price *et al.*, 1984; Desarmenien *et al.*, 1984). GABA receptors have been sub-classified as $GABA_A$ and $GABA_B$ (Bowery *et al.*, 1983), where $GABA_A$ receptors are bicuculline-sensitive and coupled to chloride channels and $GABA_B$ are bicuculline-insensitive and modulate calcium and potassium conductances (Dunlap & Fischbach, 1981; Gahwiler & Brown, 1985). With specific regard to PANs, $GABA_B$ receptors inhibit calcium channel currents, probably via a G protein(s), into cultured rat dorsal root ganglion (DRG) neurones (Dolphin & Scott, 1986; 1987) and inhibit substance P (SP) release from embryonic chick DRG cell cultures (Holz *et al.*, 1989). We have used a multi-superfusion system to study the control of SP-like immunoreactivity (LI) release from rat trachea and have investigated the effect of GABA receptor activation on spontaneous-, potassium- and capsaicin-stimulated SP-LI release using selective agonists and antagonists. Preliminary data have been presented to the British Pharmacological Society (Ray *et al.*, 1989).

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Methods

Experimental protocol

Male albino rats (Wistar 350–400 g) were obtained from the departmental colony in Bristol. Animals were killed by a terminal injection of sodium pentobarbitone (100 mg kg^{-1}, i.p.). Eight or ten spirally cut tracheae (Constantine, 1965) were mounted in parallel surfasil (Pierce Chemical Co.)-coated glass chambers and superfused (Watson-Marlow 202S/AA) at 37°C with oxygenated (95% O_2:5% CO_2) physiological salt solution (composition (mM): NaCl 120, KCl 4.7, $CaCl_2$ 2.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1) at 5 ml min^{-1} to allow equilibration. After at least 60 min, bovine serum albumin (BSA 1 mg ml^{-1}) and three peptidase inhibitors were added to the physiological salt solution and the flow rate adjusted to 1 ml min^{-1}. Peptidase inhibitors were phosphoramidon (1 μM), captopril (100 μM) and bacitracin (20 mg ml^{-1}; Turner *et al.*, 1985). After a further 15 min four fractions of 5 min from each trachea were simultaneously collected in vials containing a final concentration of 0.1% trifluoroacetic acid (TFA), ethylenediaminetetracetic acid, disodium salt, dihydrate (EDTA, 1 mM) and dithiothreitol (DTT, 1 mM). Drugs were added to the superfusion fluid reservoir for 4 min during the third fraction. Where the KCl concentration was raised the NaCl concentration was correspondingly reduced to maintain the isotonicity of the superfusing medium. GABA receptor agonists were present 1 min prior to and during exposure to stimulants of SP-LI release. GABA receptor antagonists were present throughout the equilibration and superfusion periods.

Substance P-like immunoreactivity extraction and radioimmunoassay

Superfusates were applied to Sep-Pak C_{18} cartridges (Waters Associates) and washed with 0.1% TFA. Peptides were eluted with acetonitrile-0.1% TFA (80:20 v/v) and evaporated to

0.5 ml under gaseous nitrogen. The final SP-LI content of the tracheae was extracted by plunging each trachea into a micro-centrifuge tube containing 400 μl of boiling homogenizing medium (0.1% TFA, 1 mM EDTA, 1 mM DTT) for 10 min. The tubes were then transferred to ice and the samples homogenized in the tubes with a motor-driven Teflon pestle. A further 200 μl of homogenizing medium was added to wash the pestle. The samples were then centrifuged for 15 min at 8000 g and the supernatants decanted and retained for radioimmunoassay (RIA). Samples were then lyophilised and stored at −70°C. RIA of SP-LI was performed with an antiserum directed against the C-terminus of the peptide, as previously described (Harmar & Keen, 1986). None of the agents employed interfered with the RIA at the concentrations used.

Calculation of substance P-like immunoreactivity release

Changes in SP-LI release were calculated as the difference in fractional release constants (FRC) between the mean of fractions one and two and the mean of fractions three and four based on the recovery of exogenous SP (40 pg ml^{-1}) determined as 62.2 ± 3.2% (n = 12).

Statistical analysis

Results are expressed as means ± s.e.mean. Differences between means were analysed by one-way analysis of variance. A P value of less than 0.05 was considered to be significant.

Drugs

Drugs and chemicals were obtained from the following sources: phosphoramidon, bacitracin, BSA, capsaicin, SP, GABA, (+)-bicuculline, 3-amino-1-propane-sulphonic acid (3-APS; Sigma Chemical Co., Poole, Dorset); captopril (E.R. Squibb & Sons Inc., Princeton, U.S.A.); EDTA, ethylene glycol *bis*-(2-aminoethyl) tetra-acetic acid (EGTA; Fisons Ltd., Loughborough); (±)-baclofen, phaclofen (Tocris, Essex); pentobarbitone (May & Baker, Dagenham).

Results

We have previously reported that neonatal treatments of rats with capsaicin (50 mg kg^{-1}, s.c.) causes a 93.2 ± 6.3% reduction in tracheal SP-LI content in the adult. Dose-related calcium-dependent increases in SP-LI release were detected during superfusion with KCl (37–90 mM) and capsaicin (100 nM–10 μM, Ray *et al.*, 1990).

Effect of GABA on spontaneous and potassium-stimulated substance P-like immunoreactivity release

GABA (1–100 μM) did not affect spontaneous release but, when present 1 min prior to and during exposure to 60 mM KCl, caused dose-related inhibition of SP-LI release: at 10 μM the inhibition was 30.8 ± 18.2% ($P > 0.05$, n = 8); at 30 μM 42.4 ± 19.7% ($P < 0.05$, n = 8); at 100 μM 77.7 ± 18.8% ($P < 0.001$, n = 8, Figure 1).

Effect of selective GABA receptor agonists on spontaneous and potassium-stimulated substance P-like immunoreactivity release

The effects of selective GABA receptor agonists were investigated. A $GABA_A$ receptor agonist, 3-APS and a $GABA_B$ receptor agonist, (±)-baclofen were employed. 3-APS (1–100 μM) had no significant effect on either spontaneous or potassium-stimulated SP-LI release (Figure 2) In contrast, (±)-baclofen (1–100 μM), whilst also not affecting spontaneous

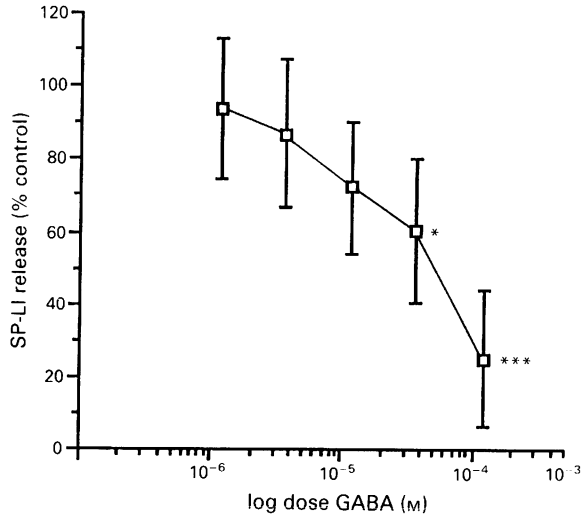

Figure 1 Effect of GABA on substance P-like immunoreactivity (SP-LI) release evoked by 60 mM KCl. Data are mean differences for 8 tracheae; vertical bars show s.e. (* $P < 0.05$; *** $P < 0.001$).

SP-LI release, mimicked the action of GABA in causing dose-related suppression of KCl-stimulated SP-LI release. At 10 μM the inhibition was 32.3 ± 15.6% ($P > 0.05$, n = 12); at 30 μM 51.8 ± 20.2% ($P < 0.05$, n = 12) and at 100 μM 52.9 ± 20.2% ($P < 0.05$, n = 12; Figure 2). Baclofen (100 μM) had no apparent effect on capsaicin (1 μM)-stimulated SP-LI release ($P > 0.05$, n = 12).

Effect of GABA receptor antagonists on baclofen-sensitive substance P-like immunoreactivity release

The effect of the $GABA_A$ receptor antagonist (+)-bicuculline, and the $GABA_B$ receptor antagonist, phacolfen, on baclofen suppression of 60 mM KCl-stimulated SP-LI release was investigated. Bicuculline (10 μM) when present throughout the equilibration and superfusion periods had no apparent effect on KCl-stimulated release. Furthermore, we were unable to detect significant antagonism of the inhibitory effect of baclofen (30 μM). Baclofen caused a 49.7 ± 12.4% inhibition of KCl-stimulated release, whereas, in the presence of bicuculline the reduction was 42.1 ± 12.0% (Figure 3). Phaclofen (100 μM)

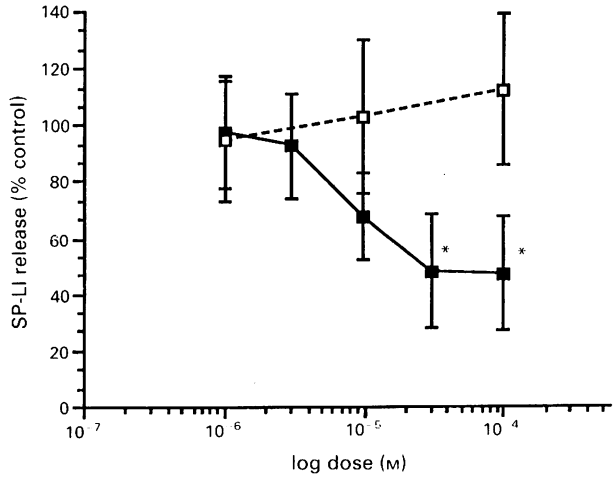

Figure 2 Effect of 3-amino-1-propane-sulphonic acid (3-APS, □) and (±)-baclofen (■) on substance P-like immunoreactivity (SP-LI) release evoked by 60 mM KCl. Data are mean differences for 8–12 tracheae; vertical bars show s.e. (* $P < 0.05$).

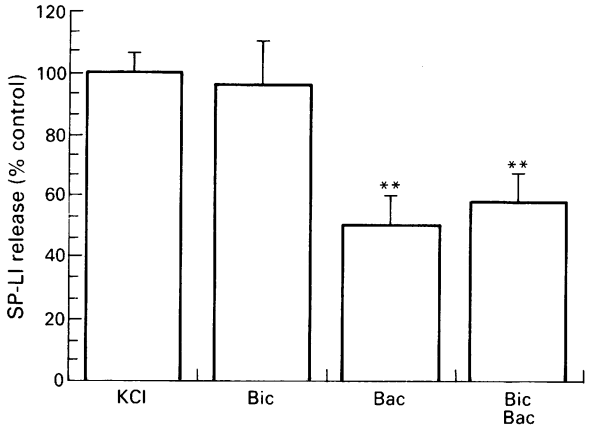

Figure 3 Effect of bicuculline (Bic; 10 μM) on baclofen (Bac; 30 μM)-sensitive substance P-like immunoreactivity (SP-LI) release stimulated by 60 mM KCl. Data are mean expressed as a percentage of 60 mM KCl-stimulated control; vertical bars show s.e. (* $P < 0.01$).

also had no effect on 60 mM KCl-stimulated release but, in contrast to bicuculline, significantly antagonized the suppression of release by baclofen (30 μM). In this experiment, baclofen caused a 64.5 $\pm$ 19.6% inhibition of KCl-stimulated release ($P < 0.01$, $n = 10$). In the presence of phaclofen, however, the degree of inhibition by baclofen was 22.5 $\pm$ 13.5% ($P > 0.05$, $n = 10$) (Figure 4).

Discussion

We have investigated the effect of GABA on SP-LI release from the superfused rat trachea *in vitro*. We have shown that GABA suppresses the increase in SP-LI release stimulated by isotonically elevated potassium ions.

Several studies with capsaicin, the pharmacological tool which when administered neonatally causes the selective destruction of chemosensitive C-fibre PANs (Jancso *et al.*, 1977), have provided considerable evidence that the vast majority of rat tracheal SP-LI content is localized to C-fibres. Neonatal treatment of rats with capsaicin prevents vagally mediated neurogenic plasma extravasation *in vivo* (Lundberg & Saria, 1982b), causes an almost complete loss of SP-immunoreactive fibres from the airways (Lundberg *et al.*, 1984) and reduces tracheal SP-LI content by at least 90% (Ray *et al.*, 1990).

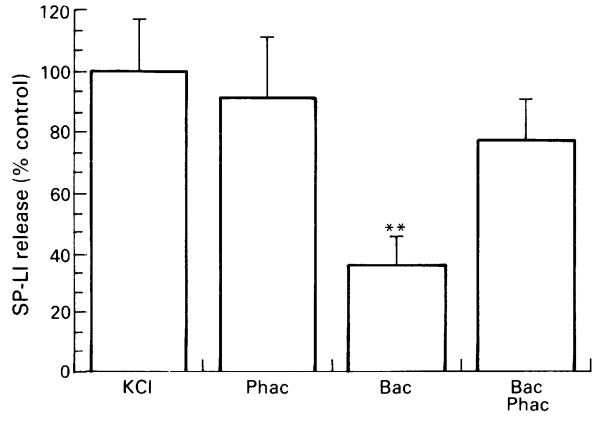

Figure 4 Effect of phaclofen (Phac; 100 μM) on baclofen (Bac; 30 μM)-sensitive substance P-like immunoreactivity (SP-LI) release stimulated by 60 mM KCl. Data are mean expressed as a percentage of 60 mM KCl-stimulated control; vertical bars show s.e. (* $P < 0.01$).

GABA receptors have been classified as bicuculline-sensitive ($GABA_A$) and bicuculline-insensitive ($GABA_B$) subtypes (Bowery *et al.*, 1983). We have pharmacologically characterized the GABA receptor mediating inhibition of KCl-stimulated SP-LI release using selective agonists and antagonists. The action of GABA was mimicked by the $GABA_B$ receptor agonist, baclofen, but not the $GABA_A$ receptor agonist, 3-APS. Furthermore, the effect of baclofen was unaffected by bicuculline but partially and significantly antagonized by the $GABA_B$ receptor antagonist, phaclofen (Kerr *et al.*, 1987). Since baclofen does not inhibit capsaicin-stimulated SP-LI release, we conclude that the suppression of release to KCl is via a relatively selective mechanism. We are unable to provide direct evidence for the precise localisation of the $GABA_B$ receptor identified. Autoradiographic studies have shown that GABA receptors of both subtypes are present on PAN terminals of the rat spinal cord (Price *et al.*, 1984). Desarmenien and associates have provided electrophysiological evidence that, in adult rat dorsal root ganglion (DRG) neurones, $GABA_A$ receptors mediate depolarization of C-fibres whereas $GABA_B$ receptor activation shortens the calcium component of action potentials (Desarmenien *et al.*, 1984). PANs are pseudo-unipolar neurones and have no known means of selectively transporting material synthesized in the cell body to the central or peripheral terminus. Indeed, in a previous study we have shown that approximately 75% of synthesized material is transported to the peripheral termini (Harmar & Keen, 1982). We therefore conclude that GABA receptors may also be present at the peripheral termini of PANs in the rat trachea and that the action of GABA and baclofen represents presynaptic inhibition of SP-LI release. Consistent with this hypothesis is the finding that GABA and baclofen inhibit electrically-stimulated SP-LI release from embryonic chick DRG neurons (Holz *et al.*, 1989). Furthermore, functional studies have shown that $GABA_B$ receptor activation inhibits vagally mediated non-adrenergic, non-cholinergic (NANC) neural bronchoconstriction in guinea-pig airways *in vivo* via a bicuculline-insensitive mechanism (Belvisi *et al.*, 1989). This action of GABA was also insensitive to naloxone or phentolamine, suggesting that an indirect action via release of endogenous opioids or catecholamines is unlikely (Belvisi *et al.*, 1989). The neurotransmitter(s) mediating NANC bronchoconstriction is unknown but SP and neurokinin A (NKA), released from sensory nerve endings, have been proposed as candidates (Barnes, 1986). Potassium-induced transmitter release is caused by a prolonged depolarization of the neuronal membrane. This initiates a brief phase of calcium influx through voltage-operated channels leading to a rise in intracellular calcium and consequent neurotransmitter release. We have previously demonstrated that KCl-stimulated release of SP-LI from the trachea is calcium-dependent (Ray *et al.*, 1990). $GABA_B$ receptors may be coupled to calcium and potassium conductances (Dunlap & Fishbach, 1981; Gahwiler & Brown, 1985). In hippocampal neurones, baclofen has been shown to hyperpolarize neurones by increasing an outward potassium conductance (Gahwiler & Brown, 1985). Such a mechanism could not be expected to operate during depolarization caused by the elevation of the extracellular potassium concentration to 60 mM. The most likely mechanism of action is therefore via modulation of calcium currents. With regard to PANs, $GABA_B$ receptor activation shortens the duration of calcium-dependent action potentials in adult rat DRG cell bodies *in vitro* (Desarmenien *et al.*, 1984) and reduces the peak amplitude of the slowly inactivating calcium current in isolated cat DRG neurones (Robertson & Taylor, 1986). In the rat, this mechanism is modulated by guanine nucleotides and may reflect coupling of the $GABA_B$ receptor to calcium channels by a G-protein(s) (Dolphin & Scott, 1987; Dolphin *et al.*, 1989). We postulate that, in the trachea, activation of $GABA_B$ receptors inhibits calcium influxes, rendering the ion less available for depolarization-secretion coupling, with consequent attenuation of SP-LI release to high potassium concentrations. A physiological role for tracheal $GABA_B$ receptors is

not known. However, GABAergic nerves are present in the peripheral nervous system, notably in the myenteric plexus of the gastrointestinal tract (GI) (Jessen *et al.*, 1986). Since the GI and respiratory tracts are derived from a common embryological origin it is plausible that a GABAergic innervation will be found in the airways. Local or axon reflexes, with consequent release of inflammatory neuropeptides, including SP, have been implicated in the aetiology of asthma (Barnes, 1986). It is possible that GABAergic innervation may have an endogenous neuromodulatory function in neurogenic inflammation. Irrespective of this hypothesis, $GABA_B$ receptor agonists may have therapeutic potential in the treatment of asthmatic disorders.

We are grateful to E.R. Squibb & Sons, Inc. for the gift of captopril. N.J.R. is supported by the Medical Research Council.

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(Received February 22, 1990
Revised September 17, 1990
Accepted September 19, 1990)

Br. J. Pharmacol. (1991), **102**, 805–810

Comparison of endothelium-dependent responses of monkey cerebral and temporal arteries

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1 Endothelium-dependency of vasodilator responses was compared in helical strips of monkey cerebral and superficial temporal arteries contracted with prostaglandin $F_{2\alpha}$. Acetylcholine produced an endothelium-dependent relaxation in the temporal arteries, but did not consistently alter the tone of cerebral arteries.

2 Adenosine 5'-triphosphate (ATP) produced a transient contraction followed by a relaxation in the temporal and cerebral arteries; removal of the endothelium partially attenuated the relaxation of the cerebral arteries and markedly suppressed the relaxation in the temporal arteries. The dependency of adenosine 5'-diphosphate (ADP)-induced relaxations on the endothelium was also greater in temporal arteries than in cerebral arteries.

3 Histamine-induced relaxations in the temporal arteries were independent of the endothelium and were reversed to contractions by cimetidine. Cerebral arterial relaxations induced by histamine were partly dependent on the endothelium. Relaxations caused by substance P were reversed to contractions by removal of the endothelium in the temporal arteries, whereas the peptide did not consistently alter the tone of cerebral arteries.

4 The Ca^{2+} ionophore, A23187, relaxed the temporal and cerebral arteries to a similar extent; removal of the endothelium abolished these relaxations. Glyceryl trinitrate elicited similar relaxations of cerebral and temporal arteries, and these were independent of the endothelium.

5 These findings clearly indicate heterogeneity in the endothelium-dependency of several vasodilator responses in monkey intra- and extracranial arteries, although the ability of these arteries to respond to A23187 and glyceryl trinitrate does not appear to differ. The heterogeneous responses observed so far could therefore be due to different distributions of receptors or to variation in receptor-effector coupling in endothelial cells.

Keywords: Monkey cerebral artery; monkey temporal artery; endothelium-dependent relaxation; acetylcholine; ATP; histamine

Introduction

In patients with unilateral vascular headache, increased heat loss from the affected frontotemporal region was thermographically observed during attacks which were relieved by compression of the superior temporal artery (Drummond & Lance, 1984). Drummond & Lance (1983) concluded that dilatation of the temporal arteries and their branches contributes substantially to migraine headache in the majority of migraine patients. On the other hand, migraine headache is considered to be associated with dilatation of extracerebral, cranial vasculature, and GR43175, a 5-hydroxytryptamine$_1$ (5-HT$_1$)-like receptor agonist (Humphrey *et al.*, 1988), aborts acute migraine attacks, possibly by its cerebral vasoconstrictor effect (Saxena & Ferrari, 1989). Endogenous vasodilator substances, such as prostaglandin I_2 (PGI_2), PGE_2 and histamine, may be involved in the vasodilatation responsible for migraine headache (Parantainen & Vapaatalo, 1983; Meyer *et al.*, 1987). Non-steroidal anti-inflammatory agents that inhibit cyclo-oxygenase activity are effective in preventing the attack, possibly by suppression of the production of prostaglandin (Diamond & Freitag, 1989). In any case, extra- and intracranial vasodilatation seems to play a crucial role in the genesis of the headaches. Since the discovery by Furchgott & Zawadzki (1980), the functional importance of endothelium-derived relaxing factor (EDRF) as a powerful, physiological vasodilator has been widely accepted. Thus, EDRF, together with the other endogenous vasodilators, may participate in the vasodilatation responsible for vascular headache. Histamine, adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP) locally released could produce localized vasodilatation that might participate in migraine headache. These substances dilate monkey and human coronary arteries (Toda, 1986; 1987) and dog arteries (De Mey & Vanhoutte, 1982) through the mediation of EDRF.

Chemical and physical stimulation of endothelium liberates EDRF in many vasculatures from experimental animals (Furchgott, 1983). Information on human cerebral arteries (Toda, 1977a; 1990b; Edvinsson *et al.*, 1987; Whalley *et al.*, 1987; Kanamaru *et al.*, 1989; Hatake *et al.*, 1990) is relatively scarce, because of a shortage of supply of fresh, non-pathological material. Responses to chemical stimuli of intracranial arteries frequently differ from those of extracranial arteries (Toda, 1977b; Owman *et al.*, 1978; Toda *et al.*, 1990). However, little information is available concerning the EDRF-releasing action of endogenous substances in primate cerebral arteries, as compared to that in extracranial arteries.

Therefore, the present study aimed to compare the responses to acetylcholine, ATP, ADP, histamine, substance P, glyceryl trinitrate and the Ca^{2+} ionophore, A23187, of cerebral and superficial temporal arteries obtained from Japanese monkeys, and to determine their endothelium-dependency. It has been observed that the magnitude and the mechanism of action of noradrenaline, dopamine, ouabain and histamine are quite similar in monkey and human cerebral arteries, whereas these clearly differ in primate and dog arteries (Toda, 1985; 1990b).

Methods

Japanese monkeys (*Macaca fuscata*) of either sex, weighing 6 to 10 kg, were anaesthetized with intramuscular injections of ketamine (40 mg kg^{-1}) and killed by bleeding from the carotid arteries. The brain was rapidly removed. Middle cerebral and basilar arteries (0.6 to 0.8 mm outside diameter) were isolated from the brain. The superficial temporal arteries (0.5 to 0.8 mm) were isolated. The arteries were cut helically into

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strips approximately 20 mm long, with special care being taken to avoid endothelial damage. The tissue was fixed vertically between two hooks in a muscle bath containing a modified Ringer-Locke solution, which was maintained at 37 ± 0.3°C and aerated with a mixture of 95% O_2 and 5% CO_2. The hook anchoring the upper end of the strip was connected to the lever of a force-displacement transducer (Nihon-Kohden Kogyo Co., Tokyo, Japan). The resting tension was adjusted to 1.0 g, which is optimal for inducing the maximal contraction. The composition of the bathing medium was as follows (mM): NaCl 120, KCl 5.4, $CaCl_2$ 2.2, $MgCl_2$ 1.0, $NaHCO_3$ 25.0 and dextrose 5.6. The pH of the solution was 7.35 to 7.42. All preparations were allowed to equilibrate for 60 to 90 min in the bathing medium, during which time the bath solution was replaced three times every 10 to 15 min.

Isometric contractions and relaxations were displayed on an ink-writing oscillograph (Nihon-Kohden Kogyo Co.). The contractile response to 30 mM K^+ was first obtained and the artery strips were repeatedly washed with fresh medium and re-equilibrated. The strips were partially contracted with $PGF_{2\alpha}$ (10^{-7} to 10^{-6} M), the contraction being in a range between 20 and 35% of the contraction induced by 30 mM K^+. Concentration-response curves for acetylcholine, histamine, ATP, ADP, Ca^{2+} ionophore and glyceryl trinitrate were obtained by addition of the agents directly to the bathing medium in cumulative concentrations. At the end of the experiment, papaverine (10^{-4} M) was applied to attain the maximal relaxation, which was taken as a standard for relaxation. Contractions induced by acetylcholine, ATP, histamine and Ca^{2+} ionophore were expressed as percentage values relative to the contraction caused by 30 mM K^+. In those experiments in which antagonists were used, the artery strips were treated for approximately 30 min, before the concentration-response curve for agonists was obtained. The endothelium of artery strips was removed by gently rubbing the intimal surface with a cotton pellet. Removal of the endothelium was determined by abolition or marked suppression of relaxations induced by the Ca^{2+} ionophore A23187 for the cerebral arteries and by acetylcholine and substance P for the temporal arteries, and was histologically confirmed by the silver staining procedure (Abrol et al., 1984). Paired comparisons of the responses were made in endothelium-intact and -denuded strips obtained from the same monkeys.

The results shown in the text and figures are expressed as the mean ± s.e.mean of n observations from different animals. Statistical analyses were made with Student's paired and unpaired t test and Tukey's method after one-way analysis of variance. Drugs used were acetylcholine chloride (Daiichi Seiyaku Co., Tokyo), histamine hydrochloride (Kanto Chemical Co., Tokyo), adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), Ca^{2+} ionophore A23187 (Boehringer Mannheim, FRG), glyceryl trinitrate (Millisrol 5 mg 10 ml^{-1}; Nihon Kayaku Co., Tokyo), substance P (Peptide Institute, Minoh, Japan), cimetidine (Fujisawa Pharmaceutical Co., Osaka, Japan), (+)-chlorpheniramine maleate (Schering Corp., Kenilworth, NJ, U.S.A.), indomethacin (Sigma, St. Louis, MO, U.S.A.), hexamethonium bromide (Yamanouchi Co., Tokyo, Japan), atropine sulphate (Tanabe Pharmaceutical Co., Osaka, Japan), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Ono Co., Osaka, Japan) and papaverine hydrochloride (Dainippon Pharmaceutical Co., Osaka). The solvent of glyceryl trinitrate in the amount used did not alter the tone of $PGF_{2\alpha}$-contracted arteries. Oxyhaemoglobin (OxyHb) was prepared by the method described by Martin et al. (1985). Dog Hb (Sigma) was reduced by $Na_2S_2O_4$, which was removed by extensive dialysis against distilled water.

Results

Effects of acetylcholine

In superficial temporal artery strips partially contracted with $PGF_{2\alpha}$, the addition of acetylcholine in concentrations ranging from 10^{-8} to 10^{-5} M produced a dose-related relaxation (Figure 1b); the mean value of the apparent median effective concentration (EC_{50}) was $3.31 \pm 0.66 \times 10^{-8}$ M ($n = 20$). The relaxation was abolished or markedly suppressed by removal of the endothelium. Typical responses are illustrated in Figure 2. The relaxation was also suppressed by treatment with 1.6×10^{-5} M OxyHb ($n = 3$). In cerebral artery strips, acetylcholine in concentrations up to 10^{-4} M did not consistently alter the tension; slight relaxations occurred in 4 out of 11 strips, slight contractions in the other 4 strips, and transient relaxations followed by sustained contractions were seen in the remaining 3 strips from separate monkeys. In each of the cerebral artery strips, endothelium integrity was determined from the relaxant response to the Ca^{2+} ionophore, A23187 (10^{-7} M). Average changes by acetylcholine in the tension of the stabilized level did not differ in the arteries with and without endothelium (Figure 1a). Transient relaxations induced by acetylcholine were not inhibited by removal of the endothelium or by treatment with hexamethonium (10^{-5} M) or

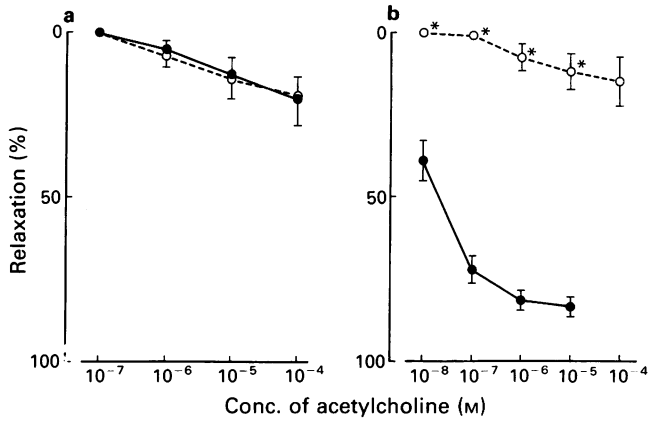

Figure 1 Concentration-relaxant response curves for acetylcholine in monkey cerebral (a) and temporal artery strips (b) with (●) and without the endothelium (○). The strips were partially contracted with prostaglandin $F_{2\alpha}$ (1 to 8×10^{-7} M). Relaxations induced by 10^{-4} M papaverine were taken as 100%; mean absolute values in cerebral arteries with and without the endothelium were 501 ± 101 mg and 465 ± 109 mg ($n = 10$), respectively, and those in temporal arteries were 410 ± 33 mg and 435 ± 44 mg ($n = 20$), respectively. Paired comparisons were made in the endothelium-intact and -denuded arteries from the same monkeys. Significantly different from the value in endothelium-intact arteries, * $P < 0.001$. Points indicate means and bars represent s.e.mean.

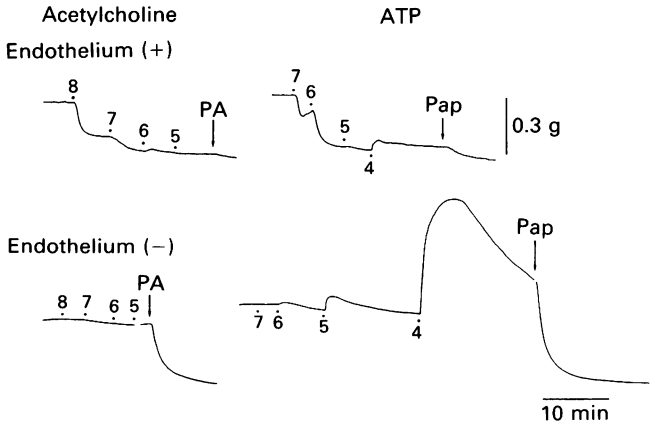

Figure 2 Typical recordings of the response to acetylcholine and ATP in temporal artery strips with and without the endothelium, obtained from the same monkey. The strips were partially contracted with prostaglandin $F_{2\alpha}$ (2×10^{-7} M). Concentrations of acetylcholine and ATP from 8 to 4 = 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M, respectively; PA = 10^{-4} M papaverine.

indomethacin (10^{-6} M), but were markedly attenuated by atropine (10^{-7} M). Sustained contractions in 3 strips were reduced by endothelium denudation.

Effects of ATP and ADP

In temporal arteries contracted with $PGF_{2\alpha}$, ATP (10^{-7} to 10^{-5} M) elicited a concentration-dependent relaxation which was frequently preceded by a transient contraction. The mean EC_{50} value for the relaxant effect of ATP was $6.85 \pm 1.89 \times 10^{-7}$ M ($n = 13$). The relaxation was markedly suppressed by removal of the endothelium (Figure 3b) or by treatment with 1.6×10^{-5} M OxyHb (from $54.9 \pm 6.4\%$ relaxation to $5.1 \pm 1.6\%$ contraction at 10^{-6} M ATP, $n = 7$, $P < 0.001$). Mean values of the transient contraction induced by 10^{-6}, 10^{-5} and 10^{-4} M ATP from the level of stabilized relaxation attained at a preceding dose were 1.5 ± 0.7, 6.9 ± 3.5 and $20.4 \pm 7.8\%$, respectively, relative to contractions caused by 30 mM K^+ in the strips with endothelium, and those were 2.7 ± 1.1, 9.8 ± 2.1 and $35.8 \pm 9.8\%$, respectively, in the arteries denuded of endothelium ($n = 10$). In the experiment shown in Figure 2, removal of the endothelium markedly potentiated the contractions. Such potentiation was seen in 4 out of 10 strips from different monkeys. In cerebral artery strips contracted with $PGF_{2\alpha}$, ATP also produced a transient contraction followed by a sustained relaxation; the EC_{50} value for relaxation average $5.98 \pm 1.07 \times 10^{-7}$ M ($n = 15$). The relaxation was significantly attenuated by removal of the endothelium (Figure 3a). Treatment with OxyHb (1.6×10^{-5} M) also attenuated the relaxation caused by 10^{-6} M ATP from $52.9 \pm 7.2\%$ to $23.5 \pm 6.1\%$ ($n = 8$, $P < 0.01$). Endothelial integrity was determined by the addition of 10^{-7} M A23187. Contractions induced by 10^{-6}, 10^{-5} and 10^{-4} M ATP (8.0 ± 1.6, 19.2 ± 4.8 and $69.2 \pm 14.0\%$, respectively, $n = 15$) were unaffected by removal of the endothelium (7.9 ± 1.6, 23.4 ± 5.9 and $63.9 \pm 12.6\%$, respectively, $n = 15$).

Temporal arteries responded to ADP (10^{-7} and 10^{-6} M) with dose-dependent relaxations. At 10^{-5} M, ADP produced a transient contraction followed by a relaxation. Removal of the endothelium markedly suppressed the relaxation (Figure 4b). In cerebral arteries, ADP (10^{-7} to 10^{-5} M) produced a concentration-related relaxation; at 10^{-4} M, transient contractions were followed by sustained relaxations. Relaxations induced by 10^{-7} and 10^{-6} M ADP were significantly attenuated by removal of the endothelium (Figure 4a). Attenuation of the response was greater in temporal arteries than in cerebral arteries.

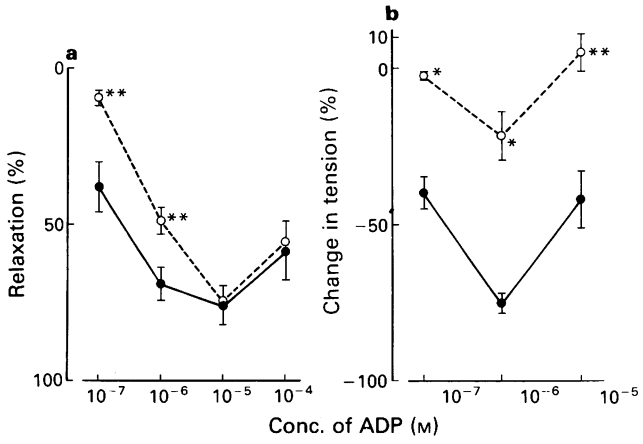

Figure 4 Concentration-relaxant response curves for ADP in monkey cerebral (a) and temporal artery strips (b) with (●) and without the endothelium (○). The strips were partially contracted with prostaglandin $F_{2\alpha}$ ($2–7 \times 10^{-7}$ M). Relaxations induced by 10^{-4} M papaverine were taken as 100%; mean absolute values in cerebral arteries with and without the endothelium were 459 ± 67 mg and 437 ± 71 mg ($n = 11$), respectively, and those in temporal arteries were 337 ± 24 mg and 320 ± 30 mg ($n = 7$), respectively. Contractions induced by 30 mM K^+ were taken as 100%; the mean absolute value in temporal arteries denuded of the endothelium was 1143 ± 182 mg ($n = 7$). Paired comparisons were made in the endothelium-intact and -denuded arteries from the same monkeys. Significantly different from the value in endothelium-intact arteries, * $P < 0.001$; ** $P < 0.01$. Points indicate means and bars represent s.e.means.

Effects of histamine and substance P

Histamine-induced relaxations in temporal artery strips were not reduced by removal of the endothelium (Figure 5b). The relaxations to histamine at concentrations of 10^{-7} to 2×10^{-6} M were abolished or reversed to contractions by treatment with 10^{-5} M cimetidine (Figure 6). The contraction

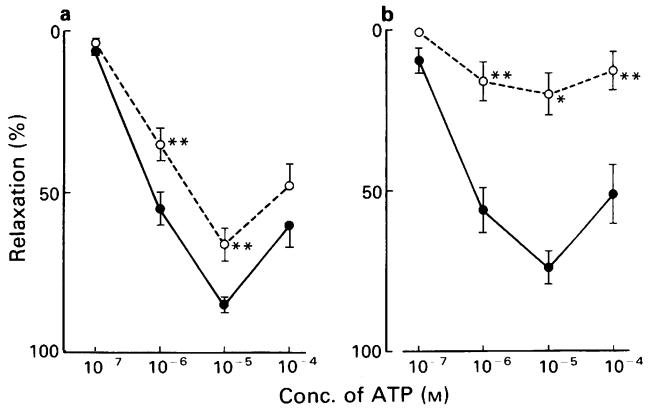

Figure 3 Concentration-relaxant response curves for ATP in monkey cerebral (a) and temporal artery strips (b) with (●) and without the endothelium (○). The strips were partially contracted with prostaglandin $F_{2\alpha}$ ($2–10 \times 10^{-7}$ M). Relaxations induced by 10^{-4} M papaverine were taken as 100%; mean absolute values in cerebral arteries with and without the endothelium were 338 ± 40 mg and 290 ± 42 mg ($n = 8$), respectively, and those in temporal arteries were 367 ± 41 mg and 355 ± 57 mg ($n = 13$), respectively. Paired comparisons were made in the endothelium-intact and -denuded arteries from the same monkeys. Significantly different from the value in endothelium-intact arteries, * $P < 0.001$; ** $P < 0.01$. Points indicate means and bars represent s.e.mean.

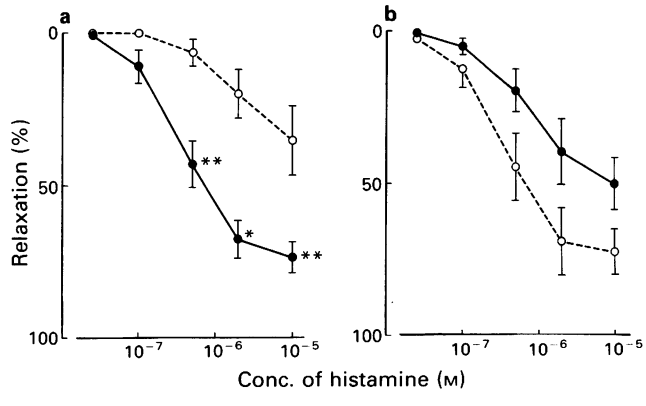

Figure 5 Concentration-relaxant response curves for histamine in monkey cerebral (a) and temporal artery strips (b) with (●) and without the endothelium (○). The strips were partially contracted with prostaglandin $F_{2\alpha}$ ($3–8 \times 10^{-7}$ M). Relaxations induced by 10^{-4} M papaverine were taken as 100%; mean absolute values in cerebral arteries with and without the endothelium were 406 ± 51 mg and 384 ± 57 mg ($n = 8$), respectively, and those in temporal arteries were 389 ± 42 mg and 392 ± 48 mg ($n = 11$), respectively. Paired comparisons were made in the endothelium-intact and -denuded arteries from the same monkeys. Significantly different from the value in endothelium-intact arteries, * $P < 0.001$; ** $P < 0.01$. Points indicate means and bars represent s.e.mean.

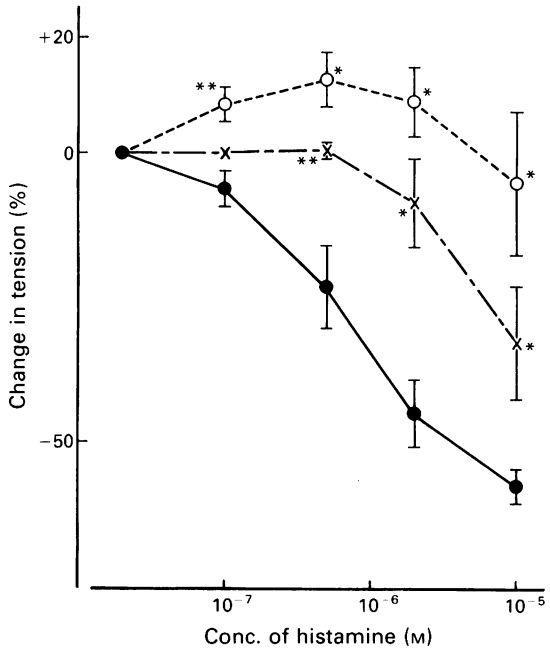

Figure 6 Modifications by cimetidine (○) and cimetidine plus chlorpheniramine (×) of the response to histamine in monkey temporal artery strips with the endothelium. The strips were partially contracted with prostaglandin $F_{2\alpha}$ ($3–8 \times 10^{-7}$ M). Relaxations induced by 10^{-4} M papaverine were taken as 100% relaxation; mean absolute values in control strips (●) and those treated with cimetidine (10^{-5} M) and cimetidine plus chlorpheniramine (10^{-6} M) were 337 ± 31 mg ($n = 6$), 303 ± 30 mg ($n = 6$) and 323 ± 107 mg ($n = 4$), respectively. Contractions induced by 30 mM K^+ were taken as 100% contraction; the mean absolute value in strips treated with cimetidine was 1710 ± 325 mg ($n = 6$). Significantly different from control, * $P < 0.01$; ** $P < 0.05$ (Tukey's method). Points indicate means and vertical bars represent s.e.mean.

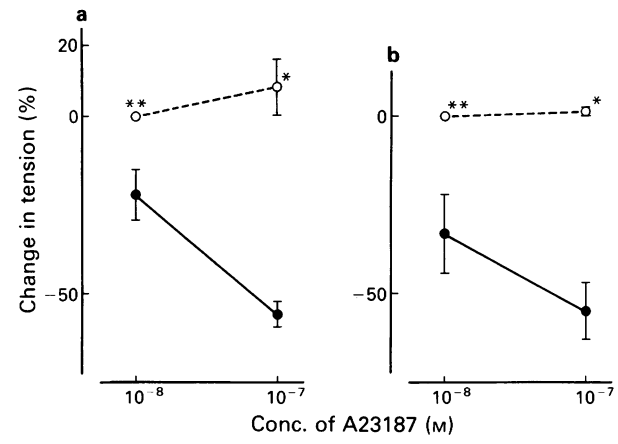

Figure 7 Responses to Ca^{2+} ionophore A23187 in monkey cerebral (a) and temporal artery strips (b) with (●) and without the endothelium (○). The strips were partially contracted with prostaglandin $F_{2\alpha}$ ($1–7 \times 10^{-7}$ M). Relaxations induced by 10^{-4} M papaverine were taken as 100% relaxation; mean absolute values in cerebral arteries with and without the endothelium were 399 ± 57 mg and 326 ± 49 mg ($n = 10$), respectively, and those in temporal arteries were 365 ± 77 mg and 384 ± 60 mg ($n = 11$), respectively. Contractions induced by 30 mM K^+ were taken as 100% contraction; mean absolute values in cerebral and temporal arteries without the endothelium were 798 ± 112 mg ($n = 4$) and 1276 ± 244 mg ($n = 3$), respectively. Paired comparisons were made in the endothelium-intact and -denuded arteries from the same monkeys. Significantly different from the value in the endothelium-intact arteries, * $P < 0.001$; ** $P < 0.01$. Points indicate means and bars represent s.e.mean.

was abolished by 10^{-6} M chlorpheniramine. Treatment with indomethacin (10^{-6} M) did not significantly alter the histamine-induced relaxation ($n = 4$). Monkey cerebral arteries responded to histamine with relaxations, which were greater than those seen in the temporal arteries (cf. control dose-response curves in (a) and (b) of Figure 5). The maximal relaxations at 10^{-5} M in the cerebral and temporal arteries were $73.5 \pm 5.1\%$ ($n = 8$) and $49.9 \pm 8.6\%$ ($n = 11$), respectively (significantly different, $P < 0.05$), and the EC_{50} values were $4.6 \pm 1.0 \times 10^{-7}$ M ($n = 8$) and $9.5 \pm 1.6 \times 10^{-7}$ M ($n = 11$), respectively ($P < 0.05$). The cerebral arterial relaxation was significantly reduced by removal of the endothelium (Figure 5a), as already demonstrated in an earlier report (Toda, 1990a).

Temporal artery strips responded to 10^{-7} M substance P with a moderate relaxation ($46.2 \pm 9.0\%$, $n = 6$), which was reversed to a contraction of $22.5 \pm 8.1\%$ ($n = 6$) by removal of the endothelium; the difference was statistically significant ($P < 0.001$). OxyHb (1.6×10^{-5} M) abolished the relaxation in 2 strips. In cerebral arteries, substance P did not consistently alter the tension; slight contraction in 6 strips, slight relaxation in 3 strips and no change in the other 2 in 11 strips from different monkeys, averaging a contraction of $5.2 \pm 3.9\%$.

Effects of Ca^{2+} ionophore A23187 and glyceryl trinitrate

The addition of A23187 in concentrations of 10^{-8} and 10^{-7} M elicited similar magnitudes of relaxation in the temporal and cerebral arteries, which was abolished or reversed to a contraction by removal of the endothelium (Figure 7). The A23187-induced relaxation was not affected by 10^{-6} M indomethacin ($n = 4$) but was abolished by 1.6×10^{-5} M OxyHb ($n = 5$) in these arteries. In contrast, relaxations caused by glyceryl trinitrate (10^{-9} to 10^{-6} M) in these arteries were not influenced by removal of the endothelium (Figure 8). EC_{50} values of glyceryl trinitrate in the temporal and cerebral arteries were $3.03 \pm 0.80 \times 10^{-9}$ M ($n = 9$) and $5.13 \pm 1.31 \times 10^{-9}$ M ($n = 10$), respectively, which were not significantly different.

Discussion

The present study revealed that responses to acetylcholine, ATP, ADP, histamine and substance P and their

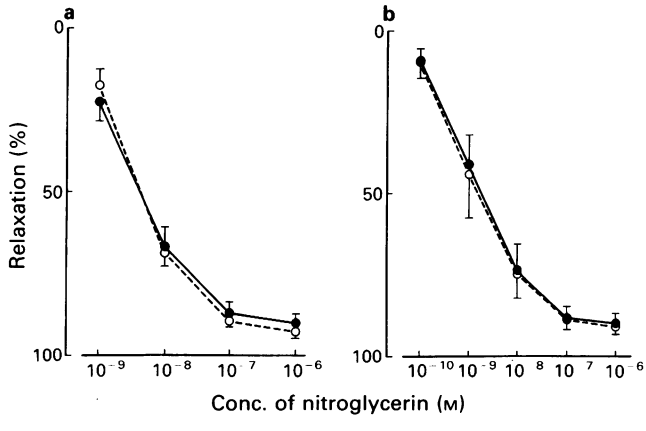

Figure 8 Concentration-relaxant response curves for glyceryl trinitrate in monkey cerebral (a) and temporal artery strips (b) with (●) and without the endothelium (○). The strips were partially contracted with prostaglandin $F_{2\alpha}$ ($3–9 \times 10^{-7}$ M). Relaxations induced by 10^{-4} M papaverine were taken as 100%; mean absolute values in cerebral arteries with and without the endothelium were 432 ± 70 mg and 382 ± 52 mg ($n = 11$), respectively, and those in temporal arteries were 367 ± 61 mg and 354 ± 39 mg ($n = 9$), respectively. Paired comparisons were made in the endothelium-intact and -denuded arteries obtained from the same monkeys. Points indicate means and bars represent s.e.mean.

endothelium-dependency markedly differed in temporal and cerebral arteries isolated from Japanese monkeys. In the temporal artery strips, acetylcholine produced a relaxation, which was suppressed by removal of the endothelium and by treatment with OxyHb, suggesting the involvement of an EDRF, as observed in many blood vessels from a variety of animals (Furchgott, 1983). In contrast, acetylcholine did not consistently alter the cerebral arterial tone. Dependence of the relaxant response to acetylcholine on the endothelium was not verified, although a component of the acetylcholine-induced contractions may be dependent on the endothelium, as suggested for the responses of monkey and dog cerebral arteries to angiotensin II, substance P, arachidonic acid, PGH_2, acetylcholine, stretch, etc. (Toda *et al.*, 1990; Onoue *et al.*, 1988; Fujiwara *et al.*, 1989; Katusic *et al.*, 1987).

Relaxations induced by ATP in temporal arteries were dependent on the endothelium and were suppressed by treatment with OxyHb, suggesting the involvement of an EDRF. Most published studies indicate endothelium-dependent relaxations by ATP in a variety of arteries from experimental animals (De Mey & Vanhoutte, 1982; Angus & Cocks, 1989) and from human pulmonary arteries (Greenberg *et al.*, 1987). However, in the present study, relaxations induced by ATP and ADP in monkey cerebral arteries were dependent only slightly on the endothelium, whereas those of temporal arteries markedly depended on the endothelium. The release of an EDRF by ATP from human umbilical vessels is reported to be much less than that by histamine and A23187 (Van de Voorde *et al.*, 1987). It appears that relaxations caused by ATP and ADP in monkey cerebral arteries are associated mainly with activation of purinoceptors (Houston *et al.*, 1987) in smooth muscle and only slightly with EDRF liberated by endothelial receptor activation. Contractions of the temporal and cerebral artery strips stimulated by ATP did not depend on endothelium, although contractions to some other agonists, such as angiotensin II and OxyHb, in monkey cerebral arteries are supposed to be mediated by constrictor substances released from endothelium (Toda *et al.*, 1990; Toda, 1990a).

Temporal artery relaxations caused by histamine were reversed to contractions by cimetidine, and the contraction was abolished by additional treatment with chlorpheniramine, suggesting that the relaxation is mediated by histamine H_2 receptors and the contraction by H_1 receptors. Whether the relaxations seen in the presence of cimetidine and chlorpheniramine are due to an incomplete inhibition of the receptors or to mechanisms other than those mediated by H_1 and H_2 receptors are not determined. Since removal of the endothelium did not inhibit the relaxation, the H_2 receptor is located in subendothelial tissues, possibly smooth muscle. On the other hand, monkey cerebral artery relaxations to histamine were significantly attenuated by removal of the endothelium, as are those in dog mesenteric arteries (Toda, 1984) and monkey and human cerebral and coronary arteries (Toda, 1986; 1987; 1990b). Detailed analyses in our laboratory of the mechanism of histamine actions in monkey cerebral arteries indicate that the relaxation is mediated via H_1 receptors located on endothelial cells and H_2 receptors on smooth muscle (Toda, 1990b). Relaxations by histamine were greater in the cerebral arteries than in the temporal arteries, and the magnitude of responses in endothelium-denuded cerebral arteries resembled that in the temporal arteries. The lesser degree of relaxation in the temporal arteries may therefore be associated with a lack of release of relaxing substance from endothelium. In contrast to monkey cerebral arteries, histamine produces a vasodilatation in cerebral arteries from subprimate mammals, such as cats and dogs, possibly by activation of H_2 receptors in smooth muscle (Edvinsson & Owman, 1975; Wahl & Kuschinsky, 1979; Toda *et al.*, 1984), as seen in monkey temporal arteries in the present study.

Monkey cerebral arteries did not consistently respond to substance P, and slight contraction and relaxation induced were independent of the endothelium, whereas relaxations by the peptide were dependent on the endothelium in temporal arteries, as are those in most blood vessels from different species (Angus & Cocks, 1989), including human cerebral (Toda, 1990b) and coronary arteries (Toda & Okamura, 1989).

Vascular headache may be associated with dilatation of cranial, extracerebral (Saxena & Ferrari, 1989) and extracranial arteries, such as the temporal artery (Drummond & Lance, 1983). In addition to PGI_2, substances liberating EDRF are expected to be involved in the genesis of the headache. The present study revealed that actions and mechanisms of action of the substances released possibly from mast cells (histamine), platelets (ADP) and nerves (acetylcholine, ATP and substance P) differed in monkey cerebral and temporal arteries. These findings may provide information for the detailed analysis of mechanisms underlying vascular headache in patients, as to whether EDRF as well as PGI_2 are involved in vascular headache, the different responsiveness participates in provoking the headache due to cranial or extracranial artery dilatation, and the arteries involved in the headache depending on the substances released.

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(Received September 3, 1990
Revised November 10, 1990
Accepted November 21, 1990)

Br. J. Pharmacol. (1991), **102**, 811–816

Investigation of the 5-hydroxytryptamine receptor mechanism mediating the short-circuit current response in rat colon

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1 5-Hydroxytryptamine (5-HT) stimulated an increase in short-circuit current (SCC) in rat isolated colonic mucosa with an EC_{50} value of approximately 4 μM. The purpose of the present study was to investigate the 5-HT receptor mechanism(s) involved in this response.

2 The relatively selective 5-HT receptor agonists 5-carboxamidotryptamine (5-CT) and α-methyl-5-HT stimulated SCC and were 6 to 8 times less potent than 5-HT. 2-Methyl-5-HT was inactive both as an agonist and an antagonist.

3 The following compounds produced no significant inhibition of the SCC response to 5-HT: ketanserin (1 μM), methysergide (1 μM), methiothepin (0.3 μM), GR38032F (0.3 μM), tetrodotoxin (0.3 μM) and sulpiride (1 μM).

4 Both metoclopramide (3 and 10 μM) and cisapride (0.1 and 1 μM) inhibited the SCC responses to 5-HT in a concentration-related manner, and the higher doses similarly inhibited the responses to 5-CT. With both agonists the inhibitory effects of metoclopramide and cisapride were insurmountable. However, these inhibitory actions appeared to be selective since neither metoclopramide nor cisapride affected the basal SCC or the SCC response to prostaglandin E_2.

5 The SCC responses to 5-HT and 5-methoxytryptamine were selectively inhibited by ICS205-930 at 3 μM, and respective pK_B values of 6.0 and 6.6 were calculated.

6 It is concluded that 5-HT stimulates an SCC response in rat colon via a receptor mechanism that cannot be clearly identified as 5-HT_1-like, 5-HT_2 or 5-HT_3. This receptor is selectively antagonized by ICS205-930 and by the benzamides, metoclopramide and cisapride. The 5-HT receptor in rat colon therefore exhibits some of the properties associated with the so-called 5-HT_4 receptor.

Keywords: Rat colon; secretion; 5-hydroxytryptamine receptor

Introduction

Large amounts of 5-hydroxytryptamine (5-HT) are present in the gastrointestinal tract (Thompson, 1971); most of this 5-HT is present in the enterochromaffin cells of the mucosa (Erspamer, 1954), although smaller amounts are found in the myenteric plexus (summarized by Branchek & Gershon, 1987). 5-HT is a potent secretagogue both *in vivo* (Donowitz *et al.*, 1977; Cassuto *et al.*, 1982) and *in vitro* (Cooke & Carey, 1985; Moriarty *et al.*, 1987), and is probably one of the endogenous secretagogues responsible for the diarrhoea in patients with carcinoid syndrome (Donowitz & Binder, 1975; Ahlman, 1985).

In guinea-pig ileum the short-circuit current (SCC; indicative of electrogenic chloride secretion) response induced by 5-HT is inhibited by relatively low concentrations of ICS205-930 in a manner consistent with the blockade of 5-HT_3 receptors (Baird & Cuthbert, 1987), and in rat ileum the SCC response to 5-HT is reported to be mediated by 5-HT_2 and/or 5-HT_3 receptors (Moriarty *et al.*, 1987; Ball *et al.*, 1988b). In the present study, experiments were carried out to determine the 5-HT receptor type mediating SCC in rat colon. This work was of particular interest to us since unlike the ileum of the guinea-pig and rat (see above), the 5-HT receptors mediating the increase in SCC in rat colon could not be clearly designated as 5-HT_1-like, 5-HT_2 or 5-HT_3 (for a summary of 5-HT receptor classification see Bradley *et al.*, 1986). A preliminary account of this work was presented to the British Pharmacological Society (Ball *et al.*, 1988a).

Methods

Experimental procedure

Male albino rats, weighing approximately 200 g, were killed by cervical dislocation and the descending colon removed. The colonic segment was opened along the mesenteric border, placed in oxygenated Krebs-Henseleit solution and pinned mucosa side downwards on a wax block. The overlying muscle layers were dissected away from the mucosa and two adjacent (paired) pieces of mucosa were used for each experiment. The mucosa was mounted in an Ussing chamber (window area 0.8 cm^2) and was prepared for measurement of short-circuit current (SCC) as described in detail elsewhere (Bunce & Spraggs, 1988). The mucosa was bathed on both sides with Krebs-Henseleit solution containing (in mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.8, KH_2PO_4 1.2, glucose 11.1; and gassed with 95% O_2/5% CO_2 and maintained at 37°C. The tissues were short-circuited by application of external current with compensation for fluid resistance, by use of a DVC amplifier (DVC-1000 World Precision Instruments, CT, USA) and the SCC was continuously recorded.

All drugs were added to both sides of the mucosa; this was done to facilitate equilibration. Antagonists were added 30 min before construction of an agonist concentration-response curve. All agonist concentration-response curves were constructed in a cumulative manner.

Compounds

The compounds used were: 5-hydroxytryptamine creatinine sulphate (5-HT; Sigma), 2-methyl-5-HT hydrochloride (Glaxo), α-methyl-5-HT maleate (Glaxo), 5-carboxamidotryptamine maleate (5-CT; Glaxo), GR38032F (1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1*H*-imidazol-1-yl)methyl]-4*H*-carbazol-4-one hydrochloride, $2H_2O$; Glaxo), ICS205-930 (3α-tropanyl-1H-indole- 3-carboxylic acid ester; Glaxo), ketanserin tartrate (Janssen), metoclopramide hydrochloride (Sigma), methysergide maleate (Sandoz), methiothepin maleate (Roche), tetrodotoxin (Sigma), sulpiride base (DEL Chemical Co.). Cisapride base was purchased from Janssen and prepared as the tartrate salt in our own laboratories. These compounds

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were dissolved directly in Krebs solution. ICS205-930 and sulpiride base were dissolved in 0.1 ml of 1 N HCl. Prostaglandin E_2 (Upjohn) was dissolved at 1 mg ml^{-1} in 1% w/v $NaHCO_3$ solution. Forskolin (Sigma) was dissolved at 1 mM in 50% ethanol solution. These latter compounds were then further diluted with Krebs solution as required. Control tissues received the appropriate solvent (Krebs, dilute HCl or dilute ethanol) and no changes in SCC were observed.

Expression of results

EC_{50} values were determined graphically. EC_{50} values and EC_{50} ratios were expressed as geometric mean with 95% confidence limits. Percentage inhibitions are expressed as mean ± s.e.mean. pK_B values were calculated from: $pK_B = \log_{10}$ (concentration-ratio − 1) − $\log_{10}$ (antagonist concentration).

Results

Basal conditions

If any tissue exhibited a steady basal SCC of less than 30 μA cm^{-2}, then that tissue pair (see below) was discarded. Based on this criterion, the basal SCC of the selected tissues was 51.4 ± 10.8 μA cm^{-2} (n = 8) (lumen side negative).

5-Hydroxytryptamine-induced secretion

Addition of a single dose of 5-HT to the preparation stimulated a rapid increase in SCC which exhibited fade, an example of which is shown in Figure 1a. Because of the nature of these responses it may appear preferable *prima facie* to have constructed sequential concentration-response curves with thorough washing between doses. However, such a protocol was unsuitable in this epithelial preparation since the repeated draining and re-addition of the bathing solution frequently caused disruption to the tissue so that it could not be voltage-clamped. It was therefore decided to construct cumulative concentration-response curves, an example of which is shown in Figure 1b; the next dose of agonist was added on obtaining

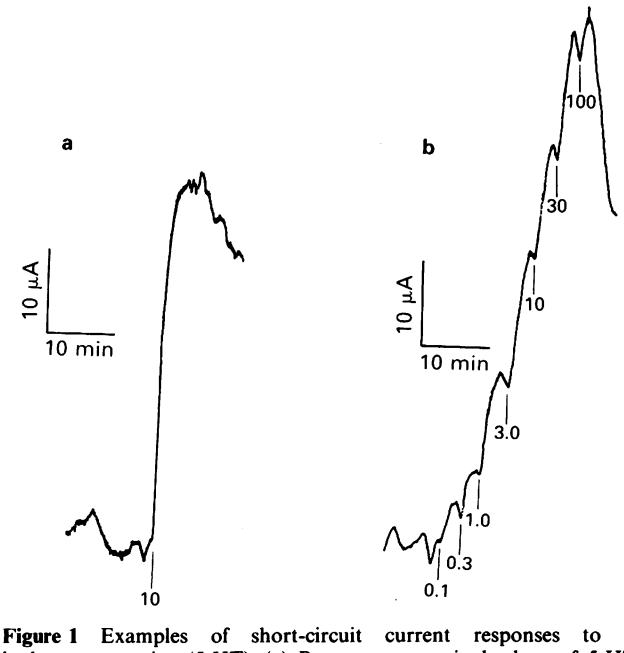

Figure 1 Examples of short-circuit current responses to 5-hydroxytryptamine (5-HT). (a) Response to a single dose of 5-HT, 10 μM. (b) Cumulative concentration-response curve to 5-HT; doses in μM.

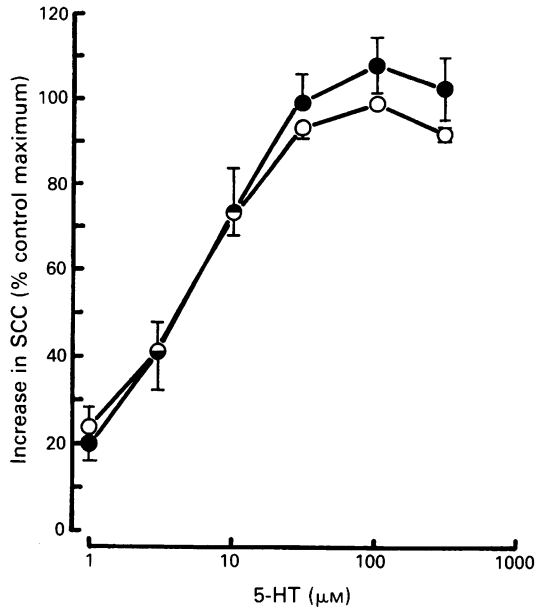

Figure 2 The short-circuit current (SCC) responses to 5-hydroxytryptamine (5-HT) in paired preparations of rat isolated colonic mucosa: (○) = first tissue; (●) = paired partner; tissues were dissected from adjacent portions of rat colon. Values are mean (n = 6 throughout) with s.e.mean shown by vertical bars. The maximum SCC response (100%) to 5-HT (first tissue) was 127.3 ± 11.3 μA cm^{-2}.

the maximum response to the previous dose. With this procedure, in an initial series of experiments 5-HT (0.1–100 μM) stimulated concentration-dependent increases in SCC with a maximum increase in current above basal of 181 ± 28 μA cm^{-2} (n = 8) at 100 μM. It was originally intended to construct repeated 5-HT concentration-response curves in each preparation but preliminary experiments showed that this was not possible since the maximum response to 5-HT in the second curve was only 66% of that in the first curve. Because of this problem all subsequent experiments were controlled by use of paired preparations taken from adjacent portions of descending colon, and the results are shown in Figure 2. With six paired sets of tissue the mean EC_{50} values for 5-HT were 3.9 (2.0–7.7) μM for the first tissue and 5.2 (2.2–11.9) μM for the paired partner ($P > 0.05$), with no significant difference in the maximum responses to 5-HT ($P > 0.05$). Although some desensitization occurred during the construction of the cumulative concentration-response curve, it was considered that the use of paired preparations provided the best protocol for the quantitative pharmacological analysis of agonist and antagonist activities. Thus the experimental design adopted was, (i) for agonist studies one tissue received 5-HT and the paired partner received the test agonist, (ii) for antagonist studies one tissue received agonist alone and the paired partner received agonist plus antagonist.

Agonists

Three agonists were tested, viz. 5-carboxamidotryptamine, α-methyl-5-HT and 2-methyl-5-HT which show some selectivity of 5-HT_1-like, 5-HT_2 and 5-HT_3 receptors respectively (Bradley *et al.*, 1986). The data are summarised in Table 1 with the results for the two active compounds, α-methyl-5-HT and 5-CT, being given in Figure 3. 2-Methyl-5-HT was inactive both as an agonist and an antagonist (against 5-HT) up to concentrations of 300 μM; 5-CT and α-methyl-5-HT were partial agonists and slightly less potent than 5-HT.

Antagonists

A number of antagonists were tested against the SCC response to 5-HT. Several of the compounds were inactive;

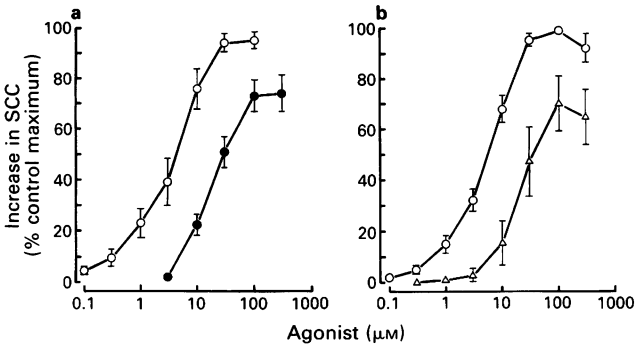

Figure 3 The short-circuit current (SCC) responses to 5-carboxamidotryptamine (5-CT) (a) and α-methyl-5-HT (b) in rat isolated colonic mucosa: (○) = 5-HT control; (●) = 5-CT, (△) = α-methyl-5-HT. The equipotent molar ratios calculated from these graphs are given in Table 1. Values are mean ($n = 4$ throughout) with s.e.mean shown by vertical bars. The maximum SCC responses (100%) to 5-HT were 166.6 ± 26.1 and 163.4 ± 17.4 μA cm^{-2} respectively for the 5-CT and α-methyl-5-HT experiments.

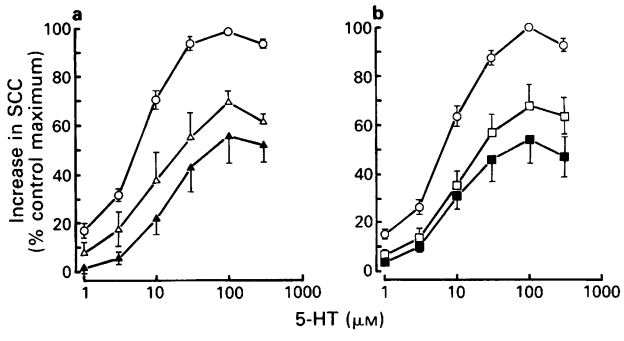

Figure 4 The effects of metoclopramide (a) and cisapride (b) on the short-circuit current (SCC) responses to 5-hydroxytryptamine (5-HT) in rat isolated colonic mucosa: (○) = 5-HT control; (△) = metoclopramide, 3 μM; (▲) = metoclopramide, 10 μM; (□) = cisapride, 0.1 μM; (■) = cisapride, 1 μM. Values are mean ($n = 4$–8) with s.e.mean shown by vertical bars. The maximum SCC responses (100%) to 5-HT were 146.2 ± 18.9 and 158.7 ± 13.5 μA cm^{-2} respectively for the metoclopramide and cisapride experiments.

these results were given in Table 2 and they show that high concentrations of ketanserin, methysergide, methiothepin, GR38032F, tetrodotoxin and sulpiride had no significant effect on the SCC response to 5-HT.

Two benzamide derivatives were identified which inhibited the SCC response to 5-HT, viz. metoclopramide and cisapride, and the results are given in Figure 4. These data show that both compounds produced a concentration-related but non-surmountable inhibition of the 5-HT-induced SCC response. The inhibitory effects of metoclopramide and cisapride appeared to be independent of the agonist used since at the higher concentrations tested (10 and 1 μM respectively) both compounds also produced a non-surmountable inhibition of the SCC response to 5-CT (Figure 5). Because of this pattern of inhibition the selectivity of the inhibitory effects of metoclopramide and cisapride was examined by determining the effects of the compounds on the increase in SCC induced by prostaglandin E_2 (PGE_2). The results are given in Figure 6 and they show that metoclopramide (10 μM) and cisapride (1 μM) failed to inhibit the response to PGE_2. Further evidence that metoclopramide and cisapride exhibited selective antagonism is derived from the observation that neither compound (at 10 and 1 μM respectively) affected (stimulation or inhibition) the basal SCC in rat colon.

In addition to the benzamides, the inhibitory action of ICS205-930 was also tested, and the result is given in Figure 7. Doses of ICS205-930 of 3' and 10 μM produced a concentration-related inhibition of the SCC response to 5-HT. At 3 μM ICS205-930 produced a significant ($P < 0.05$) inhibition of the 5-HT response [EC_{50} values of 5.0 (4.0–6.2) and 20.3 (11.2–36.8)] with a concentration-ratio of 4.1 (2.4–7.0) from which a pK_B value of 6.0 (5.7–6.3) was calculated. The

Table 1 The effect of agonists on the short-circuit current response in rat isolated colonic mucosa

Compound	*Equipotent molar ratio*‡	*% 5-HT maximum*†
5-Hydroxytryptamine	1	100
2-methyl-5-HT	*NSE up to 300 μM	—
α-methyl-5-HT	6.6 (1.3–33.0)	70 ± 11
5-Carboxamidotryptamine	8.2 (2.3–29.7)	74 ± 7

* NSE = no significant effect as an agonist or antagonist (vs. 5-HT).
‡ Geometric mean, 95% confidence limits.
† Arithmetic mean ± s.e.mean ($n = 4$ throughout).

Table 2 5-Hydroxytryptamine (5-HT)-induced secretion in rat isolated colonic mucosa: a summary of inactive antagonist compounds

Compound (μM)	*Receptor*	**5-HT EC_{50}* (μM) (Control)	**5-HT EC_{50}* (μM) (plus antagonist)	*EC_{50} ratio*	*% 5-HT maximum*‡ (plus antagonist)
Ketanserin (1.0)	5-HT_2	4.8 (3.2,7.1)	6.7 (3.0,15.1)	1.4	88 ± 11
Methysergide (1.0)	5-HT_1-like 5-HT_2	5.6 (3.8,8.4)	7.4 (4.1,13.2)	1.3	121 ± 16
Methiothepin (0.3)	5-HT_1-like 5-HT_2	5.4 (3.2,8.7)	14.5 (2.8,75.8)	2.7	95 ± 16
GR38032F (0.3)	5-HT_3	3.3 (2.3,4.8)	5.4 (3.9,7.4)	1.6	97 ± 14
Tetrodotoxin (0.3)	Neuronal blocker	3.9 (2.1,7.4)	8.6 (3.4,21.4)	2.2	97 ± 12
Sulpiride (1.0)	Dopamine D_2	6.6 (3.9,10.9)	11.6 (4.0,33.3)	1.8	79 ± 8

* Geometric mean, 95% confidence limits. Log_{10} EC_{50} values were compared by an unpaired t test. None of the antagonists tested produced a significant shift ($P > 0.05$) of the control 5-HT curve.
‡ Arithmetic mean ± s.e.mean. $n = 4$–7.

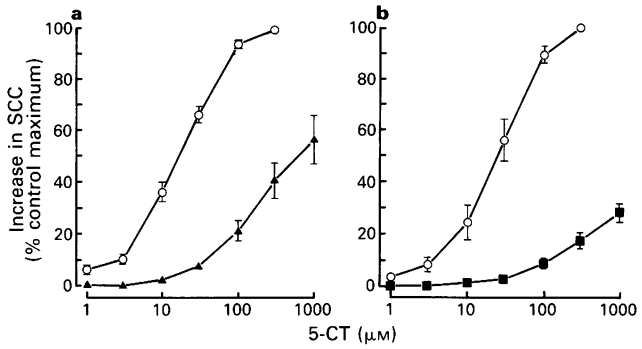

Figure 5 The effects of metoclopramide (a) and cisapride (b) on the short-circuit current (SCC) responses to 5-carboxamidotryptamine (5-CT) in rat isolated colonic mucosa: (○) = 5-CT control; (▲) = metoclopramide, 10 μM; (■) = cisapride, 1 μM. Values are mean (n = 4 throughout) with s.e.mean shown by vertical bars. The maximum SCC responses (100%) to 5-CT were 120.6 ± 17.4 and 119.7 ± 22.2 μA cm^{-2} respectively for the metoclopramide and cisapride experiments.

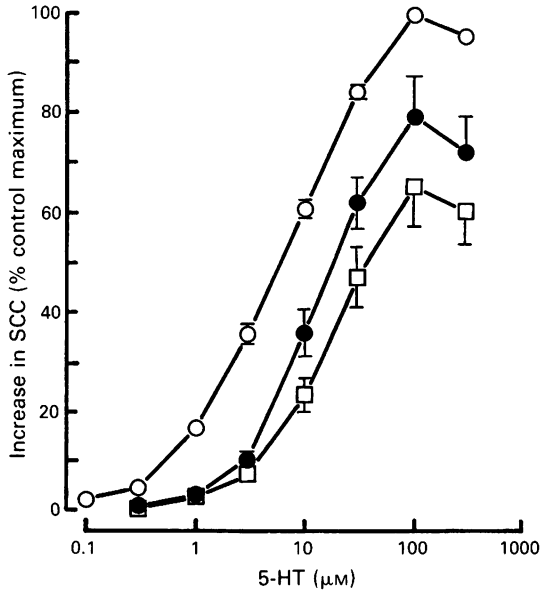

Figure 7 The effect of ICS205-930 on the short-circuit current (SCC) responses to 5-hydroxytryptamine (5-HT) in rat isolated colonic mucosa: (○) = 5-HT control; (●) = ICS205-930, 3 μM; (□) = ICS205-930, 10 μM. Values are mean (n = 4–7) with s.e.mean shown by vertical bars. The maximum SCC response (100%) to 5-HT was 176.1 ± 18.4 μA cm^{-2}.

inhibitory effect of ICS205-930 appeared to be agonist-independent since a concentration of 3 μM also antagonized the SCC responses to 5-methoxytryptamine (0.3–300 μM), a relatively non-selective agonist, with a concentration ratio of 14.5 (13.8–15.2) from which a pK_B value of 6.6 (6.5–6.8) was calculated. At 3 μM ICS205-930 was selective since it did not affect the SCC response to forskolin (0.01–3 μM) with a concentration-ratio of 1.7 (1.1–2.6). The effect of ICS205-930 at 10 μM was not analysed further since this concentration was non-selective and inhibited the SCC response to forskolin (0.01–3 μM) with a concentration-ratio of 6.4 (1.8–23.4) and a depression of the maximum response of 27.4 ± 11.6%.

Discussion

The present study confirms previous observations that 5-HT is a secretagogue in rat isolated colon (Zimmerman & Binder, 1984). It was clearly shown by Zimmerman & Binder (1984) that the increase in SCC in this tissue caused by 5-HT was due entirely to stimulation of electrogenic chloride secretion and this aspect of the work was not pursued further. Rather, the main purpose of this study was to investigate the receptor type mediating the secretory response to 5-HT in rat colon. This was first done by comparing the rank order of agonist potencies. In this context it is important to point out that ideally, if agonist relative potencies are to be compared, then these agonists should stimulate the same maximum responses (Furchgott, 1972). However, in the present study both α-methyl-5-HT and 5-CT stimulated maximum responses which were approximately 70% of that produced by 5-HT (Figure 3). With this reservation in mind the rank order of agonist potencies have been compared, and this is shown in Table 3. In rat colon the observation that 5-CT and α-methyl-5-HT were agonists may suggest that the SCC responses to these compounds, and by inference to 5-HT also, involved 5-HT_1-like and/or 5-HT_2 receptors. However, other results obtained make this suggestion unlikely. Firstly, the observation that 5-HT > 5-CT suggests that 5-HT_1-like receptors were not involved, and this contention is supported by the failure of the antagonists methysergide and methiothepin to inhibit significantly 5-HT-induced secretion. Secondly, although the result in rat colon that 5-HT > α-methyl-5-HT = 5-CT may be consistent with the presence of 5-HT_2 receptors (see Humphrey, 1984), this deduction is not supported by the failure of ketanserin, methysergide or methiothepin to inhibit the response to 5-HT. Thus, the overall body of available data indicates that neither 5-HT_1-like nor 5-HT_2 receptors play a principal role in the SCC response to 5-HT, although because of the agonist responses to 5-CT and α-methyl-5-HT we cannot exclude the possibility that small populations of these latter receptors made a minor contribution to the SCC responses in these studies. In addition, the observation that high concentrations of 2-methyl-5-HT were inactive as an agonist (and as an antagonist) suggests that neuronal 5-HT_3 receptors were not involved and this conclusion is corroborated by the lack of effect of the 5-HT_3 receptor antagonist, GR38032F, and tetrodotoxin on the response to 5-HT. Thus in the present study the receptors mediating the secretory response to 5-HT in rat colon could not be clearly designated 5-HT_1-like, 5-HT_2 or 5-HT_3. This latter result contrasts with studies in other secretory tissues; for example, in the ileum of the guinea-pig and rat at least part of the response to 5-HT appears to be mediated by 5-HT_3 receptors as indicated by the antagonism of GR38032F or relatively low concentrations of ICS205-930 (Baird & Cuthbert, 1987; Ball *et al.*, 1988b). It is interesting to note that although

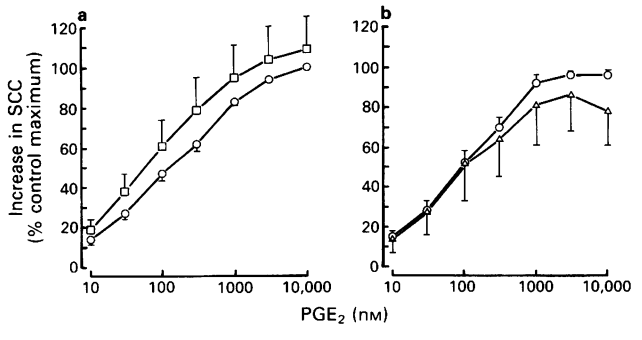

Figure 6 The effects of metoclopramide (a) and cisapride (b) on the short-circuit current (SCC) responses to prostaglandin E_2 (PGE_2) in rat isolated colonic mucosa. (○) = PGE_2 control; (□) = metoclopramide, 10 μM; (△) = cisapride, 1 μM. Values are mean (n = 6–8) with s.e.mean shown by vertical bars. The maximum SCC responses (100%) to PGE_2 were 85.9 ± 17.9 and 87.7 ± 11.0 μA cm^{-2} respectively for the metoclopramide and cisapride experiments.

Table 3 A comparison of the rank orders of agonist potencies at 5-hydroxytryptamine (5-HT) receptors

Tissue/receptor	*Agonist rank order*
†Rat colonic mucosa (this study)	5-HT (1) > α-methyl-5-HT (6.6) = 5-CT (8.8) > 2-methyl-5-HT (>77)
*5-HT_1-like	5-CT > 5-HT
*5-HT_2	5-HT > α-methyl-5-HT

† Results are equipotent molar ratios (5-HT = 1).
* Data are from Humphrey (1984) and Bradley *et al.* (1986).

the 5-HT receptor mechanism identified in the present study appears to be located on enterocytes, as opposed to enteric neurones, Gaginella *et al.* (1983) failed to identify 5-HT binding sites on plasma membranes prepared from rat colonic epithelial cells; this point remains to be resolved.

In the present work cisapride inhibited 5-HT-induced secretion in rat colon but, as discussed above, the 5-HT receptor type involved could not be identified. Allbee & Gaginella (1985) have also described the inhibitory action of cisapride against 5-HT in rat colon and attributed this to antagonism of 5-HT_2 receptors, although clearly this suggestion was not substantiated by our own work. Cisapride has also been reported to inhibit the SCC response to 5-HT in the ileum of the rat and guinea-pig (Cooke & Carey, 1985; Moriarty *et al.*, 1987). However, in these latter tissues ICS205-930 or GR38032F inhibited the 5-HT-induced SCC at doses that would be expected to be selective for 5-HT_3 receptors (Baird & Cuthbert, 1987; Ball *et al.*, 1988b), and since cisapride is a 5-HT_3 receptor antagonist (Nelson & Thomas, 1989) it is likely that cisapride inhibited such responses at least in part by antagonism of 5-HT_3 receptors. Unlike the ileum of the rat and guinea-pig, 5-HT_3 receptors were not involved in the SCC response to 5-HT in rat colon (see above), although both cisapride and metoclopramide still inhibited 5-HT-induced secretion in rat colon in a concentration-dependent manner. In each case, the antagonism was non-competitive with depression of the maximum responses, and these effects appeared to be agonist-independent (5-HT and 5-CT) and selective since neither of these benzamides affected the basal SCC or the SCC response to an E prostaglandin which, like the response to 5-HT, is due to electrogenic chloride secretion (Racusen & Binder, 1980). Similarly, experiments in rat ileum have also shown that cisapride does not affect epithelial ion transport in a non-selective manner (Moriarty *et al.*, 1987). Although the inhibitory action of cisapride against 5-HT in rat colon has been reported previously (Allbee & Gaginella, 1985) as far as the authors are aware, this is the first report of such an effect of metoclopramide.

The receptor type mediating the SCC response to 5-HT in rat colon was further examined by use of ICS205-930. At a concentration of 3 μM, ICS205-930 produced a small rightward shift of the agonist concentration-response curve from which a pK_B value of 6.0–6.6 was calculated, which is consistent with the presence of the so-called 5-HT_4 receptor (Clarke *et al.*, 1989). At this concentration (3 μM) the antagonism of the SCC responses by ICS205-930 was agonist independent (vs. 5-HT and 5-methoxytryptamine) and selective (no effect against forskolin). However, the data generated with ICS205-930 are tentative because of the small shift produced with a single concentration of compound (3 μM). In addition, at 3 μM ICS205-930 did cause a small depression of the 5-HT maximum (Figure 7) which may be attributable to some effect of ICS205-930 at high concentration on ion conductances (Scholtysik, 1987). Nevertheless, since the latter concentration of ICS205-930 did not affect the SCC response to forskolin it appeared selective and was therefore used for the calculation of pK_B values. However, at 10 μM ICS205-930 was clearly non-selective, presumably because of large effects on ion conductances and was not used for quantitative analysis.

Since metoclopramide is an antagonist at dopamine D_2 receptors (for example, Alphin *et al.*, 1986) the possible role of these receptors in the secretory response to 5-HT was determined. However, the benzamide dopamine D_2 receptor antagonist, sulpiride (Alphin *et al.*, 1986) had no effect on the SCC response to 5-HT (Table 2), and this result is corroborated by the observation that cisapride has a low affinity for dopamine D_2 receptors (Megens *et al.*, 1986) while being more potent than metoclopramide as an inhibitor of secretion in rat colon. At high concentrations, 5-HT is reported to be an agonist at α_1-adrenoceptors (Apperley *et al.*, 1976), but it is unlikely that 5-HT was stimulating secretion through such a mechanism in this study since ketanserin, an antagonist at α_1-adrenoceptors as well as 5-HT_2 receptors (Feniuk *et al.*, 1985), was without effect (Table 2).

Although both metoclopramide and cisapride inhibited the SCC response to 5-HT, it can be seen, particularly for cisapride (Figure 3) that increasing the concentration of antagonist (from 0.1 to 1 μM for cisapride) did not produce a substantially greater inhibitory effect. This result may indicate that part of the secretory response to 5-HT was resistant to metoclopramide and cisapride.

Beubler *et al.* (1990) have reported that in rat ileum cisapride inhibited the 5-HT-induced increase in potential difference (electrogenic chloride secretion) without affecting the accompanying inhibition of electroneutral NaCl absorption. In contrast, ketanserin inhibited the changes in NaCl absorption without affecting PD. Thus it is possible that in rat colon different 5-HT receptors control secretory and absorptive processes; in the present study the results obtained measuring SCC relate only to 5-HT and electrogenic chloride secretion.

In conclusion, 5-HT stimulates an SCC response in rat colon via a receptor mechanism that cannot clearly be identified as 5-HT_1-like, 5-HT_2 or 5-HT_3. This receptor is antagonized by the substituted benzamides, cisapride and metoclopramide in a selective but non-surmountable manner, and also by ICS205-930 with a pK_B value of 6.0–6.6. A receptor which cannot be designated 5-HT_1-like, 5-HT_2 or 5-HT_3 and is sensitive to the benzamides and high concentrations of ICS205-930 has been identified in other tissues, namely mouse colliculi neurones (Dumuis *et al.*, 1989), guinea-pig hippocampus (Bockaert *et al.*, 1990) and guinea-pig ileum (Craig & Clarke, 1990; Eglen *et al.*, 1990) and tentatively termed 5-HT_4 (Clarke *et al.*, 1989). However, some discrepant observations have been made. For example, cisapride is a potent full agonist in the mouse colliculi (Dumuis *et al.*, 1989), a relatively weak agonist in guinea-pig ileum (Craig & Clarke, 1990), a very weak partial agonist in guinea-pig hippocampus (Bockaert *et al.*, 1990) and an antagonist in rat colon mucosa (present study). The reason for these different results is not entirely clear, but may be attributable to the fact that cisapride is an agonist of low efficacy whose agonist profile is determined by tissue parameters, viz. receptor number and/or efficacy of stimulus-response coupling (see Kenakin, 1984, for discussion). Alternatively, the antagonist activity of cisapride in rat colon mucosa may be attributable to the presence of 5-HT_4-like receptor, although selective agonists and antagonists would be required to substantiate this contentious statement.

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(Received October 5, 1989
Revised October 4, 1990
Accepted November 21, 1990)

Br. J. Pharmacol. (1991), **102**, 817–822

Run-down of neuromuscular transmission during repetitive nerve activity by nicotinic antagonists is not due to desensitization of the postsynaptic receptor

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1 Whether the function of the postsynaptic acetylcholine receptor is use-dependently affected by repetitive nerve stimulation in the presence of competitive antagonists was studied in the mouse phrenic nerve-hemidiaphragm preparation.

2 For electrophysiological experiments, the preparation was immobilized by synthetic μ-conotoxin, which preferentially blocks muscular Na-channels causing neither depolarization of the membrane potential, inhibition of quantal transmitter release, nor depression of nicotinic receptor function.

3 High concentrations of cobratoxin depressed indirect twitches and endplate potentials (e.p.ps) without inducing waning of contractilities or run-down of trains of e.p.ps evoked at 10–100 Hz. However, waning and run-down were accelerated after washout of the toxin despite diminished postsynaptic receptor blockade. Once the run-down of e.p.ps was produced by washout or low concentrations of cobratoxin, further depression of e.p.p. amplitude with high concentrations of cobratoxin did not attenuate the e.p.p. run-down.

4 The degrees of waning of tetanus and trains of e.p.ps produced by a very high concentration of tubocurarine (20 μM) were also less than that caused at a 100 fold lower concentration, albeit the amplitudes of twitches and the first e.p.p. were depressed more rapidly and markedly.

5 Tubocurarine, like cobratoxin, depressed the amplitude of miniature endplate potentials (m.e.p.ps) more than e.p.ps.

6 In contrast to the steepened run-down of successive e.p.ps in the presence of low concentrations of either nicotinic antagonists, the amplitude of m.e.p.ps observed during repetitive stimulation was uniform and was not different from that before stimulation.

7 The results suggest that the e.p.p. run-down and tetanic fade induced by nicotinic antagonists are due to a slow kinetic blockade of presynaptic receptors and confirm that the e.p.p. run-down is not produced by a use-dependent failure of postsynaptic nicotinic receptors. The roles of the presynaptic nicotinic receptor in positive or negative feedback modulations of transmitter release are discussed.

Keywords: μ-Conotoxin; tubocurarine; acetylcholine release; endplate potential; nicotinic receptor; tetanic fade

Introduction

Characteristic phenomena observed after neuromuscular blockade by competitive nicotinic antagonists are the waning to instantaneous fade of already reduced tetanic contraction and the rapid run-down of successive endplate potentials (e.p.ps) or endplate currents during repetitive stimulation (Paton & Zaimis, 1952; Blaber, 1973; Hubbard & Wilson, 1973; Glavinovic, 1979; Magleby *et al.*, 1981). The degree of e.p.p. run-down caused by tubocurarine and other competitive antagonists is somehow in parallel with the depression of the amplitude of the first e.p.p., at least in *in vitro* experiments after equilibrium incubation (Gibb & Marshall, 1984; Chang *et al.*, 1988). The extent of the tetanic fade and the train-of-four inhibition, however, differs between antagonists and between the phases of drug action, being more prominent at the phase of recovery than at the phase of ongoing of drug action (Bowman & Webb, 1976; Sugai *et al.*, 1976; Bowman *et al.*, 1986). The most typical is the group of peptides known as α-neurotoxins isolated from snake venoms which often do not cause fade of tetanus when neuromuscular transmission is blocked by high concentrations of toxins (Chang & Lee, 1963; Bowman *et al.*, 1986; Gibb & Marshall, 1986). Interestingly, however, the fade becomes more marked after washout of α-neurotoxins (Bradley *et al.*, 1987; 1990; Chang & Hong, 1987). This unique action of α-neurotoxins can be substantiated by their effect on trains of e.p.ps, i.e., little run-down despite the depressed amplitude of the first e.p.p. at the phase of ongoing action vs marked run-down but with restored amplitude of the first e.p.p. on washout of the toxin (Chang & Hong, 1987).

The run-down of e.p.ps in the presence of a nicotinic antagonist has been attributed to a successive decline in the release of acetylcholine (ACh) (Bowman, 1980; Bowman *et al.*, 1986; Chang *et al.*, 1988) since the postsynaptic response to exogenously applied ACh remains unchanged immediately after the run-down of e.p.ps as compared to that before high frequency stimulation (Otsuka *et al.*, 1962). Moreover, tubocurarine depresses uniformly the responses to trains of ionophoretically applied ACh without causing any run-down (Gibb & Marshall, 1984). The presynaptic hypothesis suggests that the release of ACh during repetitive stimulation is automodulated by the released ACh acting through presynaptic nicotinic receptors. A blockade of this positive feedback modulation by nicotinic antagonists decreases the quantal contents of successive e.p.ps and results in run-down of e.p.ps. The differences in the degree of tetanic fade or e.p.p. run-down caused by different nicotinic receptor antagonists could be due to differences in the affinities or binding kinetics of pre- and postsynaptic receptors (*cf.* Bowman *et al.*, 1986; Chang & Hong, 1987). Recently, these findings have been explained by an agonist-antagonist cooperation at the postsynaptic site (Bradley *et al.*, 1987; 1990). It is hypothesized that when the high affinity site of the postsynaptic nicotinic receptor is selectively occupied by a low concentration of antagonist, not

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agonist, subsequent binding of the agonist to the low affinity site initiates, after activation of the receptor, a chain of events which leads to desensitization of the receptor.

These hypotheses have been tested by measuring any changes in the activity of spontaneous miniature endplate potentials (m.e.p.ps), which are reliable indicators of the sensitivity of the postsynaptic nicotinic receptor, during repetitive stimulation when antagonists had induced run-down of trains of e.p.ps. Changes of m.e.p.p. amplitudes rather than changes in the amplitude of iontophoretic ACh potentials were followed because alterations of junctional nicotinic receptors may not be equally manifest in the response to endogenous and exogenous neurotransmitters (Pennefather & Quastel, 1981).

Methods

Muscle contractions

Phrenic nerve-hemidiaphragm preparations were isolated from ICR strain mice 20–30 g. The organ bath contained 8–15 ml Tyrode (composition in mM: NaCl 137, KCl 2.8, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.33, $NaHCO_3$ 11.9 and glucose 11.2) maintained at 37°C and oxygenated with 95% O_2 and 5% CO_2. The phrenic nerve was stimulated with 0.03 ms rectangular pulses at the frequencies indicated. Muscle contractions were recorded via an isometric transducer (BG 25) on a physiorecorder (Gould 2200S).

Intracellular recordings

Intracellular recordings of m.e.p.ps and e.p.ps and current injections were carried out by conventional techniques with a high impedance amplifier (Dagan 8100). Microelectrodes were filled with 3 M KCl and resistances of 3–10 MΩ. The potentials were d.c.-coupled (Gould PB200) and hardcopied onto an electrostatic recorder (Gould ES2000). The recording unit has a bandwidth of 35 kHz (−3 dB). Diaphragms were immobilized with synthetic μ-conotoxin (1.2 μM), which like the natural one (Cruz *et al.*, 1985; Hong & Chang, 1989) blocks the Na-channel of muscle preferentially against that of nerve. Undistorted e.p.ps without the complication of muscle action potential can be evoked from the normal non-depolarized resting membrane potential. M.e.p.ps can be monitored accurately even after binding of about 50% of the ACh receptors with nicotinic antagonists. The effect of μ-conotoxin on evoked quantal release was studied on the cut diaphragm muscle preparation (Barstad & Lilleheil, 1968) prepared by damaging both ends of muscle fibres (Hong & Chang, 1989).

M.e.p.ps were recorded continuously from 10 s before and for up to 10 s after 15 s trains of pulses. For the sake of accuracy of measurement only those endplates with averaged m.e.p.p. amplitude (before trains of pulses) greater than 0.3 mV (>2 fold noise level) were selected. M.e.p.ps with amplitude greater than 2.5 fold average amplitude (giant m.e.p.ps) were discarded. Quantal contents of e.p.ps were estimated from e.p.ps and m.e.p.ps recorded in the same endplate according to the direct method (del Castillo & Katz, 1954). The amplitudes of e.p.ps in these calculations were corrected for non-linear summation (McLachlan & Martin, 1981; Chang *et al.*, 1986) with the correcting factor set at 0.56.

Statistics

In order to correlate changes of m.e.p.ps with e.p.ps during trains of stimulation, statistical analyses of data were sampled from two groups of endplates based arbitrarily on the extent of run-down of e.p.ps in trains: endplates with plateau e.p.ps (the average amplitude of the 51st to the 150th e.p.ps) maintained at greater than 75% of the first e.p.p. (see Results) were classified as those with a normal physiological run-down, while those which declined to less than 70% were classified as significant run-down. According to this sorting criterion, the majority of endplates (19 out of 21) belonged to the physiological run-down group when the preparations were not treated with nicotinic antagonists. The degree of e.p.p. run-down is defined as $(e.p.p_f - e.p.p_p)/e.p.p_f$, where $e.p.p_f$ and $e.p.p_p$ represent amplitudes of the first and the plateau e.p.ps, respectively.

All data are expressed as means ± s.e. of recordings obtained from more than 15 endplates in at least 3 preparations. Differences between group means were analysed by Student's t test. n refers to the total number of endplates studied.

Drugs

Tubocurarine chloride and tetrodotoxin were purchased, respectively, from Sigma (St. Louis, U.S.A.) and Calbiochem (San Diego, U.S.A.). μ-Conotoxin GIIIB (a 22 amino acid protein) was synthesized by the Peptide Institute (Osaka, Japan). Cobratoxin (a 62 amino acid protein) was purified from crude venom of *Naja naja atra* to homogeneity by repeated chromatography on CM-Sephadex columns (Pharmacia, Sweden).

Results

Effect of synthetic μ-conotoxin

Like the natural geographutoxin II, synthetic μ-conotoxin, at a concentration of 1.2 μM, changed neither the resting membrane potential (−80.7 ± 0.2 vs −83.4 ± 0.2 mV, $n \geqslant 65$) nor the m.e.p.p. amplitude (1.4 ± 0.1 vs 1.7 ± 0.2 mV, $n \geqslant 87$) or frequency of occurrence (1.8 ± 0.2 vs 1.4 ± 0.2/s, $n \geqslant 87$) while providing good recordings of e.p.ps with amplitude as large as 26.3 ± 1.0 mV ($n = 20$). After μ-conotoxin, the muscle membrane exhibited little, if any, active regenerative properties when the intracellularly injected current, up to 40 nA, induced a large electrotonic membrane depolarization of 25–30 mV and this was verified by further treatment with tetrodotoxin 0.5 μM. The amplitudes of successive e.p.ps evoked at 10–100 Hz declined to a plateau of 20.8 ± 0.9 mV ($n = 20$) within 20 pulses. The relative amplitude of e.p.ps at plateau in comparison to the first e.p.p. was not different from that obtained in cut muscle preparations, the degree of run-down being 0.22 ± 0.01 vs 0.24 ± 0.02 ($n = 29$). In cut muscle preparations, μ-conotoxin affected neither the amplitude nor the run-down of e.p.ps. The pharmacological profile and potency of synthetic μ-conotoxin on skeletal muscle are generally the same as those of natural geographutoxin II (Hong & Chang, 1989).

Effects of cobratoxin

It is known that short-term after washout of cobratoxin or erabutoxin the tetanic fade and run-down of e.p.ps were more prominent than before washout despite a significant recovery of the twitch response, peak tetanic tension and e.p.p. amplitude (Bradley *et al.*, 1987; 1990; Chang & Hong, 1987). On the other hand, similar tetanic fade and rundown of e.p.ps could be produced if the preparation was treated with very low concentrations of cobratoxin (0.0015–0.0045 μM) for a prolonged time (3–5 h), suggesting that the process of washout of toxin is not essential for the development of fade and run-down. To save time, waning was initially induced by incubating preparations with 0.45 μM cobratoxin for 30 min, a procedure which completely abolished e.p.ps and muscle contractions. Preparations were then washed twice with an interval of 5 min between washes. Further washing resulted in a too rapid and more complete recovery. Typical indirect twitch experiments are illustrated in Figure 1. A greater degree of waning of contractility was found after washout of cobratoxin than before washing. For intracellular recordings, μ-conotoxin at a con-

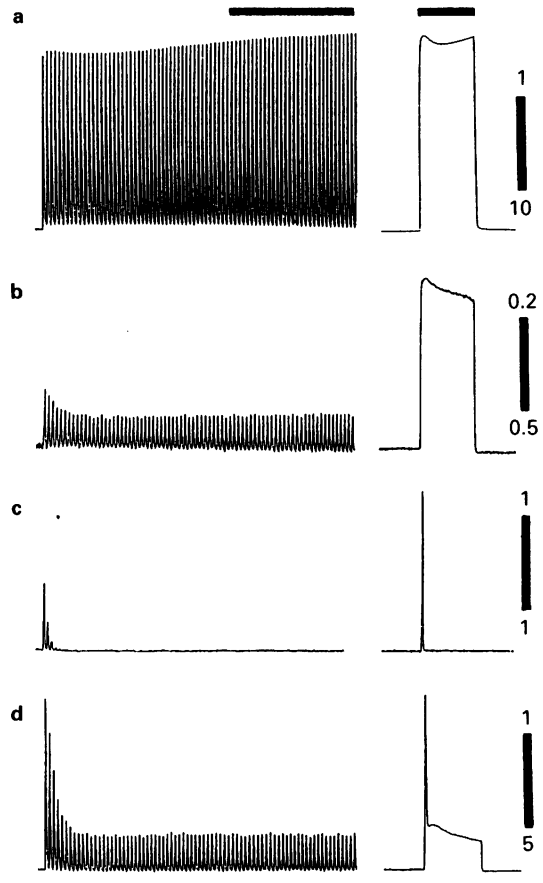

Figure 1 Effects of cobratoxin on wanings of indirect contractility of mouse phrenic nerve-hemidiaphragm preparation. The phrenic nerve was stimulated with trains of pulses (10 Hz for 7 s, left column) and 60 s later, at 100 Hz for 3 s (right column). (a) Control; (b) 12 min after treatment with cobratoxin 0.45 μM. (c and d) 10 and 50 min, respectively, after washout of cobratoxin (the preparation had been incubated with cobratoxin for 30 min). Note the more marked wanings of contractility after washout of cobratoxin in spite of the restoration of the initial contractions. The paired upper and lower numerals to the right of each panel represent the force calibrations for twitches at 10 Hz and tetanus at 100 Hz, respectively. The time scales on the right top corner of each column represent 3 s.

centration of 1.2 μM was added immediately following wash. After these treatments, the response of the nicotinic receptors recovered gradually and m.e.p.ps began to appear 15–20 min after the last wash. At endplates where m.e.p.ps were greater than 0.3 mV, repetitive nerve stimulation at 10 Hz was applied for 15 s to see the effect of stimulation on m.e.p.ps and e.p.ps. In agreement with previous reports, most of the endplates (27 out of 30) exhibited significant pharmacological run-down of e.p.ps 30–50 min after washout of cobratoxin (Figure 2 and Table 1). The degree of e.p.p. run-down increased from 0.22 ± 0.01 to 0.42 ± 0.01. In contrast, there was no run-down of m.e.p.ps during nerve stimulation (Figure 2 and Table 2).

With time, the amplitudes of e.p.ps and m.e.p.ps grew while the degree of run-down of e.p.ps decreased. Sixty to 80 min after washout of cobratoxin, the amplitude of both m.e.p.ps and the first e.p.p. in a train of stimuli recovered to 65 ± 6 and 84 ± 5% of controls, respectively, and the population of endplates showing significant e.p.p. run-down at 10 Hz decreased to about 42% (14 out of 33 endplates). Two h after washing, less than 20% of endplates (4 out of 27) showed significant run-down of e.p.ps. Whether there was run-down of e.p.ps or not, repetitive nerve stimulation did not induce a progressive decline of m.e.p.p. amplitude.

M.e.p.p. frequency was also studied to determine whether some m.e.p.ps might be depressed and lost in the noise during repetitive nerve stimulation which might have been inadvertently excluded. Nerve stimulation at 10 Hz increased m.e.p.p. frequency by about 30% either in control or in cobratoxin treated preparations, indicating that the unchanged amplitude of m.e.p.ps during repetitive stimulation is not due to an overestimate (Table 2). It is unlikely that a use-dependent block of the postsynaptic receptor occurred. When the stimulation rate was increased to 50–100 Hz, cobratoxin still caused a run-down of e.p.ps. Under these experimental conditions, m.e.p.ps were difficult to measure because of the increased incidence of superimposition of m.e.p.ps on e.p.ps. Nevertheless, measurements indicated that m.e.p.p. amplitude was not reduced during stimulation.

The above results, together with those obtained with tubocurarine (see next heading), indicate that high concentrations of nicotinic antagonists not only depress e.p.p. amplitude rapidly but also prevent run-down of trains of e.p.ps. High concentrations of cobratoxin (0.45 μM) did not attenuate the already established run-down of trains of e.p.ps that was produced either after washout of a high concentration of cobratoxin (Figure 3) or by prolonged incubation with low concentrations (0.0045 μM) of the toxin (not shown).

Effects of tubocurarine

From the results obtained with α-neurotoxins it appears that a rapid blockade of the receptor provoked little tetanic fade or e.p.p. run-down. It is of interest to see if tubocurarine, a competitive antagonist with rapid association/dissociation kinetics, produced less waning at high than at low concentrations like cobratoxin. The preparation was pretreated with

Table 1 Effects of nicotinic antagonists on the amplitude of endplate potentials (e.p.ps) evoked by repetitive nerve stimulation

Treatment			*The first e.p.p.*[a]	*E.p.ps at plateau*[b]		
Antagonist	(μM)	(min)	(mV)	(mV)	*E.p.p. run-down*	n
Control			25.1 ± 0.7	19.8 ± 0.4**	0.22 ± 0.01	44
Cobratoxin[c]	0.45	30				
	washout	0–5	0	0	0	15
		30–50	16.9 ± 0.5*	9.9 ± 0.4**	0.42 ± 0.01*	27
		60–80	21.3 ± 0.9*	13.9 ± 0.6**	0.35 ± 0.02*	14
Tubocurarine[d]	0.1–0.2	60–120	19.1 ± 0.6*	12.0 ± 0.8**	0.37 ± 0.02*	43

[a] E.p.ps were evoked by trains of pulses (10 Hz for 15 s) at various times after washout of cobratoxin or addition of tubocurarine as indicated.

[b] The amplitude of e.p.ps at plateau was the mean of e.p.ps generated during the last 10 s of stimuli. The degree of e.p.p. run-down was defined as the fractional depression of the plateau e.p.p.

[c] Mouse phrenic nerve-hemidiaphragm preparations were pretreated with cobratoxin 0.45 μM for 30 min, washed twice to remove cobratoxin and finally immobilized with μ-conotoxin 1.2 μM.

[d] The nerve-muscle preparations were pre-immobilized with μ-conotoxin 1.2 μM for 60 min.

Control experiments followed identical procedures but without addition of cobratoxin or tubocurarine. The results from these two groups were not different and were pooled together.

n: endplates studied. * $P < 0.05$ vs control; * $P < 0.05$ vs the first e.p.p.

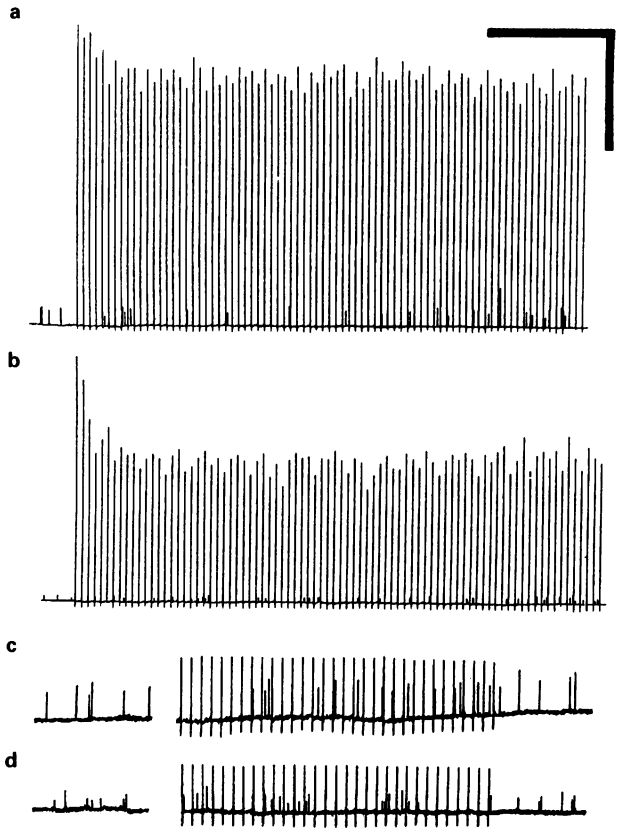

Figure 2 Effects of repetitive nerve stimulation and cobratoxin on amplitudes of endplate potentials (e.p.ps) and miniature endplate potentials (m.e.p.ps). Mouse phrenic nerve-hemidiaphragm preparations were treated with cobratoxin 0.45 μM for 30 min, washed twice and immobilized with μ-conotoxin 1.2 μM (b, d). Control experiments (a, c) followed the same procedure but in the absence of cobratoxin. (a) and (b) show e.p.ps evoked by a train of pulses at 10 Hz. (c and d) show high gain amplification recordings of m.e.p.ps just prior to and during the last 3 s of a 15 s train of pulses. The constant downward and upward deflections indicate stimulus artifacts and evoked e.p.ps (amplitude cut off), respectively. The resting membrane potentials of the 4 different endplates were in the range of −78 to −86 mV. Note that cobratoxin induced significant run-down of e.p.ps but not of m.e.p.ps. Calibrations: 2 s and 10 mV (a, b) or 1.25 s and 5 mV (c, d).

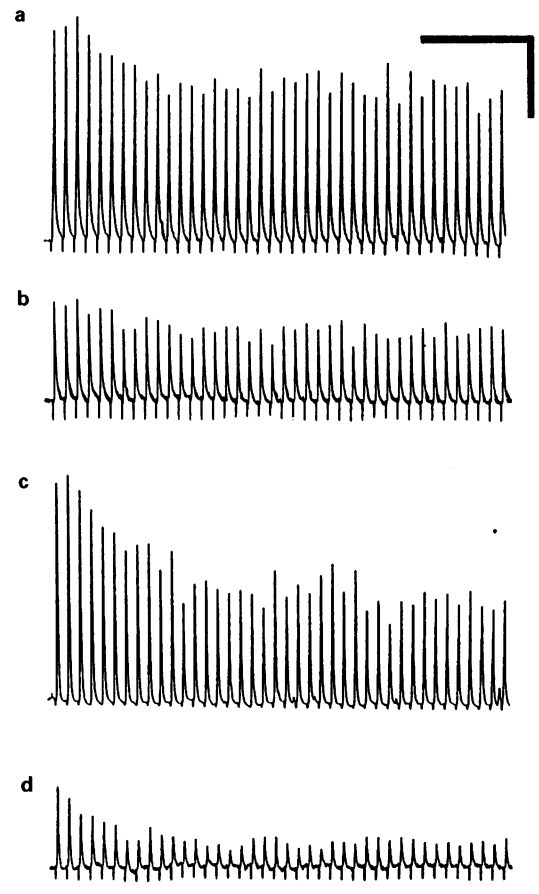

Figure 3 Effects of a high concentration of cobratoxin on run-down of endplate potentials (e.p.ps). The mouse phrenic nerve-hemidiaphragm preparation was immobilized with μ-conotoxin 1.2 μM. E.p.ps were evoked by a train of pulses at 100 Hz. (a) Control; (b) about 5 min after incubation with a high concentration of cobratoxin 0.45 μM; (c) 80 min after washout of cobratoxin (the preparation had been incubated with cobratoxin 0.45 μM for 30 min); (d) the same endplate as (c) about 5 min after a further addition of a high concentration of cobratoxin 0.45 μM. Note that cobratoxin depressed e.p.p. without attenuating run-down of e.p.ps (b). The resting membrane potentials at the time of recordings were −78, −84, −84 and −82 mV, respectively. Calibrations: 100 ms and 10 mV (a, c) or 2.5 mV (b, d).

1.2 μM μ-conotoxin for 60 min and then with 20 μM tubocurarine, a concentration that rapidly depressed e.p.p. amplitude at a rate one thousand fold faster than cobratoxin. The degree of e.p.p. run-down on tetanic stimulation was far less prominent compared to that after washout or after treatment with a 100 fold lower concentration of tubocurarine (Figure 4 and Table 1). The results are compatible with *in vivo* experiments in which tubocurarine caused virtually no tetanic fade when administered by close-arterial bolus injection (Bowman *et al.*, 1986). In order to record e.p.ps as well as m.e.p.ps at steady states and to avoid blocking the ACh ion channel (cf. Colquhoun, 1986) lower concentrations of tubocurarine were used in the following experiments. After 60 min of equilibration with 0.1–0.2 μM tubocurarine, the amplitudes of sampled e.p.ps and m.e.p.ps were 76 ± 1 and 46 ± 1% ($n = 43$), respectively, of those measured before tubocurarine. Repetitive stimulation at 10 Hz induced a significant run-down of e.p.ps (0.37 ± 0.02) (Table 1). Again, the amplitude of m.e.p.ps was not suppressed by nerve stimulation (Table 2). These results were similar to those obtained with cobratoxin.

Quantal release before and during stimulation

The effect of nicotinic antagonists on evoked transmitter release was estimated (Table 3). Repetitive stimulation in the presence of antagonists caused a progressive decline in the

Table 2 Effects of repetitive nerve stimulation on the amplitude and frequency of miniature endplate potentials (m.e.p.ps).

	Treatment[a]		*Amplitude* (mV)[b]		*Frequency* (s^{-1})		
Antagonist	(μM)	(min)	*before stimulation*	*during stimulation*	*before stimulation*	*during stimulation*	n
Control			1.41 ± 0.05	1.34 ± 0.05	1.39 ± 0.06	1.76 ± 0.06	44
Cobratoxin	0.45	30					
	washout	30–80	0.63 ± 0.02*	0.58 ± 0.02*	1.09 ± 0.03	1.42 ± 0.06	41
Tubocurarine	0.1–0.2	60–120	0.65 ± 0.01*	0.61 ± 0.02*	1.02 ± 0.06	1.37 ± 0.06	43

[a] Mouse phrenic nerve-hemidiaphragm preparations were treated with μ-conotoxin, cobratoxin or tubocurarine as described in Table 1.
[b] M.e.p.ps were taken 10 s before or during the last 10 s of trains of pulses (10 Hz for 15 s).
* $P < 0.05$ vs control.

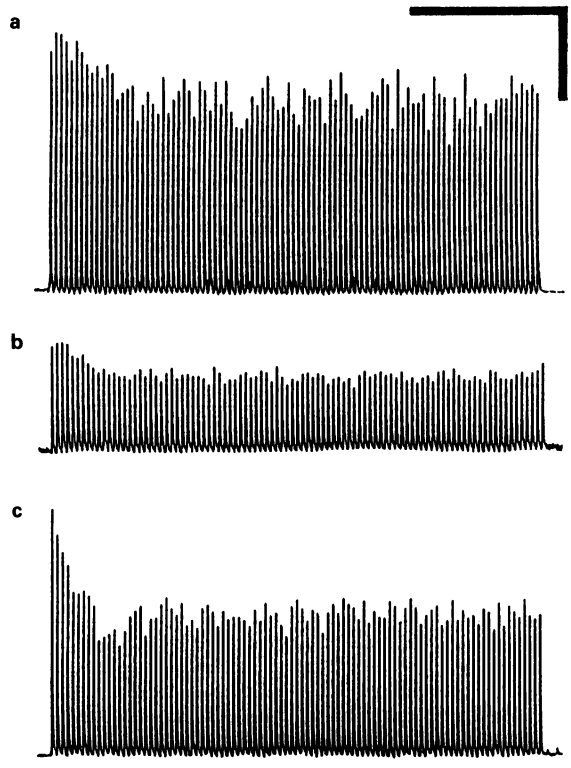

Figure 4 Effects of a high concentration of tubocurarine on run-down of endplate potentials (e.p.ps). The mouse phrenic nerve-hemidiaphragm preparation was immobilized with μ-conotoxin 1.2 μM. E.p.ps were evoked by a train of pulses at 100 Hz. (a) Control; (b) 10 s after a bolus addition of 20 μM tubocurarine (the organ bath contained 8 ml Tyrode solution). (c) 200 s after washout of tubocurarine (the preparation had been incubated with tubocurarine for 25 s). (a), (b) and (c) were from the same endplate. The resting membrane potentials at the time of recordings were −86, −86 and −78 mV, respectively. Note that tubocurarine depressed e.p.p. rapidly without causing significant run-down of e.p.ps (b). Calibrations: 300 ms and 10 mV (a, c) or 5 mV (b).

amplitude of trains of e.p.ps but had no effect on m.e.p.ps. It is evident that the quantal content of plateau e.p.ps declined. Cobratoxin and tubocurarine, on the other hand, appeared to increase the quantal content of the first e.p.p. by about 50% since both antagonists depressed the amplitude of m.e.p.ps more than e.p.ps.

Discussion

We have shown that the synthetic μ-conotoxin affected neuromuscular transmission qualitatively and quantitatively in a manner similar to natural geographutoxin II. This toxin has proven to be a useful tool for studies of neuromuscular transmission in that it does not depolarize the muscle, change the cellular ion composition or block ACh receptors (Hong & Chang, 1989).

The present results demonstrate that, in contrast to the enhanced run-down of trains by e.p.ps by nicotinic antagonists, the quantal response (m.e.p.p.) of the postsynaptic ACh receptor was not depressed further after repetitive bombardment of 'junctional' receptors with nerve-released ACh. If nicotinic antagonists do, in cooperation with nerve-released agonists, cause a use-dependent failure of the postsynaptic receptor ion channel (desensitization) as suggested by Bradley *et al.* (1990), the amplitude of the m.e.p.p. should be depressed more or less in parallel to the run-down of e.p.ps. From the present experiments, it seems evident that junctional receptors did not functionally change during the period of repetitive stimulation. The finding that further applications of high concentrations of cobratoxin did not attenuate the e.p.p. run-down once it was produced, seems to disfavour the idea that e.p.p. run-down results from selective binding of nicotinic antagonists to high affinity sites on ACh receptors with slow dissociation kinetics (Marchot *et al.*, 1988) as proposed by Bradley *et al.* (1990). Voltage clamp data also indicate that the concentration of tubocurarine used is too low to produce a significant open-channel block of the ACh receptor (Magleby *et al.*, 1981; Gibb & Marshall, 1984; Colquhoun, 1986). The unchanged function of the postsynaptic site throughout repetitive stimulation supports the hypothesis that e.p.p. run-down originates in the presynaptic site (Hubbard *et al.*, 1973; Glavinovic, 1979; Bowman, 1980; Magleby *et al.*, 1981; Wilson, 1982; Gibb & Marshall, 1984; Bowman *et al.*, 1986; Chang *et al.*, 1988; Ferry & Kelly, 1988). The relatively slow induction and dissipation of e.p.p. run-down and tetanic fade vs the rapid depression and restoration of e.p.p. amplitude and twitch tension suggest that the presynaptic receptor has a higher affinity but slower association/dissociation kinetics than the postsynaptic one.

The present findings seem to give further support to the positive feedback hypothesis that an appropriate activation of the nerve terminal nicotinic receptor enhances the transmitter release under physiological conditions, while a negative feedback modulation predominates when the concentration of ACh in the synaptic cleft piles up in the presence of anticholinesterase agents (Bowman *et al.*, 1986; Chang *et al.*, 1988). The apparent increase of the quantal content, estimated by the direct method, of either single e.p.ps or the first e.p.p. in the train by nicotinic antagonists (Wilson, 1982; Ferry & Kelly, 1988; present experiment) seems to contradict the above hypothesis. The lesser inhibition of single e.p.ps than of m.e.p.ps by nicotinic antagonists can be accounted for partially by the non-linear summation of ACh responses (McLachlan & Martin, 1981). This view is supported by the finding that tubocurarine suppressed the amplitudes of single e.p.ps and m.e.p.ps proportionately if the amplitude of e.p.ps was first reduced to less than 4 mV by lowering the Ca^{2+} concentration in Tyrode to 0.2 mM (unpublished). Moreover, in voltage clamp experiments (Harbone *et al.*, 1988) tubocurarine did not increase the quantal content of the first endplate current but reduced those of subsequent endplate currents. The view concerning the negative-feedback role of presynaptic nicotinic receptor during a low rate of nerve stimulation in the presence of intact acetylcholinesterase implies that a majority of the nicotinic receptor is tonically stimulated by the non-

Table 3 Effects of nicotinic antagonists on the estimated quantal content of trains of endplate potentials (e.p.ps)

	Treatment[a]		*Quantal contents*[b]		
Antagonist	(μM)	(min)	*The first e.p.p.*	*E.p.ps at plateau*	n
Control			21.3 ± 0.9	16.9 ± 0.7**	44
Cobratoxin	0.45	30			
	washout	30–80	33.7 ± 0.9*	21.2 ± 0.6**	41
Tubocurarine	0.1–0.2	60–120	34.4 ± 0.7*	21.9 ± 0.6**	43

[a] Mouse phrenic nerve-hemidiaphragm preparations were treated with μ-conotoxin, cobratoxin or tubocurarine as described in Table 1.
[b] Quantal contents of e.p.ps were estimated from data in Tables 1 and 2 by dividing e.p.p. with m.e.p.p. The amplitudes of e.p.ps were normalized to a standard resting membrane potential of −82 mV with the correcting factor set at 0.56 (see Methods).
* $P < 0.05$ vs control; ** $P < 0.05$ vs the first e.p.p.

evoked leakage of ACh from the nerve terminal which is unlikely. It is also incompatible with the observations that anticholinesterase agents, which can cause a remarkable build up of ACh in the synaptic cleft (Zemkova *et al.*, 1990), did not reduce transmitter release to the first stimulus in a train (Wilson, 1982; Ferry & Kelly, 1988). Taken together, the inference that blockades of presynaptic nicotinic receptors may enhance evoked transmitter release (Wilson, 1982; Ferry & Kelly, 1988) should be reserved.

Paradoxical to the overwhelming electrophysiological findings that trains of e.p.ps or endplate currents run down within 20 pulses in the presence of nicotinic antagonists (Hubbard & Wilson, 1973; Glavinovic, 1979; Magleby *et al.*, 1981; Gibb & Marshall, 1984), the neurochemical data indicate that tubocurarine causes essentially no effect on [^{3}H]-ACh release pooled from 100 pulses but 50% depression if 200 pulses were studied (Wessler *et al.*, 1987). If this result were correct, it would mean that the release of ACh up to the 100th pulse was unchanged whereas that from the 101st pulse on was suddenly and completely abolished. This is unlikely and is not substantiated by any electrophysiological studies. For the collection of nerve released [^{3}H]-ACh to be feasible hemicholinium-3 was used which, alone, has undesired pharmacological actions such as alterations of ACh synthesis and receptor response (Takagi *et al.*, 1970) and may bring about complicated drug interactions. In addition, the measurement of [^{3}H]-ACh suffers from a low signal to background ratio, the evoked release of [^{3}H]-ACh being only some 20–100% greater than the background leakage. In these respects, one should be circumspect in interpreting the neurochemical results. Indeed, it is difficult to reconcile that the time course of inhibition of evoked [^{3}H]-ACh release by tubocurarine should lag behind the occurrence of e.p.p. run-down (Wessler *et al.*, 1987) and that α-bungarotoxin but not cobratoxin or erabutoxin reduced evoked [^{3}H]-ACh release (Wessler *et al.*, 1990).

We appreciated the generous gift of μ-conotoxin from Dr K.T. Wang (Taipei). The work was supported by the National Science Council (NSC80-0412-B002).

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(Received October 16, 1990
Revised November 20, 1990
Accepted November 26, 1990)

Br. J. Pharmacol. (1991), **102**, 823–826

Haemodynamic responses to N^G-monomethyl-L-arginine in spontaneously hypertensive and normotensive Wistar-Kyoto rats

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1 Nitric oxide (NO) is a major component of endothelium-derived relaxing factor (EDRF) the synthesis of which from L-arginine can be inhibited by N^G-monomethyl-L-arginine (L-NMMA). To assess whether basal NO tone is different in experimental hypertension, the haemodynamic effects of L-NMMA have been compared in anaesthetized spontaneously hypertensive (SH) and normotensive Wistar-Kyoto (WKY) rats in which autonomic reflexes were blocked by ganglion blockade.

2 Bolus intravenous injections of L-NMMA, 1–30 mg kg^{-1}, but not D-NMMA, 1–30 mg kg^{-1}, induced dose-related increases in mean arterial pressure and decreases in conductances in the renal, carotid, hindquarters and mesenteric vascular beds in both SH and WKY rats. Although the different vascular beds varied in their maximum responses to L-NMMA, there were neither qualitative nor quantitative differences between the two rat strains in this respect.

3 The effects of L-NMMA, 30 mg kg^{-1}, i.v. on all parameters were rapidly and completely reversed by L-arginine, 30 mg kg^{-1}, i.v., in both SH and WKY rats.

4 The results indicate that NO derived from L-arginine exerts a powerful vasodilator tone in both anaesthetized, ganglion-blocked SH and WKY rats. Although NO appears to contribute differentially to tone in the different vascular beds, there were no major differences between the two rat strains in this respect. Hence a reduced NO tone to the vasculature is unlikely to be a major factor contributing to the elevated blood pressure in the adult SH rat.

Keywords: N^G-monomethyl-L-arginine; spontaneously hypertensive rats; Wistar-Kyoto normotensive rats; endothelium-derived relaxing factor; nitric oxide; peripheral vascular tone

Introduction

The role of the endothelial cell as an important source of mediators which influence vascular reactivity is established (Angus & Cocks, 1989; Vane *et al.*, 1990). An important component of endothelium-derived relaxing factor (EDRF) is nitric oxide (NO) which is formed from the N^G (guanidino) group of L-arginine (Palmer *et al.*, 1988) by a cytosolic enzyme which can be inhibited by certain closely related derivatives of L-arginine such as N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine (see Moncada *et al.*, 1989; Ishii *et al.*, 1990).

The administration of L-NMMA to guinea-pigs (Aisaki *et al.*, 1989), rabbits (Rees *et al.*, 1989), dogs (Chu *et al.*, 1990) and rats (Whittle *et al.*, 1989; Hecker *et al.*, 1990; Tolins *et al.*, 1990) results in substantial increases in blood pressure due largely, if not entirely to an increase in peripheral resistance (Gardiner *et al.*, 1990). The effects of L-NMMA can be reversed stereoselectively by L-arginine (Whittle *et al.*, 1989; Chu *et al.*, 1990; Tolins *et al.*, 1990) suggesting a remarkable degree of NO 'tone' to the peripheral vasculature in such animals. The implications for the maintenance of peripheral resistance in man under normal circumstances (Vallance *et al.*, 1989) and in certain cardiovascular pathophysiologies such as hypertension (see Lüscher, 1990) is obvious.

A decreased responsiveness to endothelium-dependent vasodilator substances is characteristically seen in vessels taken from various models of experimental hypertension (Winquist *et al.*, 1984; Lüscher & Vanhoutte, 1986; Tesfamariam & Halpern, 1988). However, the question of whether this represents a decreased release of EDRF remains open (Van de Voorde & Leusen, 1986; Lüscher *et al.*, 1987). Indeed, bioassay of EDRF indicates no difference between vessels from SH or WKY rats with respect to their capacity to release EDRF (Lüscher *et al.*, 1987; Hoeffner & Vanhoutte, 1989).

Until now there has been no report of the effects of NO biosynthesis inhibitors in spontaneously hypertensive (SH) rats. In the present work, we have attempted to define the basal NO 'tone' of the cardiovascular system of SH rats by monitoring the haemodynamic response to L-NMMA. Comparisons have been made with age-matched, normotensive, Wistar-Kyoto (WKY) control animals as a first step towards the identification of a putative differential NO contribution to the experimental hypertensive state.

A preliminary account of these experiments was presented at the XIth IUPHAR meeting in Amsterdam (Fozard & Part, 1990).

Methods

In vivo *preparations*

Male SH rats weighing 322–354 g and age-matched WKY control animals weighing 317–329 g, supplied by Biological Research Laboratories, Füllinsdorf, Switzerland, were used in this study. Rats were anaesthetized with Inaktin, 120 mg kg^{-1} i.p. A tracheotomy was performed and catheters inserted into the right carotid artery for blood pressure and heart rate (HR) and into the jugular vein for drug administration. Pulsed Doppler flowprobes (Haywood *et al.*, 1981) were placed around the left carotid artery and, through a midline abdominal incision, around the left renal artery, the superior mesenteric artery and the lower abdominal aorta just above the iliac bifurcation (equated to hindquarters blood flow). The incisions were closed and parameters allowed to stabilise for 1 h. Following the stabilisation period, the rats were ganglion-blocked with mecamylamine (0.25 mg kg^{-1}, infused intravenously over 15 min).

Experimental design

Thirty minutes following the end of the infusion of mecamylamine, a sequence of i.v. doses of L-NMMA (1, 3, 10 and 30 mg kg^{-1}) was started; the doses were given cumulatively at 1.5 min intervals; 40.5 min following the final dose of

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L-NMMA an identical sequence of doses of D-NMMA was started. Then 22.5 min after the final dose of D-NMMA a second sequence of doses of L-NMMA identical to the first was initiated. L-Arginine was injected i.v. 1.5 min after the final ($30\,mg\,kg^{-1}$) dose in this series. This protocol was followed in both SH and WKY rats which were used on alternate days.

Drugs

N^G-monomethyl-L-arginine citrate (L-NMMA) and N^G-monomethyl-D-arginine citrate (D-NMMA) were supplied by Ultrafine Chemicals, Salford, U.K. Mecamylamine hydrochloride was the kind gift of Merck, Sharpe and Dohme. Phenylephrine hydrochloride was obtained from Serva and L-arginine from Sigma. All drugs were freshly prepared each day and diluted in 0.9% w/v NaCl solution prior to use.

Statistical analysis

All data are expressed as mean values ± s.e.mean of n individual determinations. Student's t test was used to evaluate the significance of a difference between group means.

Results

Resting circulatory variables

The mean arterial pressure (MAP) of anaesthetized SH rats after surgery but before injection of mecamylamine was 127 ± 5 mmHg and that of WKY was 100 ± 5 mmHg ($P < 0.05$; $n = 4$ in each case). The resting circulatory variables after ganglion blockade and just prior to starting the first L-NMMA injection sequence in anaesthetized SH and WKY rats are shown in Table 1. The mean value of MAP in SH rats was some 20% higher than that of WKY controls. Vascular flows and conductances were consistently less in SHR than in WKY, these differences being particularly marked in the carotid and hindquarters vascular beds (Table 1).

Effects of N^G-monomethyl-L-arginine, N^G-monomethyl-D-arginine and L-arginine

Blood pressure and heart rate Cumulative bolus injections of L-NMMA, 1–30 $mg\,kg^{-1}$, induced immediate, dose-related and similar increases in MAP in both SH and WKY rats (Figure 1). The mean change in MAP 1.5 min following the $30\,mg\,kg^{-1}$ dose was 41 ± 7 mmHg and 43 ± 7 mmHg for SH and WKY rats, respectively. The MAP response declined rapidly immediately after the $30\,mg\,kg^{-1}$ dose but remained at a level 15–20 mmHg higher than the baseline value during the succeeding 40–45 min. Predictably, since the animals were treated with a ganglion blocking agent, changes in HR were minimal (Figure 1).

Table 1 Baseline haemodynamic parameters prior to injecting N^G-monomethyl-L-arginine (L-NMMA)

	WKY (n = 4)	*SHR* (n = 4)
MAP (mmHg)	78 ± 4	93 ± 3*
HR ($b\,min^{-1}$)	341 ± 14	343 ± 9
Flows[1]		
Renal	3.11 ± 0.91	2.52 ± 0.52
Mesenteric	3.40 ± 0.61	3.07 ± 0.52
Carotid	2.32 ± 0.78	1.32 ± 0.34
Hindquarters	2.89 ± 0.40	1.31 ± 0.20*
Conductances[2]		
Renal	4.04 ± 1.29	2.70 ± 0.52
Mesenteric	4.39 ± 0.87	3.39 ± 0.62
Carotid	2.98 ± 1.04	1.40 ± 0.32
Hindquarters	3.65 ± 0.32	1.40 ± 0.17*

[1] In kHz Doppler Shift.
[2] In conductance units derived from flow divided by MAP (i.e. 100 [$kHz\,mmHg^{-1}$]).
* Significantly different from corresponding WKY value ($P < 0.05$).

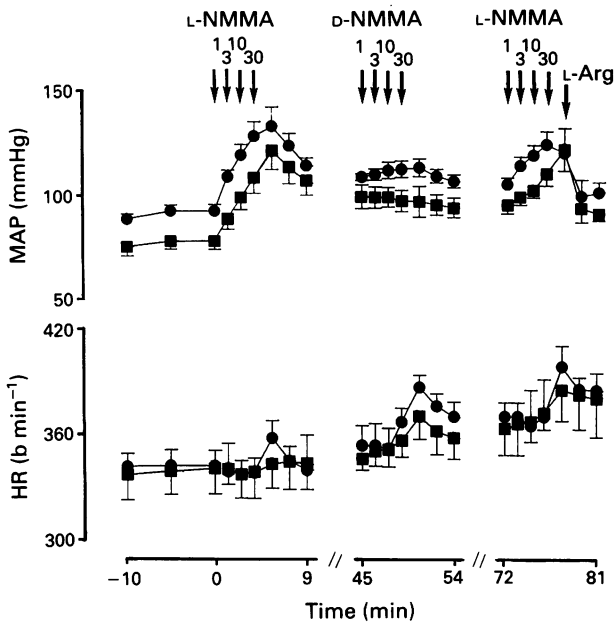

Figure 1 Effects of increasing bolus doses ($mg\,kg^{-1}$) of N^G-monomethyl-L-arginine (L-NMMA), D-NMMA and a second series of doses of L-NMMA followed by a single, $30\,mg\,kg^{-1}$, dose of L-arginine (L-Arg) on mean arterial pressure (MAP) and heart rate (HR) in anaesthetized, ganglion-blocked SH (●) or WKY (■) rats. The abscissa scale represents the time scale of the experiment with time 0 the point at which the first dose of L-NMMA was given. Points represent mean values (with s.e.mean) of 3–4 individual observations.

In contrast to the effects seen with L-NMMA, a series of bolus injections of D-NMMA, 1–30 $mg\,kg^{-1}$, started 40.5 min after the final dose of L-NMMA had little effect on MAP; however, small, dose-related increases in heart rate were seen in both SH and WKY rats following the two highest doses (Figure 1).

A second sequence of doses of L-NMMA started 22.5 min after the final dose of D-NMMA again gave dose-related increases in MAP in both SH and WKY rats. In this series, the absolute changes in MAP were less than those seen in the first series, probably reflecting the higher baseline values at the start of the second injection sequence (Figure 1). A clear tendency for HR to increase was seen in both rat strains following the highest dose of L-NMMA. A dose of L-arginine given 1.5 min after the $30\,mg\,kg^{-1}$ dose of L-NMMA induced in both SH and WKY rats an immediate fall in MAP to a level slightly below the values prior to the start of the injection sequence (Figure 1).

Changes in regional vascular conductance These data are presented as absolute changes induced by the first sequence of L-NMMA doses in Figure 2 and as percentage changes (for L-NMMA and D-NMMA) in Figures 3 and 4. L-NMMA, 1–30 $mg\,kg^{-1}$ i.v., induced dose-related decreases in conductance in each vascular bed (Figures 2–4). Analysis of the normalized data indicates no significant difference between SH and WKY rats in this respect (Figures 3 and 4). However, in each rat strain, the maximum decreases in conductance were greater in the renal and carotid than in the mesenteric and hindquarters vascular beds. Consistent with the observations on MAP, D-NMMA had minimal effects on peripheral vascular conductance in either SH or WKY rats (Figures 3 and 4).

The responses to the second series of L-NMMA injections were somewhat attenuated compared to the initial series due probably to the lower baseline values at the start of this injection series (data not illustrated). Injection of L-arginine,

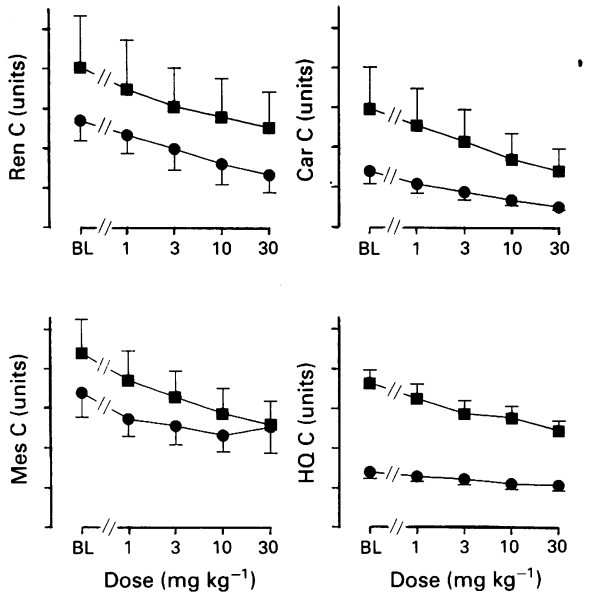

Figure 2 Changes in vascular conductance (C; units = 100 [kHz mmHg^{-1}]) in renal (Ren), mesenteric (Mes), carotid (Car) and hindquarters (HQ) beds of anaesthetized, ganglion-blocked, SH (●) and WKY (■) rats in response to increasing bolus doses of N^G-monomethyl-L-arginine (L-NMMA). Points represent mean values of 3–4 individual observations; s.e.mean shown by vertical bars.

30 mg kg^{-1}, 1.5 min after the 30 mg kg^{-1} dose of L-NMMA resulted in immediate and complete reversal of the conductance decrease in each of the vascular beds under study and in each rat strain (Figures 3 and 4).

Discussion

The present experiments were carried out in animals treated with a ganglion blocking agent. The dose of mecamylamine used can be considered adequate since neither in the present experiments (with L-NMMA) nor in previous studies with

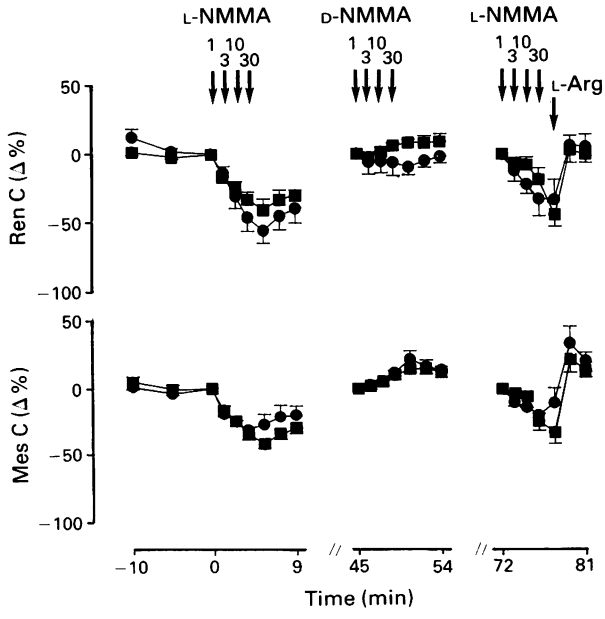

Figure 3 Changes in vascular conductance (C, %) in renal (Ren) and mesenteric (Mes) beds of anaesthetized, ganglion-blocked, SH (●) or WKY (■) rats in response to N^G-monomethyl-L-arginine (L-NMMA), D-NMMA and a second series of doses of L-NMMA followed by a single dose (30 mg kg^{-1}) of L-arginine (L-Arg). For further details, see legend to Figure 1.

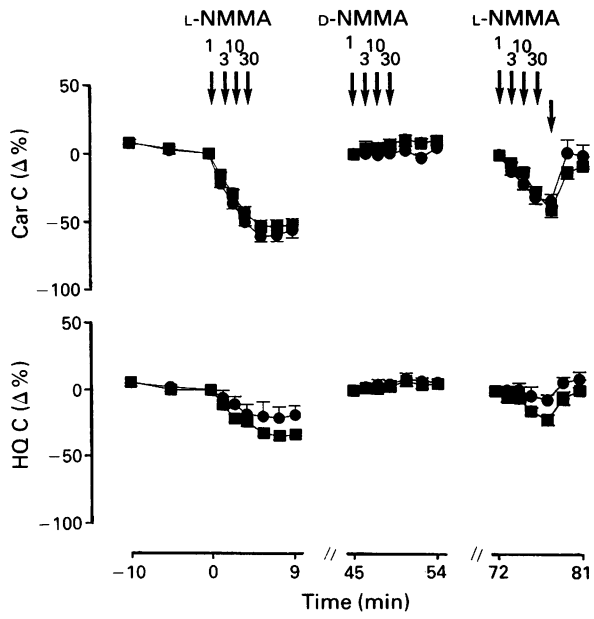

Figure 4 Changes in vascular conductance (C, %) in carotid (Car) and hindquarters (HQ) beds of anaesthetized, ganglion-blocked, SH (●) or WKY (■) rats in response to N^G-monomethyl-L-arginine (L-NMMA), D-NMMA and a second series of doses of L-NMMA followed by a single dose (30 mg kg^{-1}) of L-arginine (L-Arg). For further details, see legend to Figure 1.

other vasoconstrictor or vasodilator stimuli in the same preparation (Wright & Fozard, 1988; 1990) did reflex changes in heart rate accompany the blood pressure changes. The data can thus be interpreted without complications arising from baroreceptor reflex activation which is known to be attenuated in experimental hypertension (Korner, 1989). Under these conditions, the evidence is consistent with a major contribution of NO to peripheral vascular tone in both SH and WKY rats. Thus, increase in blood pressure was associated with a decrease in conductance in each of the vascular beds under study following administration of L-NMMA. That the effect was rapidly and completely reversed by L-arginine and stereoselective (D-NMMA was ineffective) implicates inhibition of NO synthesis as the basis of the constrictor response. These data confirm and extend previous findings in guinea-pigs (Aisaki *et al.*, 1989), rabbits (Rees *et al.*, 1989), dogs (Chu *et al.*, 1990) and both anaesthetized (Whittle *et al.*, 1989; Hecker *et al.*, 1990; Tolins *et al.*, 1990) and conscious (Gardiner *et al.*, 1990) normotensive rats.

Although the constrictor effects of L-NMMA in the different vascular beds were manifested over the same dose range, the maximum changes achieved were different. For instance, the maximum decrease in hindquarters conductance following 30 mg kg^{-1} L-NMMA was only half that seen in the carotid vascular bed. The result does not reflect a difference in speed of onset of the response in the different vascular beds since conductance changes had reached a plateau before subsequent doses were given (data not illustrated). The data suggest that under the conditions of our experiments, NO contributes differentially to tone in the different vascular beds. It bears emphasis that in the only other detailed haemodynamic study with L-NMMA reported to date (Gardiner *et al.*, 1990) carried out in conscious Long-Evans rats, the maximum conductance changes induced by L-NMMA in renal, mesenteric, hindquarters and internal carotid vascular beds were similar. Differential compensation by the baroreceptor reflexes of the local vasoconstrictor effects of L-NMMA would be expected to regulate the maximum effects achieved and could be the basis for the differences between animals with intact and attenuated reflexes.

The major finding of the present study is that the haemodynamic responses of age-matched WKY and SH rats to

L-NMMA are qualitatively and quantitatively similar. Predictably, (see Korner *et al.*, 1989) due to the vascular hypertrophy present in SH rats (Folkow, 1982; and indicated in the present study by the higher blood pressure and lower peripheral vascular conductances following ganglion blockade, Table 1), a given dose of L-NMMA produced a greater decrease in the individual conductance values in WKY than in SH rats (Figure 2). However, the normalized dose-response curves (Figures 3 and 4) were superimposable in the two rat strains demonstrating that it is the influence of the hypertrophic vasculature rather than a difference in sensitivity to the vasoconstrictor stimulus which accounts for the difference in absolute response changes. Thus, under the conditions of our experiments, the basal activity of NO appears to be similar in SH and WKY rats.

The physiological stimuli responsible for the basal release of NO are unknown. However, since the haemodynamic changes induced by L-NMMA in the anaesthetized, ganglion-blocked animals in the present study were broadly similar to those seen in conscious animals (Gardiner *et al.*, 1990), a major role for the autonomic nervous system can be ruled out. Physical factors such as shear stress and pulsatile flow (Griffith *et al.*, 1987) are the most likely factors controlling basal NO biosynthesis.

In conclusion, our data indicate an important role for NO generated from L-arginine in the control of peripheral resistance in anaesthetized, autonomically compromized SH and WKY rats. Although NO appears to contribute differentially to tone in the different vascular beds, there was no major difference between the two rat strains in this respect. Hence, a reduced NO tone to the vasculature is unlikely to be a major factor contributing to the elevated blood pressure in the adult SH rat.

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(Received October 1, 1990
Revised November 21, 1990
Accepted November 26, 1990)

Br. J. Pharmacol. (1991), **102**, 827–830

Enhanced contractility of the rat stomach during suppression of angiotensin converting enzyme by captopril *in vitro*

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1 Intragastric pressure (IGP) was used as an index, of the effect of serosal application of captopril (SQ 14,225; D-3-mercapto-2-methylpropanoyl-L-proline) on the contractility of rat stomach *in vitro*.

2 Captopril, at concentrations $>0.3\,\mu$M, enhanced the spontaneous gastric motility (GM) in a concentration-dependent manner whereas concentrations $<0.3\,\mu$M selectively potentiated 4 nM bradykinin (BK)-evoked gastric contractions without significantly affecting the spontaneous GM.

3 The kallikrein inhibitor, aprotinin ($100\,u\,ml^{-1}$), markedly antagonized the enhanced GM to $1.4\,\mu$M captopril and BK (4 nM)-evoked contractions, without affecting the contractions evoked by angiotensin 1 (10 nM) and acetylcholine ($0.4\,\mu$M). The angiotensin II antagonist, saralasin ($50\,\mu$M) failed to mimic aprotinin.

4 The enhanced GM to captopril was markedly inhibited by tetrodotoxin ($1\,\mu$M), and partially inhibited by atropine ($1\,\mu$M).

5 These results indicate that *in vitro*, captopril ($>0.3\,\mu$M) enhances gastric contractility through kininase/ACE inhibitory action, presumably by increasing the concentration of undegraded tissue kinins and substance P. This motor response seems to be predominantly due to activation of the cholinergic neurones but non-cholinergic excitatory neurones are also involved.

Keywords: Gastrointestinal motility; captopril; aprotinin; saralasin; kininase-II or ACE inhibitor, kinins

Introduction

Captopril (SQ 14,225; D-3-mercapto-2-methylpropanoyl-L-proline) is a potent inhibitor of angiotensin converting enzyme (ACE) or kininase II (Ondetti *et al.*, 1977; Cushman *et al.*, 1982), that catalyses the cleavage of bradykinins (Ferreira & Bakhle, 1977) and substance P (Cascieri *et al.*, 1983), besides converting angiotensin I (A-I) to angiotensin II (A-II) (Erdos, 1976; Rubin *et al.*, 1978).

Captopril has been shown to potentiate selectively bradykinin (BK)-evoked contractions in the guinea-pig ileum (Rubin *et al.*, 1978) and ferret tracheal smooth muscle (Dusser *et al.*, 1988) *in vitro*. The demonstration of kinin-generating and degrading enzymes viz., kallikreins (Kobayashi *et al.*, 1979; Frankish & Zeitlin, 1980; Kobayashi & Ohata, 1981) and kininase II (Roth *et al.*, 1969; Zeitlin & Smith, 1973) in the gut, led to the proposal that the BK-potentiating effect of captopril must be associated with the accumulation of undegraded endogenous kinins. However, this BK-potentiating effect could be due to either the increased tissue concentration of endogenous kinins or substance P (Cascieri *et al.*, 1983), or to decreased A-II activity (Carretero *et al.*, 1981). It seemed important, therefore, to identify the peptide(s) involved.

The aim of this work, therefore, has been to discern the endogenous peptides mediating captopril-induced contractility of the rat stomach *in vitro*, by use of specific inhibitors for kinins and angiotensin. Further, an attempt has been made to investigate the nature and mechanism of action of captopril on gastric contractility.

Methods

Animals and preparation

Stomach preparations, isolated from rats (Charles-Foster) of either sex weighing 150–200 g, were used. Animals were fasted overnight before they were killed by cervical dislocation and exsanguination. Th stomach was cannulated with a balloon-tipped catheter according to the procedure detailed elsewhere (Rani & Rao, 1988). In brief, the procedure involves: flushing the gastric lumen with warm saline through a nick made in the wall of the cardiac end of the fundus, and introducing a balloon (1 ml)-tipped polyvinyl catheter (2 mm o.d.) into the gastric lumen, such that the tip of the deflated balloon lay 1 cm proximal to the gastroduodenal junction. After securing the catheter in place, the gastro-oesophageal and gastroduodenal junctions were tied. The whole stomach along with the catheter was excised and mounted horizontally in an organ-bath (25 ml). The serosal surface of the stomach was superfused with Tyrode solution having the following composition (mM): NaCl 137, KCl 3.7, $MgCl_2$ 0.05, $CaCl_2$ 1.02, NaH_2PO_4 0.32, $NaHCO_3$ 11.9 and glucose 5.0. In the bath, the fluid was continuously gassed with a mixture of 95% O_2 and 5% CO_2 and maintained at 36 ± 1°C. The bath fluid was changed every 15 min, unless specified otherwise.

Recording of intragastric pressure (IGP)

The pressure in the intragastric balloon was initially made atmospheric and then 1 ml of Tyrode solution was introduced into the balloon, which enabled us to record phasic contractions of moderate vigour (Statham P23-AC pressure transducer; curvilinear pen recorder). The transducer output was calibrated against a water manometer. Preparations were allowed to stabilize 30–45 min in the bath before the control IGP was recorded. In earlier studies a good agreement was found between the IGP recorded with balloon-tip catheter and open-tip catheter (Rao, 1980; Rani & Rao, 1988).

Pretreatments

The effect of various pharmacological blockers on the enhanced gastric motility (GM) elicited by a test dose of captopril ($1.4\,\mu$M) was examined in four different groups of preparations, pretreated with one of the following antagonists: aprotinin ($100\,u\,ml^{-1}$; contact time 45 min), saralasin (50–$100\,\mu$M; 45 min) tetrodotoxin (TTX $1\,\mu$M; 15 min) and atropine ($1\,\mu$M; 15 min).

For transmural stimulation (TMS), trains of pulses, from a Grass (S-88) stimulator, were delivered once every 4 min

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through a pair of Ag-$AgCl_2$ wire electrodes; one placed in the gastric lumen and another in the bathing medium. A single strain (10 s) of 0.2 ms pulses at 10 Hz was used to evoke nerve-mediated gastric contractions.

Drugs and solutions

Acetylcholine chloride (ACh), bradykinin triacetate (BK), angiotensin-1 (A-I), saralasin (1-sar-8-Ala-angiotensin II) and tetrodotoxin (TTX) were from Sigma Chemicals, St. Louis, Missouri, U.S.A. Captopril (SQ 14,225) and aprotinin were from Squibb Institute for Medical Research, New Jersey, U.S.A. Atropine sulphate was from Merck. Other reagents were of analytical grade. All solutions were made in glass-distilled water and prepared fresh except for the stock solution of BK which was stored at −16°C for up to two weeks.

Data analysis

Intragastric pressure, reflecting the algebraic sum of tonic and phasic contractions of whole-stomach, was used as an index of gastric motor activity. The area (cm^2) under the IGP curve measured (with reference to atmospheric pressure) over a period of 12 min was regarded as gastric motility (GM) and was used for quantitating the effect of captopril. The EC_{50} was determined using probit values of % responses, with the maximal IGP response observed at 6 μM captopril regarded as the 100% response.

The evoked contractile responses to BK, A-I and ACh were presented as the area (cm^2) under the IGP curve measured (with reference to the pre-existing IGP) over a period of 2 min from the onset of the response. The effect of captopril and other drugs on these evoked contractions was expressed as a percentage of control responses evoked before the addition of captopril.

Data are presented as mean ± s.d. Significant differences between the means were assessed by use of Student's t test for paired or unpaired data. A P value of <0.05 was considered statistically significant.

Results

Potentiation of gastric motor activity by captopril

The time course of IGP changes in the whole stomach preparation caused by serosal application of a single dose (1.4 μM; contact time 15 min) of captopril is shown in Figure 1a. The basal IGP (8.4 cmH_2O) and the amplitude of superimposed phasic contractions (0.9–1.8 cmH_2O) increased to a maximum of 13.8 and 23.4 cmH_2O, respectively, within 8–12 min following application of captopril and recovered after 15 min wash.

As shown in Figure 1b, the area under the IGP curve

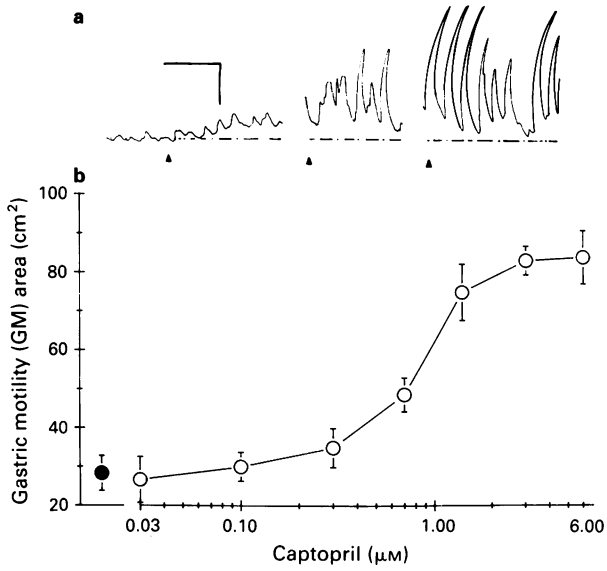

Figure 1 Representative intragastric pressure (IGP) tracing (a) illustrates the time-course of contractions in the rat excised whole stomach, in response to a single dose of captopril (contact time = 15 min). The solid triangles (left to right) indicate zero min, 5 min and 8 min after serosal application of captopril (1.4 μM). The horizontal interrupted line denotes the control basal pressure (8.4 cmH_2O). Vertical calibration 7.2 cmH_2O, horizontal calibration 1 min. In (b), cumulative concentration-response relationship for enhanced gastric motility (GM) by captopril (○) is shown and also GM before captopril (●). The GM (ordinate scale) represents the area (cm^2) under IGP curve recorded over a period of 12 min at each concentration of captopril, which was applied (every 15 min) in an ascending order. Data are mean obtained from five preparations with s.d. shown by vertical bars.

(12 min) representing the gastric motility increased in a dose-dependent manner to cumulative concentrations of captopril between 0.3 μM and 3.0 μM. The maximum cumulative response at 6 μM captopril was 55.7 ± 6.91 cm^2 (n = 5) and the EC_{50} was 0.77 ± 0.27 μM captopril.

A single exposure to a submaximally-effective concentration (1.4 μM) of captopril enhanced the GM by 155 ± 14.9% (Table 1). This submaximally-effective concentration was used in all subsequent experiments.

To test if the drug had any desensitizing effect, four preparations were re-exposed to the same concentration (1.4 μM) of captopril, after 30 min wash following the first. The IGP response (GM) to the second exposure was found to be 58.5 ± 14.9 cm^2 against 43 ± 9.7 cm^2 of the first response, suggesting a marginal but not statistically significant increase in the sensitivity and/or cumulative effect of the stomach to captopril. therefore, stomach preparations were exposed only

Table 1 Effect of aprotinin (100 u ml^{-1}; 45 min), saralasin (50 μM; 45 min), tetrodotoxin (TTX, 1 μM; 15 min), and atropine (1 μM; 15 min) on captopril (1.4 μM)-induced gastric motility (GM) *in vitro*

	Gastric motility (area; cm^2)			
Condition (n)	*Before captopril*	*After captopril*	*Change in GM* (Area; cm^2)	(%)
Control (9)	29.2 ± 2.11	74.5 ± 6.39[b]	45.3 ± 4.36	155 ± 14.9
Aprotinin (6)	25.7 ± 3.17[a]	32.1 ± 2.50	6.4 ± 0.95	25 ± 3.7
Saralasin (5)	25.0 ± 2.24[a]	75.0 ± 6.04[b]	50.0 ± 4.12	200 ± 16.5
Atropine (7)	28.5 ± 1.75	41.7 ± 2.93[b]	13.1 ± 2.02	46 ± 7.1
TTX (5)	15.8 ± 1.14[a]	17.1 ± 1.03	1.3 ± 0.34	8 ± 2.2

Data are mean ± s.d. and n denotes the number of preparations. The GM refers to the area under the IGP curve, measured with reference to atmospheric pressure, over 12 min immediately before (after pretreatment) or after captopril.

[a] Significantly different from the control GM, recorded in preparations without pretreatment; unpaired t test ($P < 0.05$).

[b] Significantly different from the control GM, recorded before captopril; t test for paired data ($P < 0.001$).

In all the conditions, percentage change in GM caused by captopril is significantly ($P < 0.001$; unpaired t test) different from that of the control.

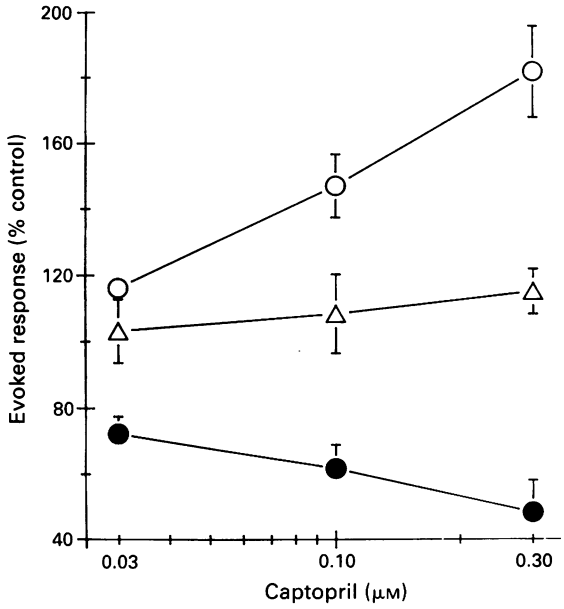

Figure 2 Effect of captopril on gastric contractions evoked by 4 nM bradykinin (BK) (○), 10 nM angiotensin-I (A-I) (●) and 0.4 μM acetylcholine (ACh) (△). The evoked contractions (ordinate scale), representing the increase in the area (cm^2) under the IGP response curve (2 min) with reference to the pre-existing basal IGP, are presented as percentage of control responses which were $4.7 \pm 0.64\,cm^2$ for BK, $3.1 \pm 0.30\,cm^2$ for A-I and $16.2 \pm 1.63\,cm^2$ for ACh (see Table 2). Data are mean obtained from five to six preparations with s.d. shown by vertical bars.

once to a single concentration (1.4 μM) of captopril in the subsequent experiments.

The subthreshold concentrations (<0.7 μM) of captopril, which failed to enhance the GM (see Figure 1b), caused concentration-dependent augmentation of BK-evoked contractions and attenuation of A-I-evoked contractions, without significantly affecting the contractions evoked by ACh (Figure 2).

Effect of tetrodotoxin and atropine on captopril-induced gastric motility

The enhanced GM produced by captopril (1.4 μM) was reduced by 95% (155 ± 14.9% vs. 8.0 ± 2.2%) in the presence of TTX (1 μM), while the spontaneous GM was decreased by about 46% of control (Table 1; before captopril). Similarly, atropine (1 μM) inhibited the captopril-induced increase in GM by about 70%, without significantly affecting the spontaneous GM. The residual motor response caused by captopril, resistant to atropine (1 μM), was regarded as non-cholinergic in nature since a ten fold increase in atropine concentration (10 μM) did not attenuate further the captopril response ($12.3 \pm 2.55\,cm^2$; $n = 3$).

Effect of aprotinin and saralasin

Aprotinin (100 u ml^{-1}), a known competitive antagonist to the kinin-generating enzyme kallikrein (Vogel & Werle, 1970), markedly inhibited the captopril-induced GM by 84% (155 ± 14.9% vs. 25 ± 3.7%) while it attenuated the spontaneous GM marginally by about 12%.

The angiotensin antagonist, saralasin neither attenuated the spontaneous GM nor antagonized the ability of captopril to enhance GM (Table 1). Even when the concentration of saralasin was doubled to 100 μM, the captopril (1.4 μM)-induced increase in GM was still $176 \pm 25.8\,cm^2$ of control ($n = 3$).

Table 2 Effect of aprotinin, saralasin, atropine and tetrodotoxin (TTX) on evoked gastric contractions to bradykinin (BK), acetylcholine (ACh) and transmural stimulation (TMS, 100 pulses of 0.2 ms at 10 Hz)

	Evoked gastric contraction (area; cm^2)		
Antagonist	*BK* (4 nM)	*ACh* (0.4 μM)	*TMS*
Control	4.7 ± 0.65 ($n = 6$)	15.7 ± 2.17 ($n = 5$)	2.3 ± 0.69 ($n = 9$)
Aprotinin (100 u ml^{-1})	1.3 ± 0.61* ($n = 6$)	16.2 ± 1.63 ($n = 6$)	2.1 ± 0.88 ($n = 4$)
Saralasin (50 μM)	5.1 ± 0.74 ($n = 5$)	17.5 ± 3.91* ($n = 5$)	2.7 ± 0.94 ($n = 4$)
Atropine (1 μM)	1.7 ± 0.55* ($n = 5$)	Abolished ($n = 7$)	0.8 ± 0.66* ($n = 4$)
TTX (1 μM)	1.0 ± 0.67* ($n = 5$)	Abolished ($n = 3$)	Abolished ($n = 5$)

Data are mean ± s.d. and n denotes the number of preparations.
Significantly different from their respective control; * $P < 0.001$ unpaired t test.

Effect of the antagonists on contractions evoked by bradykinin, acetylcholine and transmural nerve stimulation

Data describing the action of aprotinin, saralasin, atropine and TTX, against the contractions evoked by BK (4 nM), ACh (0.4 μM), and TMS are summarized in Table 2. Aprotinin (100 u ml^{-1}; 45 min) attenuated BK-evoked contractions without significantly affecting those evoked by ACh and TMS. Saralasin (50 μM; 45 min), which attenuated the contractions to A-I (10 nM) by 77% ($0.7 \pm 0.59\,cm^2$ vs. $3.1 \pm 0.30\,cm^2$; $n = 6$), failed to attenuate the contractions to BK and ACh. TTX (1 μM; 15 min) markedly attenuated contractile responses to TMS, ACh and BK.

Discussion

The present study demonstrates that captopril (0.7–3 μM) produces a dose-dependent increase in spontaneous gastric motility (GM) *in vitro*. In addition, our findings showed a selective potentiation of BK-evoked contractions at lower concentrations (30–300 nM) of captopril, which failed to enhance the GM. This observation is consistent with the previous work which demonstrated the BK-potentiating action of captopril in the guinea-pig ileum (Rubin *et al.*, 1978) and in ferret tracheal smooth muscle *in vitro* (Dusser *et al.*, 1988). The GM induced by captopril (1.4 μM) seems to be associated with undegraded endogenous kinins, accumulated during ACE inhibitory action. Thus, aprotinin, which inhibits the generation of endogenous kinins by suppressing the activity of plasma and glandular kallikreins both *in vitro* and *in vivo* (Vogel & Werle, 1970), not only selectively attenuated BK-evoked gastric contractions, but also markedly inhibited captopril-induced GM. It must, however, be mentioned that though aprotinin inhibits plasma and glandular kallikreins, it is a polyvalent inhibitor which affects many other proteolytic enzymes (Vogel & Werle, 1970).

Even though aprotinin and captopril are proteinase inhibitors, the former *per se* failed to mimic captopril in enhancing GM. This perhaps suggests that these proteinase inhibitors are likely to act on different enzyme systems through different mechanisms. It has been suggested that the enzyme inhibition caused by some of the proteinase inhibitors, like aprotinin, may result from the allosteric effects (Geiger & Kortmann, 1977) rather than by breaking down the enzyme (Mills *et al.*, 1975).

The involvement of A-I, accumulated during the ACE inhibition, in mediating captopril-evoked GM may be ruled out

because the captopril response was resistant to saralasin, a specific competitive angiotensin II antagonist (Pals & Masucci, 1973), at a concentration (50 μM) that blocked the gastric contractile response to A-I.

The present data cannot preclude the possibility that substance P may mediate captopril-induced GM, for captopril has been shown to potentiate substance P activity, presumably by inhibiting its degradation (Cascieri *et al.*, 1983). It is also possible that kinins accumulated during kininase inhibition may sensitize intrinsic nerve plexuses, especially those containing substance P.

Captopril appears to exert a nonspecific post-junctional action, as shown by its potentiating action on the spontaneous gastric motility (Figure 1), contractions evoked by exogenous BK (Figure 2), cholinergically-mediated contractions evoked by TMS and contractions evoked by exogenous ACh (unpublished observations). However, the fact that TTX blocked neurally-mediated contractions evoked by TMS or BK and strongly antagonized the captopril-induced GM, suggests that the nonspecific action of captopril may be due to the non-selective sensitization of intrinsic neurones by accumulated substance P or related tissue kinins.

The ability of atropine to attenuate the captopril response is indicative of the involvement of cholinergic neurones. The residual, atropine-resistant response is unlikely to be due to inadequate blockade of muscarinic ACh receptors because a ten times higher concentration of atropine failed to attenuate further captopril-induced GM. Instead, the atropine-resistant responses may involve a non-cholinergic excitatory neuronal system. In particular, certain neurally mediated non-cholinergic contractions of the guinea-pig ileum may be mediated by the release of substance P (Franco *et al.*, 1979; Leander *et al.*, 1981; Bertho *et al.*, 1982). As already discussed, captopril may either enhance the action of released substance P (by preventing its degradation) or it may enhance the release of substance P (via accumulated undegraded kinins).

This work was supported by the Indian Council of Medical Research (ICMR) and R.R. was an ICMR Research Fellow. Captopril was a gift from the Squibb Institute for Medical Research, New Jersey, U.S.A.

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(Received June 26, 1990
Revised November 5, 1990
Accepted November 27, 1990)

Br. J. Pharmacol. (1991), **102**, 831–836

Effect of *p*-chlorophenylalanine on release of 5-hydroxytryptamine from the rat frontal cortex *in vivo*

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1 Rats were given *p*-chlorophenylalanine (PCPA, 150 mg kg^{-1}, i.p.) to inhibit partially 5-hydroxytryptamine (5-HT) synthesis so that its concentration in the frontal cortex fell by about half. The effects of this treatment on frontal cortex dialysate 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) concentrations were determined before and after stimulation by increasing K^+ concentration in the perfusion fluid by 100 mM for 20 min. Rates of 5-HT synthesis as indicated by the effects of 3-hydroxybenzylhydrazine (NSD 1015, 150 mg kg^{-1}, i.p.) on frontal cortex tissue and dialysate 5-hydroxytryptophan (5-HTP) and dialysate 5-HIAA were also measured in rats that had not been stimulated with K^+.

2 Dialysate 5-HT and 5-HIAA concentrations of both vehicle- and PCPA-treated rats fell into major (group 1) and minor (group 2) populations statistically distinguishable from each other by the high 5-HT and low 5-HIAA values of the latter group.

3 In group 1 animals, PCPA decreased both the dialysate 5-HT concentration and its rise following stimulation by K^+ in proportion with the decrease of 5-HT in frontal cortex tissue. 5-HIAA fell more markedly than 5-HT and in similar proportion in both tissue and dialysate. The fall of dialysate 5-HIAA on stimulation by K^+ was also attenuated to the same degree. The elevated 5-HT/5-HIAA ratios after PCPA treatment imply increased conservation of the depleted 5-HT stores.

4 PCPA decreased the above 5-HIAA values and the effects of NSD 1015 on tissue 5-HTP or dialysate 5-HIAA concentrations in similar proportion. However, PCPA had little effect on corresponding dialysate 5-HTP values.

5 The results are discussed with respect to relationships between synthesis, storage and release of 5-HT. They indicate that (under the conditions of the present study) the availability of 5-HT to receptors is directly proportional to total vesicular stores under both basal conditions and during neuronal firing.

Keywords: *p*-chlorophenylalanine; 5-hydroxytryptamine; *in vivo* dialysis

Introduction

The technique of *in vivo* dialysis permits repetitive monitoring of central extracellular transmitter concentrations and can thus be used to study mechanisms of transmitter release. Recent investigations on 5-hydroxytryptamine (5-HT) by Auerbach *et al.* (1989), Kalen *et al.* (1988) and Carboni & DiChiara (1989) show that various drug treatments influence dialysate 5-HT levels in the expected direction. Other studies reveal that levels rise during motor activity or handling (Kalen *et al.*, 1989). In our own laboratory, *in vivo* dialysis was used to study the release of brain 5-HT following its depletion by reserpine (Hutson & Curzon, 1989), to obtain direct evidence that the behavioural effects of *p*-chloroamphetamine (PCA) are mediated by release of 5-HT from the neuronal cytoplasm (Adell *et al.*, 1989) and to show that the novel antidepressant, tianeptine, attenuated K^+-evoked 5-HT release (Whitton *et al.*, 1991).

In the present study, brain 5-HT stores were partially depleted by means of the 5-HT synthesis inhibitor *p*-chlorophenylalanine (PCPA) and *in vivo* dialysis used to determine the consequences for basal dialysate 5-HT concentration and K^+-evoked 5-HT release. These investigations extend earlier findings on the effect of PCPA on behavioural responses to tryptophan (Marsden & Curzon, 1976) and to PCA (Curzon *et al.*, 1978).

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Methods

Animals

Male Sprague-Dawley rats (Charles River, U.K., weight 200–250 g) were housed individually under a 12 h light-dark cycle (lights on 06 h 00 min) at 20°C ± 1°C for 5 days before experimentation. Rat diet (Labsure, Poole, Dorset) and water were freely available. The animals were injected with *p*-chlorophenylalanine methyl ester (PCPA), 150 mg kg^{-1} i.p. in 0.9% NaCl (n = 15) or with 0.9% NaCl (n = 19) in a volume of 1.0 ml kg^{-1} at 09 h 00 min–10 h 00 min before implantation of dialysis probes at 13 h 00 min–16 h 00 min.

Probe implantation

Concentric dialysis probes (5 mm membrane length, 200 μm diameter) were made essentially as described by Hutson *et al.* (1985) except that the internal glass capillary tubes were replaced by vitreous silica tubing (SGE, Milton Keynes) and Filtral 12 (Hospal, Rugby), was used for the dialysis membrane. *In vitro* recoveries from probes of this type were as follows: 5-HTP 22%, 5-HT 32%, 5-HIAA 23% when determined as Adell *et al.* (1989) but with flow rate = 2 μl min^{-1}. Results in Figures and Tables were not corrected for recovery.

After anaesthesia with pentobarbitone (Sagatal, May and Baker, 60 mg kg^{-1}, i.p.) probes were implanted into the frontal cortex using coordinates according to Paxinos & Watson (1982) as follows: 3.2 mm anterior and 6.0 mm ventral to bregma, 1.2 mm lateral to the midline. Probes were implanted without a guide cannula. The dialysis sac was kept rigid

during implantation by slowly passing perfusion fluid through it.

Perfusion experiments

Perfusion experiments were started 24 h after giving PCPA (vehicle-treated rats, $n = 19$; PCPA-treated rats, $n = 15$) by passing fluid (of composition (mM): NaCl 125, KCl 2.5, $MgCl_2$ 1.18 and $CaCl_2$ 1.26) through the probe. A CMA pump (Carnegie Medicin, Sweden), producing a flow rate of 2 μl min^{-1} was used. The dead volume between the probe and collecting vial was approx. 5 μl which corresponded to a flow time of 2.5 min. Figures were not corrected for this. After a 20 min washout period, four 40 min dialysate samples were collected into 0.4 ml reaction vials (L.I.P., Shipley, Yorks). Samples were frozen on solid CO_2 and stored at −70°C until analysis of indolic constituents within the next 3 days.

Effect of stimulation by K^+ Perfusion was continued and the effect of stimulation by K^+ investigated by substituting KCl (100 mM) for an equimolar concentration of NaCl in the perfusion fluid of 6 vehicle- and 6 PCPA-treated rats for 20 min. A 20 min dialysate sample was taken during this period. Four further 20 min samples were taken during subsequent passage of the normal perfusion fluid.

Effect of 3-hydroxybenzylhydrazine Animals that had not been treated with K^+ but perfused throughout with normal fluid were then given the aromatic decarboxylase inhibitor 3-hydroxybenzylhydrazine (NSD 1015, 150 mg kg^{-1}, i.p.) as previously used to determine 5-hydroxytryptophan (5-HTP) accumulation as an index of the rate of 5-HT synthesis (Carlsson *et al.*, 1972). Three 10 min dialysate samples were then usually collected (though in a few cases only two samples were obtained) and the rats immediately killed by decapitation and their brains rapidly removed, the dialysis probes withdrawn and frontal cortices dissected out, frozen on solid CO_2 and stored at −70°C until analysis within the next 2 weeks.

Biochemical analysis

5-HTP, 5-HT and 5-HIAA were determined by high performance liquid chromatography (h.p.l.c.). Dialysate was analysed with an Ultrasphere 3 μm ODS analytical column (7.5 cm × 4.6 mm i.d.) (Beckman) with an 0.5 μm high resolution filter (Anachem, Upchurch, U.K.) but no guard column. The mobile phase consisted of 0.1 M potassium dihydrogen phosphate buffer, 1 mM octyl sodium sulphate and 0.1 mM Na-EDTA, pH 2.75, with 18% v/v methanol. This was passed through a 0.2 μm filter (Millipore, U.K.) under vacuum and degassed with helium before use. A typical flow rate of 1.2 ml min^{-1} gave the following retention times (min): 5-HIAA 2.50, 5-HTP 2.84, 5-HT 4.88.

An electrochemical detector (Coulochem 5100A, Severn Analytical, U.K.) with porous graphite electrodes was used. The mobile phase was conditioned by means of a model 5020 guard cell set at +0.60 V and sited on line prior to a Rheodyne 7125 (50 μl loop) injection valve. A high sensitivity analytical cell (model 5011) with detector 2 set at +0.35 V with respect to the reference electrodes was positioned immediately after the column and filter assembly.

Brain tissue samples were treated and analysed as described by Adell *et al.* (1989). Chromatographic data were recorded and concentrations determined by Hitachi Merck D2000 integrators. The detection limit for 5-HT was approx. 0.1 fmol μl^{-1} (3 × baseline noise).

Drugs

DL-*p*-Chlorophenylalanine methyl ester hydrochloride (PCPA) and *m*-hydroxybenzylhydrazine dihydrochloride (NSD 1015) were obtained from Sigma Chemical Co.

Results

Basal dialysate 5-HT and 5-HIAA concentrations

Dialysate 5-HT and 5-HIAA concentrations and 5-HT/5-HIAA ratios for individual vehicle and PCPA-treated rats were essentially constant but values for different animals varied over a wide range (Figure 1). It was noted that the four vehicle-treated rats with the highest 5-HT concentrations had 5-HIAA concentrations which were well below the overall mean value. These animals therefore had very high 5-HT/5-HIAA ratios. Most of the rats (designated as group 1) were significantly distinct from this group (group 2) with respect to all three of the above variables. The PCPA-treated rats could also be divided into groups 1 and 2, the corresponding differences for 5-HT and 5-HT/5-HIAA being significant and the mean 5-HIAA value for group 2 animals being lower than that for group 1, albeit not significantly so. The 5-HT and 5-HIAA concentrations of group 1 were significantly decreased by PCPA and the mean 5-HT/5-HIAA ratio substantially (but not significantly) increased. The 5-HT and 5-HIAA values for vehicle-treated rats (group 1) approached a significant positive correlation with each other ($r = 0.54$, $P = 0.058$, $n = 13$).

Effect of stimulation by K^+ (100 mM) on dialysate 5-HT and 5-HIAA

A 20 min pulse of K^+ (100 mM) increased 5-HT in the dialysate collected between 0 and 60 min after starting the pulse (Figure 1). Values for the vehicle pretreated rats attained a plateau at 20 to 40 min of about four times the basal values and then returned to approximately the previous levels. There was a substantial associated fall of dialysate 5-HIAA which was maximal at 40 min. Animals pretreated with PCPA showed significantly smaller effects. Area under the curve (AUC) values for the 5-HT and 5-HIAA changes are given in the legend of Figure 2.

Effect of NSD 1015 (150 mg kg^{-1}, i.p.) on dialysate 5-HTP and 5-HIAA of rats not treated with K^+

This experiment was performed to determine whether the effect of PCPA on 5-HT synthesis as indicated by tissue 5-HTP accumulation after giving NSD 1015 (Table 1) was paralleled in the dialysates. As tissue findings on group 1 and 2 were similar, animals from both groups were included. Dialysate 5-HTP concentration increased linearly in samples taken between 10 and 30 min after giving the aromatic amino acid decarboxylase and monoamine oxidase inhibitor NSD

Table 1 5-Hydroxytryptamine (5-HT) metabolism in vehicle- and *p*-chlorophenylalanine (PCPA, 150 mg kg^{-1}, i.p.)-treated rats 30 min after giving 3-hydroxybenzylhydrazine (NSD 1015, 150 mg kg^{-1}, i.p.): frontal cortex tissue

	Vehicle	*PCPA*
5-HTP (pmol g^{-1} h^{-1})	1830 ± 130 (10)	420 ± 50 (8)**
5-HT (pmol g^{-1})	1600 ± 95 (9)	700 ± 90 (8)**
5-HIAA (pmol g^{-1})	1000 ± 70 (10)	230 ± 20 (8)**
5-HT/5-HIAA	1.69 ± 0.09 (9)	3.62 ± 0.78 (8)*

Vehicle or PCPA was given 28 h 40 min before injection of NSD 1015 and the rats killed 30 min later. Values are means ± s.e. with numbers of rats in parentheses. Data on both group 1 and group 2 rats (see Figure 1) are included as their tissue values differed only negligibly. Differences from vehicle treated rats: * $P < 0.05$, ** $P < 0.001$ (Student's *t* test). 5-HTP: 5-hydroxytryptophan; 5-HIAA: 5-hydroxyindoleacetic acid.

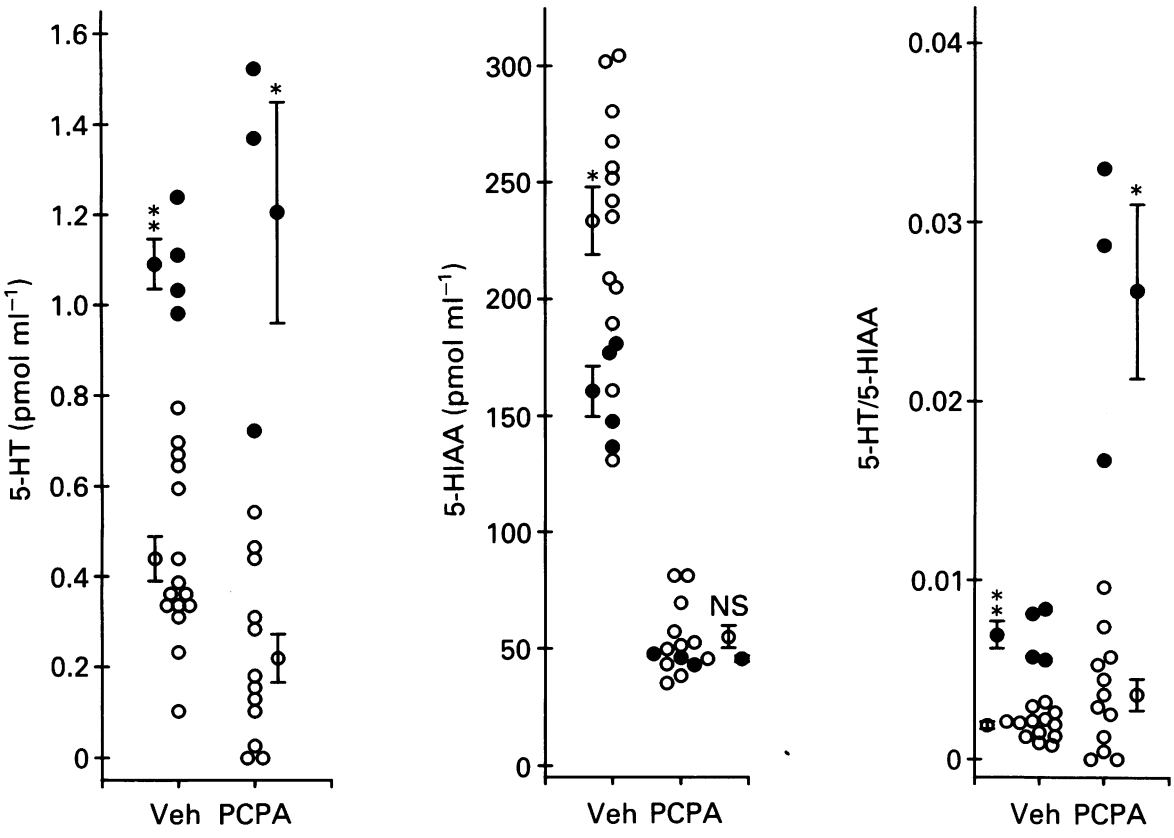

Figure 1 Basal 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations in frontal cortex dialysates: 0.9% NaCl or *p*-chlorophenylalanine (PCPA, 150 mg kg^{-1}) were given 24 h previously. (○) The main group of rats (group 1): vehicle, $n = 13$–15; PCPA, $n = 12$; (●) rats with high 5-HT and low 5-HIAA values (group 2): vehicle, $n = 4$; PCPA, $n = 3$. The bars show means ± s.e. for the two groups. Differences between groups 1 and 2: * $P < 0.05$, ** $P < 0.001$. Differences between vehicle- and PCPA-treated group 1 rats: 5-HT $P < 0.01$, 5-HIAA $P < 0.01$, 5-HT/5-HIAA non significant (Student's *t* test).

1015 (Figure 3). Rats pretreated with PCPA showed a rather lower rate of increase than the controls but the difference was not significant. Although dialysate 5-HIAA concentrations of the two groups only decreased slightly during the above period the changes were sufficient for the estimation of its rate of decrease. In the control animals, this was fairly comparable with the rate of 5-HT synthesis calculated from the rise of dialysate 5-HTP. Unlike the latter value the rate calculated from the fall of dialysate 5-HIAA was substantially and significantly decreased after PCPA treatment.

5-HT metabolism in frontal cortex tissue after giving NSD 1015 (150 mg kg^{-1}, i.p.) to rats not treated with K$^+$

Unlike the dialysate results, the frontal cortex tissue 5-HTP concentration of rats killed 30 min after giving NSD 1015 (i.e., immediately after taking the final dialysate sample) was strikingly decreased by PCPA pretreatment (Table 1). 5-HIAA was comparably decreased. As 5-HT was substantially but less markedly decreased by PCPA, the 5-HT/5-HIAA ratio rose significantly.

Comparison of effects of PCPA (150 mg kg^{-1}, i.p.) on indoles in frontal cortex dialysate and tissue

Effects of PCPA on dialysate and tissue values are shown in percentage form in Table 2. 5-HTP accumulation was strikingly decreased in brain tissue but not in dialysate. However dialysate 5-HT, its increase on K$^+$ stimulation and tissue 5-HT all fell by about half. PCPA decreased dialysate 5-HIAA, tissue 5-HIAA and the falls of dialysate 5-HIAA on both K$^+$ and NSD 1015 treatments to a greater extent than it decreased 5-HT. These 5-HIAA changes were proportionate to the fall of tissue 5-HTP. As PCPA decreased 5-HT values to a lesser extent than those of 5-HIAA, the 5-HT/5-HIAA ratios were increased in both dialysate and tissue though the change was statistically significant only in the latter case.

Table 2 Effect of *p*-chlorophenylalanine (PCPA) on indoles in the frontal cortex

	% *change*
5-HTP increase after NSD 1015	
Dialysate	−21 (NS)
Tissue	−77***
5-HT	
Dialysate (basal)	−50**
Dialysate (increase after K$^+$)	−46**
Tissue	−56***
5-HIAA	
Dialysate (basal)	−76**
Dialysate (decrease after K$^+$)	−80**
Dialysate (decrease after NSD 1015)	−82***
Tissue	−77***
5-HT/5-HIAA	
Dialysate	+90 (NS)
Tissue	+114*

The statistical significances shown refer to the differences between the values for vehicle- and PCPA-treated groups (see Figures 1, 2 and 3 and Table 1) from which the above % changes were calculated. Dialysate values are for group 1 (5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA)) and groups 1 and 2 combined (5-hydroxytryptophan (5-HTP)). Tissue values are for groups 1 and 2 combined (See Table 1). Significant changes: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NSD 1015: 3-hydroxybenzylhydrazine.

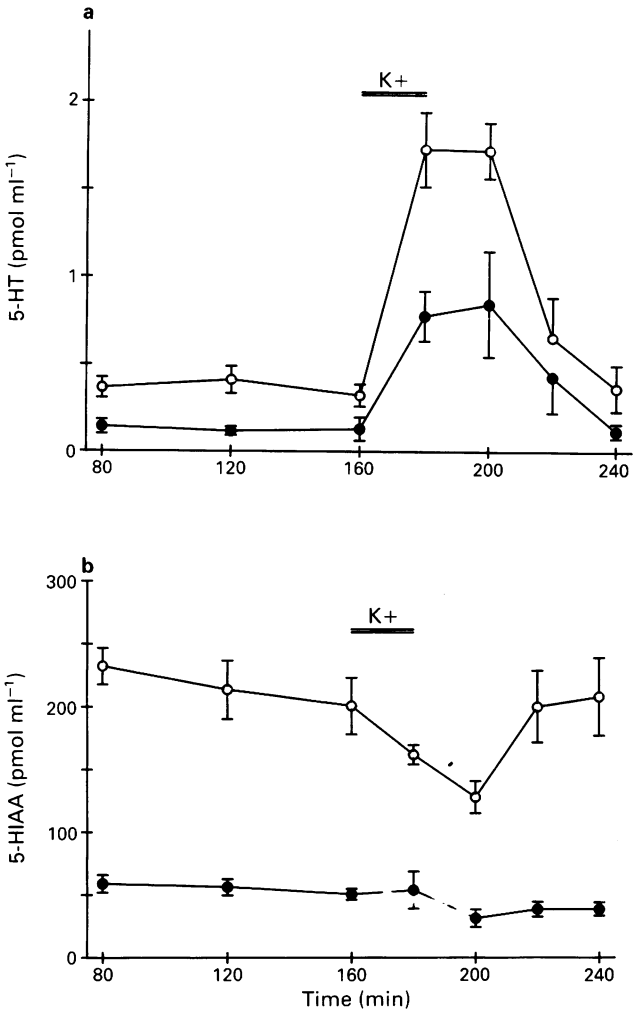

Figure 2 The effect of K^+ (100 mM, 20 min) in the perfusate on concentrations of (a) dialysate 5-hydroxytryptamine (5-HT) and (b) dialysate 5-hydroxyindoleacetic acid (5-HIAA). Treatment 27 h previously: (○) 0.9% NaCl; (●) *p*-chlorophenylalanine (PCPA, 150 mg kg^{-1}). Results are given as means with s.e. shown by vertical bars. All animals were from group 1 of Figure 1. Area under curve (AUC) values were calculated by summating changes at 180, 200 and 220 min after subtracting baseline concentrations (means of values obtained before and after the above period). AUC (pmol ml^{-1} h ± s.e. $n = 6$/group). 5-HT: 0.9% NaCl 2.82 ± 0.16, PCPA 1.53 ± 0.32, $P < 0.01$; 5-HIAA: 0.9% NaCl 151 ± 50, PCPA 30 ± 13, $P < 0.001$ (Student's *t* test).

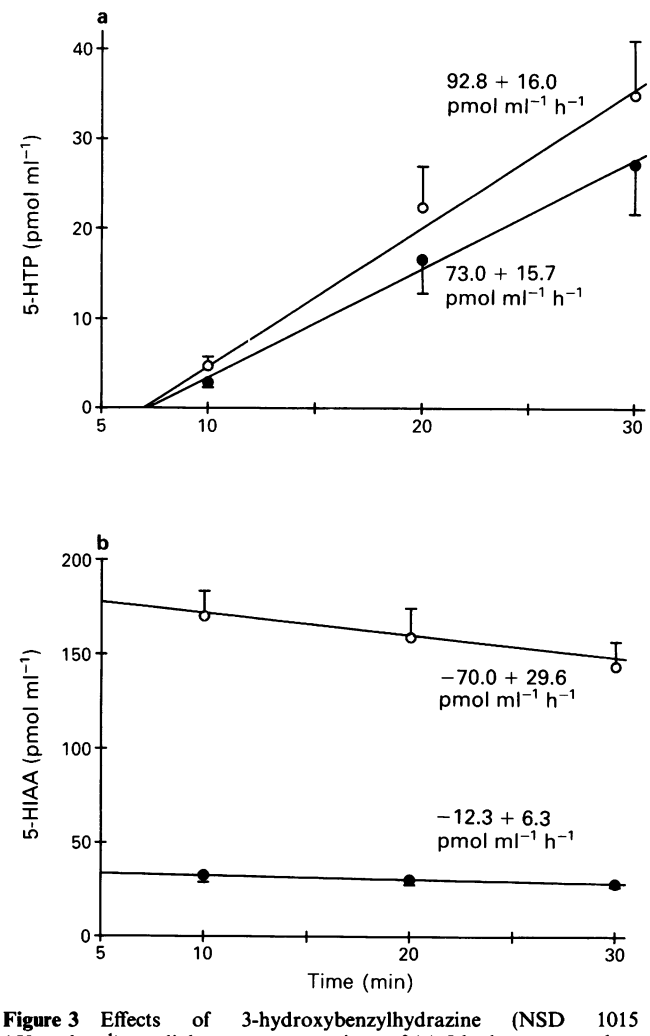

Figure 3 Effects of 3-hydroxybenzylhydrazine (NSD 1015 150 mg kg^{-1}) on dialysate concentrations of (a) 5-hydroxytryptophan (5-HTP) and (b) 5-hydroxyindoleacetic acid (5-HIAA) in rats not treated with K^+. (○) 0.9% NaCl; (●) *p*-chlorophenylalanine (PCPA, 150 mg kg^{-1}) both given 28 h 40 min previously. Values are means + or − s.e. (vertical bars). $n = 9$–10. The lines derive from the least-square analysis of best fit for values at 10, 20, 30 min after giving NSD 1015. Slopes ± s.e. are shown. The effect of PCPA on the rate of decrease of 5-HIAA was significant ($P < 0.05$), Duncan's test after 1-way ANOVA.

Discussion

Before discussing the main body of results, it is necessary to consider the small group of rats with markedly high basal dialysate 5-HT and rather low 5-HIAA concentrations (group 2, Figure 1). The data on these animals were statistically distinguishable from that obtained on the other rats (group 1, Figure 1). Its unusual characteristics could reflect particularly high basal firing rates due to intrinsic neurophysiological differences of the rats or to some non-apparent variable. Increased firing seems reasonable as high cortical dialysate 5-HT and low 5-HIAA values also occur on both handling (Kalen *et al.*, 1989) and stimulation with K^+ (Figure 2). The latter finding agrees with previous dialysis studies (Kalen *et al.*, 1988; Auerbach *et al.*, 1989; Carboni & DiChiara, 1989). It is relevant that transport of 5-HIAA from neurones has been suggested to be impaired during the depolarized state which occurs when 5-HT is actively released (Miyamoto *et al.*, 1990).

The remaining animals (group 1) had basal dialysate 5-HT concentrations of about 0.14% of the 5-HIAA values (after correction for the different *in vitro* recoveries of the two indoles) which may be compared with the value of 0.18% calculable from previous data obtained in our laboratory (Adell *et al.*, 1989). While the rats in group 2 had high basal 5-HT and low basal 5-HIAA, those in group 1 showed a positive correlation between basal 5-HT and 5-HIAA which approached statistical significance. In view of the findings of Miyamoto *et al.* (1990) this could imply 5-HT release unrelated to firing. Whether basal dialysate 5-HT reflects firing or not it probably largely originates in 5-HT vesicles as basal values are greatly decreased by reserpine (Adell *et al.*, 1989; Carboni & DiChiara, 1989) which disrupts the vesicles (Shore & Giachetti, 1978).

Numerous behavioural experiments (Harvey *et al.*, 1975; Marsden & Curzon, 1976; Curzon *et al.*, 1978; Borbely *et al.*, 1981) suggest that depletion of brain 5-HT stores by PCPA decreases the availability of the transmitter to receptors. However, it has not previously been clear whether release on neuronal firing is directly proportional to neuronal stores or whether partially depleted stores are mobilised so that they become more available for release. The present findings support the former alternative (at least with respect to the experimental conditions used) as PCPA decreased tissue 5-HT, basal dialysate 5-HT and the rise of dialysate 5-HT on

K^+ stimulation all to similar degrees (Table 2). The concentrations of 5-HIAA in both tissue and basal dialysate and 5-HIAA changes on both NSD 1015 (see below) and K^+ treatment were also decreased in proportion to each other but more markedly than the changes of 5-HT. The resultant increased 5-HT/5-HIAA ratios agree with previous findings on brains of rats given PCPA at dosages sufficient to deplete partially central 5-HT (Marsden & Curzon, 1976; Curzon *et al.*, 1978; Dickinson & Curzon, 1983).

The above tissue 5-HT and 5-HIAA values were obtained after giving NSD 1015 in order to determine the rate of 5-HT synthesis. However, as the drug not only inhibits aromatic amino acid decarboxylase but also monoamine oxidase, brain 5-HT concentration is unaltered 30 min after giving NSD 1015 (Carlsson *et al.*, 1972). This result and the slow decreases of dialysate 5-HIAA after giving the drug suggest that tissue levels of the two indoles obtained 30 min after giving NSD 1015 provide good indices of their values in its absence.

Tissue 5-HTP and 5-HIAA changes after PCPA treatment both point to essentially identical decreases of the rate of 5-HT synthesis. Corresponding decreases of 5-HT were less marked, consistent with increased conservation of the transmitter due to more effective storage in unfilled vesicles (Segawa & Fujisawa, 1972; Taber & Anderson, 1973).

Unlike the tissue values, the rate of rise of dialysate 5-HTP after treatment with NSD 1015 was only decreased by PCPA to a small and statistically insignificant extent (Figure 3) although the associated rate of fall of 5-HIAA was decreased in parallel with the other 5-HIAA changes. The anomalous 5-HTP data are explicable by analogy with the stimulation of tryptophan efflux from synaptosomes by other large neutral amino acids (Grahame-Smith & Parfitt, 1970) since it suggests that as PCPA, like 5-HTP, is a large neutral amino acid it stimulated efflux of the latter into extracellular fluid. As PCPA has a half-life of about 3 days (Koe & Weissman, 1966) its concentration in the brain would still be high at the time of the experiment. It should be noted that as 5-HTP is rapidly decarboxylated to 5-HT intraneuronally, its concentration is normally very low (Lindqvist *et al.*, 1975) so that it was detected in the extracellular fluid only when decarboxylation was prevented by NDS 1015.

The present neurochemical evidence that the availability of 5-HT to receptors on neuronal firing is proportional to brain 5-HT concentration agrees with a behavioural study of rats with 5-HT synthesis partially inhibited by PCPA (Marsden & Curzon 1976). These animals exhibited hyperlocomotion which was reversed by tryptophan in parallel with the degree of re-attainment of normal 5-HT levels. In contrast to these findings, when 5-HT was released by PCA, the decrease of behavioural response in PCPA-pretreated rats was greater than predictable from the fall of brain tissue 5-HT (Curzon *et al.*, 1978). This result is explicable by a combined *in vivo* dialysis and behavioural study of the effect of PCA on reserpine-treated rats (Adell *et al.*, 1989) which confirmed earlier *in vitro* evidence (Ross & Kelder, 1977) that PCA releases 5-HT, not from vesicles, but from the neuronal cytoplasm. Enhanced vesicular uptake of 5-HT after PCPA treatment (Segawa & Fujisawa, 1972; Taber & Anderson, 1973) would presumably result in the cytoplasmic pool being more markedly depleted than suggested by the brain tissue 5-HT changes.

The above findings, taken together, suggest that when 5-HT synthesis is partly inhibited by PCPA, both brain 5-HT and its availability to receptors are decreased in similar proportion. The relative effects on 5-HT and 5-HIAA suggest that conservation of the transmitter is concurrently facilitated by transport from the small cytoplasmic pool (where it is vulnerable to attack by monoamine oxidase) to the vesicular pool from which 5-HT is released on neuronal firing. A similar mechanism could conceivably have a compensatory role in disorders in which defective 5-HT synthesis is implicated.

The Medical Research Council and (for C.M.P.) the Centro Nazionale Ricerca are thanked for financial support.

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(Received October 10, 1990
Revised November 7, 1990
Accepted November 29, 1990)

Br. J. Pharmacol. (1991), **102**, 837–840

Influence of acute and chronic chlorimipramine treatment on the 5-HT receptor-mediated modulation of acetylcholine release from the cerebral cortex of freely moving guinea-pigs

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1 Acetylcholine (ACh) release from the cerebral cortex of freely moving guinea-pigs, implanted with epidural cups, was studied.

2 A single dose of chlorimipramine (Cl-Imip, 10 mg kg^{-1}, s.c.), reduced the cortical ACh release both in normal and in chronically (10 mg kg^{-1} daily, s.c., for 14 days) Cl-Imip-treated guinea-pigs; the 5-HT$_3$ antagonist MDL 72222 (1 mg kg^{-1}, s.c.) antagonized this effect.

3 A single dose of Cl-Imip significantly reduced the effect of the 5-HT$_{1A}$ agonist 8-hydroxy-2-(di-n-propylaminotetralin) (8-OH-DPAT, 0.1 mg kg^{-1}, s.c.), which nearly doubled the cortical ACh release in control animals. MDL 72222 restored to normal the response to 8-OH-DPAT reduced by the antidepressant.

4 A single dose of Cl-Imip did not change the inhibitory, MDL 72222-sensitive, effect induced by the 5-HT$_3$ agonist 2-methyl-5-hydroxytryptamine (2-methyl-5-HT, 500 μg, i.c.v.).

5 In chronically Cl-Imip-treated guinea-pigs, the facilitatory effect of 8-OH-DPAT was no longer present, while the inhibitory, MDL 72222-sensitive, effect of 2-methyl-5-HT was maintained.

6 These results indicate that the 5-HT$_{1A}$ receptor-mediated increase in ACh release is reduced by prolonged Cl-Imip treatment, while the 5-HT$_3$ receptor-mediated inhibition of ACh release is unaffected. The relevance of these findings to the antidepressant mechanism of Cl-Imip is discussed.

Keywords: Acetylcholine release; 5-HT receptors; antidepressants; chlorimipramine

Introduction

Antidepressant agents are known to affect the biochemistry and function of 5-hydroxytryptamine (5-HT) neurones in the brain (Heninger & Charney, 1987). The subdivision of 5-HT receptors into at least three broad classes, 5-HT$_1$, 5-HT$_2$ and 5-HT$_3$ (Bradley *et al.*, 1986), has focused interest on the role played by each subtype in the mechanism of action of antidepressant drugs. Because of the delayed onset of clinical efficacy with antidepressant drugs, most attention has been given to the adaptive changes in 5-hydroxytryptaminergic mechanisms following long-term antidepressant administration. Thus, behavioural (Goodwin *et al.*, 1985), electrophysiological (Blier *et al.*, 1988) and neuroendocrine (Wozniak *et al.*, 1989) paradigms, as well as changes in 5-HT receptor binding (Peroutka & Snyder, 1980), have been investigated.

5-HT modulation of acetylcholine (ACh) release from the cerebral cortex of freely moving guinea-pigs has been previously reported (Bianchi *et al.*, 1986). It has been shown that activation of 5-HT$_{1A}$ autoreceptors induces facilitation of ACh release (Siniscalchi *et al.*, 1990a; Bianchi *et al.*, 1990), while 5-HT$_3$ receptors inhibit ACh release (Bianchi *et al.*, 1990). Thus, the *in vivo* study of ACh release modulation provides a neurochemical correlate of 5-HT$_{1A}$ and 5-HT$_3$ receptor activation and may unmask possible changes in their functions, following antidepressant treatment. Part of the present findings have been the subject of a communication (Siniscalchi *et al.*, 1990b).

Methods

Guinea-pigs of either sex weighing 400–500 g, were treated with chlorimipramine (Cl-Imip), 10 mg kg^{-1} s.c. once a day, for 14 days. Another group of animals (controls) received only vehicle. On the 13th day, the animals were pretreated with midazolam, 7.5 mg kg^{-1} i.p. and anaesthetized with ketamine, 90 mg kg^{-1} i.p. A perspex cup (0.8 ml vol) was screwed into the right parietal bone and a stainless steel guide cannula for intracerebroventricular (i.c.v.) injection was also implanted (Beani *et al.*, 1978). Morphine, 2 mg kg^{-1} i.p., was administered on the day of the surgery. The experiments were carried out two days after surgery, i.e. 24 h after the last injection of Cl-Imip or vehicle. The release of ACh was measured by filling the epidural cups with 0.25 ml of Ringer solution containing physostigmine (0.3 mM), which was collected and renewed every 30 min. Generally, the experiments consisted of 5–6 collection periods so that any normal and drug-induced changes in ACh release could be reliably assessed.

The ACh present in the samples was bioassayed on tetrodotoxin-pretreated guinea-pig ileum (Beani *et al.*, 1978).

Drugs

The drugs used were: 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, RBI), 2-methyl-5-hydroxytryptamine (2-methyl-5-HT, Sandoz), 1αH,3α,5αH-tropan-3yl-3,5-dichlorobenzoate (MDL 72222) (Merrel Dow), midazolam (Roche), ketamine (Parke Davis), morphine HCl (Salars), chlorimipramine (Geigy) and tetrodotoxin (Sigma).

Statistical analysis

The statistical significance of the differences between the data before and after treatment, as well as between groups, was determined with ANOVA, followed by Student's *t* test for paired or non paired data.

Results

Acute administration of chlorimipramine

As shown in Table 1, Cl-Imip, 10 mg kg^{-1} s.c., acutely administered to freely moving guinea-pigs 30 min after a s.c. injection of saline, significantly reduced the cortical ACh release. Onset of the effect was slow, reaching its maximum 90 min

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Table 1 Effect of chlorimipramine (Cl-Imip, 10 mg kg^{-1} s.c.) on the release of acetylcholine (ACh) from the cerebral cortex of freely moving guinea-pigs pretreated (30 min before) with saline or MDL 72222 (1 mg kg^{-1} s.c.)

			ACh release				
					After Cl-Imip		
Pretreatment	No expts.	Before Cl-Imip (control)	30 min	60 min	90 min	120 min	150 min
Saline	5	147 ± 25	158 ± 34 (104)	122 ± 28* (81)	97 ± 17* (69)	102 ± 11* (76)	105 ± 11* (77)
MDL 72222	4	154 ± 23	155 ± 23 (102)	144 ± 24 (95)	144 ± 23 (95)	167 ± 26 (109)	—

* $P < 0.05$, significantly different from the controls, Student's t test for paired data.
Values are pmol cm^{-2} 30 min^{-1} ± s.e.mean. In parentheses the percentage of control values.

after administration, and lasted for over 2 h. Cl-Imip-induced inhibition was antagonized by the 5-HT$_3$ receptor antagonist, MDL 72222, 1 mg kg^{-1} s.c. (Table 1). MDL 72222 was devoid of any direct effect on ACh release i.e. 127 ± 11 pmol ACh cm^{-2} 30 min^{-1} before MDL 72222, and 127 ± 8, 144 ± 22, 120 ± 4 and 130 ± 12 pmol cm^{-2} 30 min^{-1} at 30, 60, 90 and 120 min respectively after MDL 72222, ($n = 5$).

Table 2 shows the facilitatory effect of the 5-HT$_{1A}$ agonist 8-OH-DPAT, 0.1 mg kg^{-1} s.c., on ACh release in control animals. Pretreatment with Cl-Imip, 10 mg kg^{-1} s.c. 30 min before 8-OH-DPAT, significantly reduced the facilitation induced by 8-OH-DPAT at 60 min (i.e. 90 min after Cl-Imip). MDL 72222 did not modify the ACh release increased by the 5-HT$_{1A}$ agonist (to 161% at 60 min, $n = 2$) and restored it to normal values in guinea-pigs acutely pretreated with Cl-Imip (Table 2).

In agreement with previous data (Bianchi *et al.*, 1990) 2-methyl-5-HT, 500 μg i.c.v., reduced cortical ACh release in control animals; this effect was antagonized by MDL 72222, 1 mg kg^{-1} s.c. (Figure 1a). Pretreatment of the guinea-pigs with Cl-Imip did not modify either the response to 2-methyl-5-HT, or the antagonism of this response by MDL 72222 (Table 3).

Chronic administration of chlorimipramine

As shown in Figure 2, in freely moving guinea-pigs, chronically (14 days) treated with Cl-Imip, 10 mg kg^{-1} daily s.c., 8-OH-DPAT, 0.1 mg kg^{-1} s.c., was no longer able to increase ACh release. However, 2-methyl-5-HT, 500 μg i.c.v., maintained its inhibitory, MDL 72222-sensitive effect (Figure 1b). In chronically treated animals, Cl-Imip, 10 mg kg^{-1} s.c., given 24 h after the last injection, still reduced ACh release: 138 ± 28 pmol ACh cm^{-2} 30 min^{-1} before Cl-Imip, reduced to 88%, 71%, 82% and 85% at 30, 60, 90 and 120 min respectively after Cl-Imip ($n = 5$). The latter values did not differ significantly from those for the saline-pretreated group (see Table 1).

Pretreatment with a single injection of Cl-Imip, 10 mg kg^{-1} s.c., did not modify the effects either of 8-OH-DPAT (Table 2), or of 2-methyl-5-HT (Table 3), challenged 24 h later.

Discussion

It is well known that Cl-Imip is a potent 5-HT uptake blocker, whereas its metabolite desmethylimipramine inhibits noradrenaline uptake (Rudorfer & Potter, 1989). The potencies and selectivities of antidepressant drugs as uptake inhibitors, on the other hand, do not appear sufficient alone to explain their clinical effect. In particular, blockade of amine uptake occurs rapidly (hours), while the clinical antidepressant effect has a long onset (weeks). Therefore, comparisons between the different effects produced by acute and chronic treatments may be useful in understanding the mechanisms involved in their antidepressant action.

ACh release from the cerebral cortex of freely moving guinea-pigs was reduced by acute administration of Cl-Imip. This effect was most likely due to the activation of 5-HT$_3$ receptors, since it was antagonized by MDL 72222. Cl-Imip may directly activate 5-HT$_3$ receptors, or, more likely, its action may be caused by increased levels of synaptic 5-HT: the delay in the onset of this Cl-Imip effect is compatible with the time required to attain uptake blockade (Rudorfer & Potter, 1989) and an increase of synaptic levels of 5-HT. Indeed, an inhibitory effect of 5-HT on ACh release, via 5-HT$_3$ receptors, has been demonstrated both *in vivo* (Bianchi *et al.*, 1990), and *in vitro* (Barnes *et al.*, 1989). When Cl-Imip was acutely administered before the 5-HT$_{1A}$ agonist 8-OH-DPAT, the facilitatory effect of the latter drug was significantly reduced.

Table 2 Effect of 8-hydroxy-2-(di-n-propylaminotetralin) (8-OH-DPAT, 0.1 mg kg^{-1} s.c.) on the release of acetylcholine (ACh) from the cerebral cortex of freely moving guinea-pigs pretreated (30 min before) with saline, chlorimipramine (Cl-Imip, 10 mg kg^{-1} s.c.) or Cl-Imip plus MDL 72222 (1 mg kg^{-1} s.c.)

			ACh release			
				After 8-OH-DPAT		
Pretreatment	No expts.	Before 8-OH-DPAT (control)	30 min	60 min	90 min	120 min
Saline[a]	8	125 ± 11	176 ± 16*** (141)	217 ± 22*** (178)	166 ± 22 (134)	116 ± 10 (94)
Cl-Imip[a]	6	106 ± 8	152 ± 15* (143)	142 ± 12° (135)	143 ± 12 (136)	135 ± 17 (129)
Cl-Imip[b]	3	132 ± 27	213 ± 12* (156)	199 ± 14* (164)	164 ± 23 (149)	159 ± 30 (135)
Cl-Imip[a] + MDL 72222	4	128 ± 6	170 ± 8* (134)	218 ± 17* (168)	158 ± 20 (122)	138 ± 22 (116)

Values are pmol ACh cm^{-2} 30 min^{-1} ± s.e.mean; in parentheses the percentage of control values. *$P < 0.05$; ***$P < 0.001$ significantly different from the corresponding controls, Student's t test for paired data. °$P < 0.05$ significantly different from saline and Cl-Imip + MDL 72222 groups, Student's t test for non paired data.
[a] 30 min before 8-OH-DPAT, [b] 24 h before 8-OH-DPAT.

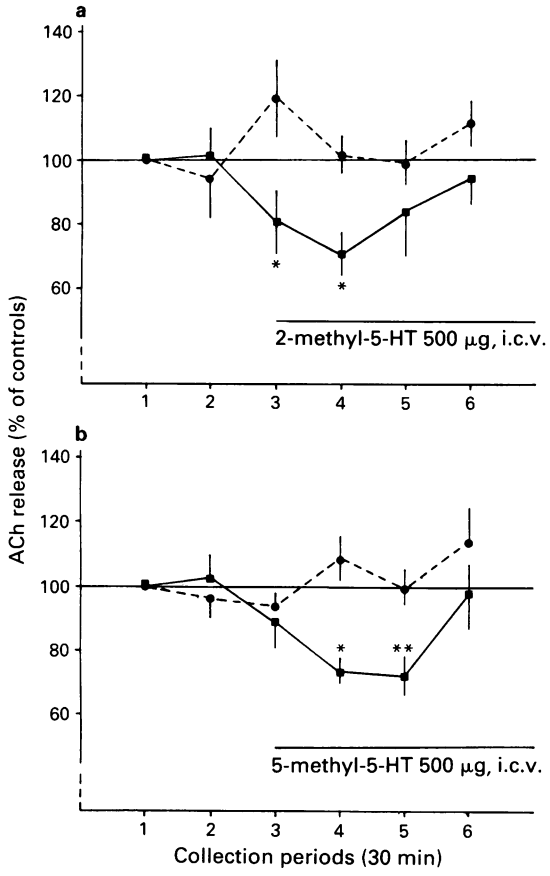

Figure 1 (a) Effect of 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) 500 μg i.c.v. (■, $n = 5$) and antagonism by MDL 72222, 1 mg kg^{-1} s.c. (●, $n = 5$) on acetylcholine (ACh) release from the cerebral cortex of control guinea-pigs. (b) Effect of 2-methyl-5-HT, 500 μg i.c.v. (■, $n = 5$) and antagonism by MDL 72222, 1 mg kg^{-1} s.c. (●, $n = 5$) on ACh release from the cerebral cortex of chronically (14 days) chlorimipramine (Cl-Imip)-treated guinea-pigs. Abscissa scales: collection periods (30 min); ordinate scales: percentage changes in ACh release. $*P < 0.05$; $**P < 0.01$ significantly different from the corresponding control, Student's t test for paired data. Control ACh release in chronically Cl-Imip-treated guinea-pigs was 127 ± 29 pmol cm^{-2} 30 min^{-1} and was not different from control guinea-pigs.

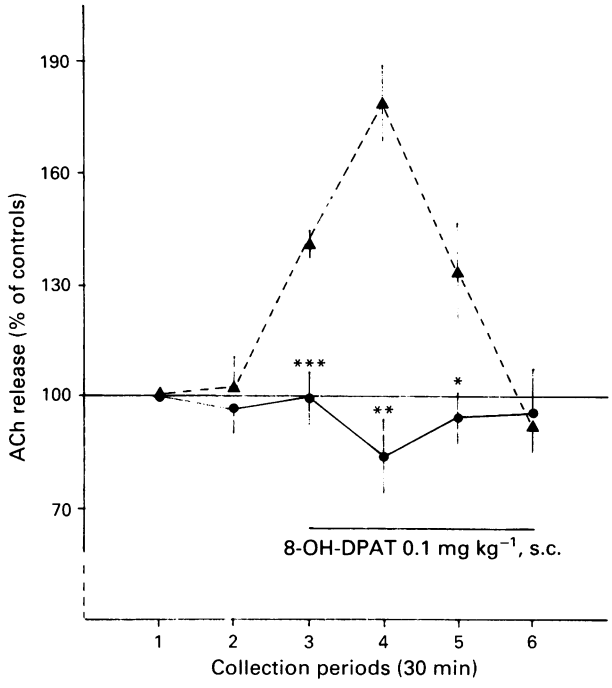

Figure 2 Lack of effect of 8-hydroxy-2-(di-n-propylaminotetralin) (8-OH-DPAT) 0.1 mg kg^{-1} s.c. on acetylcholine (ACh) release from the cerebral cortex of chronically chlorimipramine (Cl-Imip)-treated guinea pigs (●, $n = 6$). For comparison, the effect of 8-OH-DPAT in control animals (▲, $n = 8$) is shown. Abscissa scale: collection periods (30 min); ordinate scale: percentage changes in ACh release. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ significantly different from control animals, Student's t test for non paired data. Control ACh release in chronically Cl-Imip-treated guinea-pigs was 101 ± 18 pmol cm^{-2} 30 min^{-1} and was not significantly different from control guinea-pigs.

A rapid desensitization of 5-HT$_{1A}$ receptors following acute Cl-Imip administration is unlikely, because MDL 72222 restored the 8-OH-DPAT effect, indicating that the reduction was due to an opposing inhibitory effect of acute Cl-Imip, probably via 5-HT$_3$ receptors.

When Cl-Imip was acutely administered before 2-methyl-5-HT, the inhibitory effect of the latter drug on ACh release was not potentiated, suggesting a ceiling effect by 2-methyl-5-HT, 500 μg i.c.v. MDL 72222 was able to antagonize the inhibitory effect of the combination of Cl-Imip and 2-methyl-5-HT, suggesting an involvement of 5-HT$_3$ receptors.

In chronically Cl-Imip-treated guinea-pigs, 8-OH-DPAT was no longer able to produce its facilitatory effect on ACh release. The mechanism underlying this phenomenon was not the same as in acutely-treated animals, because the experiments were performed 24 h after the last Cl-Imip injection, a time compatible with the elimination half-life of the drug (Rudorfer & Potter, 1987). Moreover, a single injection of Cl-

Table 3 Effect of 2-methyl-5-hydroxytryptamine (2-methyl-5-HT, 500 μg i.c.v.) on the release of acetylcholine (ACh) from the cerebral cortex of freely moving guinea-pigs pretreated with saline, chlorimipramine (Cl-Imip, 10 mg kg^{-1} s.c.) or Cl-Imip plus MDL 72222 (1 mg kg^{-1} s.c.)

			ACh release			
		Before 2-methyl-5-HT	*After 2-methyl-5-HT*			
Pretreatment	*No expts.*	(control)	30 min	60 min	90 min	120 min
Saline[a]	5	123 ± 12	104 ± 13* (81)	83.4 ± 10* (71)	102 ± 16 (84)	110 ± 10 (94)
Cl-Imip[a]	4	109 ± 9.1	78.3 ± 11* (78)	67.9 ± 4.9* (70)	73.2 ± 7.1* (75)	89.2 ± 17 (88)
Cl-Imip[b]	3	122 ± 13	98 ± 11* (81)	109 ± 12 (88)	126 ± 14 (103)	126 ± 16 (103)
Cl-Imip[a] + MDL 72222	4	120 ± 18	122 ± 18 (102)	105 ± 12 (92)	119 ± 20 (101)	108 ± 19 (91)

[a] 30 min before 2-methyl-5-HT, [b] 24 h before 2-methyl-5-HT.
Values are pmol ACh cm^{-2} 30 min^{-1} ± s.e.mean; in parentheses the percentage of pretreatment values. $*P < 0.05$ significantly different from corresponding controls, Student's t test for paired data.

Imip, 24 h before the experiment, did not modify the 8-OH-DPAT effect. However, the possibility of accumulation of the drug, after chronic treatment, needs to be considered. Our data, although indirect, are against this possibility: basal ACh release 24 h after the last dose of Cl-Imip did not differ from normal values, and Cl-Imip still reduced ACh release, indicating that the acute inhibitory effect of the drug was extinguished, and that tolerance to this effect did not develop. Thus, it is likely that adaptive changes in 5-HT_{1A} receptors account for the lack of effect of 8-OH-DPAT in chronically Cl-Imip-treated guinea-pigs, in agreement with previous reports based on behavioural experiments (Goodwin *et al.*, 1985).

In contrast to 5-HT_{1A}-mediated effects, 5-HT_3 receptor-mediated inhibition did not seem to be affected by chronic Cl-Imip administration. In fact, the 5-HT_3 agonist, 2-methyl-5-HT, maintained its inhibitory, MDL 72222-sensitive effect in chronically treated guinea-pigs. Moreover, Cl-Imip, acutely administered to chronically treated animals, maintained its inhibitory effect as well.

The experimental approach used here does not shed light on any possible changes induced by Cl-Imip on other 5-HT receptor subtypes controlling ACh release. In fact, 5-HT_2 receptors are not involved in the control of cortical ACh release (Bianchi *et al.*, 1990) although they mediate a facilitatory effect on the basal ACh release from guinea-pig caudate nucleus slices (Bianchi *et al.*, 1989). The latter effect was prevented by chronic Cl-Imip treatment (Siniscalchi *et al.*, 1990b,c), supporting a down-regulation of 5-HT_2 receptors, in agreement with previous reports, based on binding studies (Peroutka & Snyder, 1980).

In conclusion, these results suggest that cortical ACh release is modulated in opposing ways by 5-HT_{1A} and 5-HT_3 receptor activation. In addition, it has been shown that chronic Cl-Imip treatment reduces the facilitatory response mediated by 5-HT_{1A} autoreceptors, while the inhibitory responses mediated by 5-HT_3 receptors are maintained. To our knowledge, modifications in 5-HT_3 functions or binding sites, following long-term antidepressant treatment, have not thus far been investigated. The differences in the adaptive changes shown by the various receptor classes may play a role in the antidepressant action of Cl-Imip.

We wish to thank Mr G. Marzola for the skilful technical assistance and chronic treatments.

The gift of the following substances is acknowledged: 2-methyl-5-HT (Sandoz), MDL 72222 (Merrel Dow), midazolam (Roche). This work was supported by a MURST 60% grant.

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(Received July 25, 1990
Revised November 12, 1990
Accepted November 29, 1990)

Br. J. Pharmacol. (1991), **102**, 841–846

L-Arginine induces relaxation of rat aorta possibly through non-endothelial nitric oxide formation

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1 The relaxation of rings of rat thoracic aorta induced by L-arginine and its derivatives was investigated.

2 L-Arginine (0.3–100 μM), but not D-arginine, induced relaxation of the arteries, which was detectable after 2 h and maximal after 4–6 h on its repeated application; it was endothelium-independent.

3 L-Arginine methyl ester, Nα-benzoyl L-arginine and L-homo-arginine had essentially similar effects to those of L-arginine.

4 N^G-nitro L-arginine methyl ester (L-NAME, 3 μM), N^G-nitro L-arginine (L-NNA, 1 μM) and N^G-monomethyl L-arginine (L-NMMA, 10 μM), inhibitors of nitric oxide (NO) formation from L-arginine, inhibited or reversed the L-arginine-induced relaxation, irrespective of the presence or absence of the endothelium. In contrast, N^G-nitro D-arginine was without effect.

5 Haemoglobin (Hb, 10 nM) and methylene blue (MB, 0.3 μM) inhibited or reversed the L-arginine-induced relaxation.

6 L-Arginine (1–100 μM), but not D-arginine, increased guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels in the tissues that relaxed in response to L-arginine. This effect of L-arginine was suppressed by Hb (3 μM), MB (1 μM) and L-NAME (100 μM). Removal of the endothelium did not significantly alter the L-arginine-induced cyclic GMP production.

7 These results suggest that L-arginine itself caused a slowly developing relaxation of rat aorta, possibly via formation of NO by an endothelium-independent mechanism.

Keywords: L-Arginine; thoracic aorta; relaxation; EDRF/NO; non-endothelial NO formation; cyclic GMP

Introduction

It is now generally accepted that endothelium-derived relaxing factor (EDRF, Furchgott & Zawadzki, 1980) is identical to nitric oxide (NO) (Ignarro *et al.*, 1987; Palmer *et al.*, 1987) derived from the terminal guanidino nitrogen atoms of L-arginine (Palmer *et al.*, 1988a; Schmidt *et al.*, 1988a). The proposal that formation of NO from L-arginine plays a role in the vascular tone is based on the inhibitory effects of N^G-monomethyl L-arginine (L-NMMA), an inhibitor of NO formation from L-arginine (Hibbs *et al.*, 1987; Palmer *et al.*, 1988b; Palmer & Moncada, 1989), on EDRF-mediated vasorelaxation (Palmer *et al.*, 1988b; Rees *et al.*, 1989a,b; Sakuma *et al.*, 1989; Whittle *et al.*, 1989) or acetylcholine (ACh)-induced NO release from perfused aorta (Rees *et al.*, 1989a). All these effects are reversed by L-arginine.

However, direct evidence showing that L-arginine potentiated EDRF-mediated vasorelaxation, or that L-arginine itself induced significant vasorelaxation has not been presented so far. Several reports have shown that it caused slight relaxation of up to 7%, at concentrations in the mM range (Palmer *et al.*, 1988; Rees *et al.*, 1989b; Sakuma *et al.*, 1988; Thomas *et al.*, 1989b).

Therefore, we have examined the direct actions of L-arginine and related compounds on the rat thoracic aorta and have elucidated the possible mechanism of the vasodilator action of L-arginine. While this work was in progress, several reports have shown that in bovine intrapulmonary arteries rendered refractory to A23187, L-arginine caused endothelium-dependent relaxation (Gold *et al.*, 1989b; Fukuto *et al.*, 1990; Schini *et al.*, 1990; Wood *et al.*, 1990).

Preliminary accounts of some of this work have been presented at the 63rd Annual Meeting of the Japanese Pharmacological Society, 1990 (Ueda *et al.*, 1990), and the 7th International Symposium on Vascular Neuroeffector Mechanisms, Bonn, 1990 (Moritoki *et al.*, 1990).

Methods

Organ bath experiments

Male Wistar rats, 8–9 weeks old (317.6 ± 20.9 g), were killed by a blow on the head and bled. Segments of their thoracic aorta of 3 mm length were cut with parallel razors and freed from adjacent tissues under a dissecting microscope. These ring segments were set up in a 10 ml organ bath. The Krebs solution in the bath had the following composition (mM): NaCl 115.3, KCl 4.9, $CaCl_2$ 1.46, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, disodium edetate (EDTA) 0.03, ascorbic acid 0.11 and glucose 11.1. This solution was maintained at 34°C and bubbled with O_2 containing 5% CO_2. The preparations were incubated in the bath at a resting tension of 1.0 g for 2 h before the start of experiments. Responses were recorded isometrically with a force displacement transducer (Nihon Kohden SB 1TH).

For measurement of relaxation, the arteries were first contracted with 0.3 μM phenylephrine at concentrations corresponding to the EC_{80} (the molar concentration producing 80% of the maximal response). Concentration-response curves were constructed by adding L-arginine cumulatively to the 10 ml bath in a volume of 7–20 μl, and relaxations were plotted as percentages of the contractions induced by the EC_{80} concentrations of phenylephrine.

For investigation of the effect of the vascular endothelium, the endothelium was removed by rubbing the lumen of the aorta with cotton thread, and its removal was confirmed by the loss of the relaxant response to ACh. To produce tone, endothelium-denuded preparations were contracted with 0.1 μM phenylephrine (corresponding to the EC_{80}), because removal of the endothelium shifted the concentration-response curve for phenylephrine to the left (EC_{50} value: with endothelium, 94.3 ± 6.0 nM; without endothelium, 39.8 ± 6.3 nM) with increase in the maximal tension development (with endothe-

lium, 664.6 ± 61.2 mg; without endothelium, 1087.6 ± 66.2 mg, $n = 8$).

Assay of cyclic nucleotides

A helical strip of aorta was equilibrated under 1.0 g tension in Krebs solution bubbled with O_2 containing 5% CO_2, and 10 μM L-arginine was applied at 60 min intervals until a constant relaxation to L-arginine was obtained. Then, the arteries were cut into small pieces, and equilibrated in Krebs solution without resting tension for further 60 min. The preparations were incubated with the EC_{80} concentration of phenylephrine for 2 min, and transferred to the solution containing L-arginine and phenylephrine. Inhibitors, when used, were added to the medium before and during incubation with L-arginine. At various times of incubation with L-arginine, the preparations were quickly frozen in liquid nitrogen and homogenized in ice-cold 6% trichloroacetic acid in a Potter-glass homogenizer in ice. The homogenates were centrifuged at 1700 g for 15 min at 4°C, and the supernatants were extracted with 3 volumes of water-saturated ether. Guanosine 3':5'-cyclic monophosphate (cyclic GMP) was measured by radioimmunoassay (Honmar *et al.*, 1977) with commercially available kits (Yamasa Shoyu, Chosi, Japan). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin used as a standard.

Statistical analysis

Values are expressed as means ± s.e.mean. The statistical significance of differences was analyzed by Student's unpaired t test, and P values of less than 0.05 were considered as significant.

Materials

The materials used were L-arginine hydrochloride, D-arginine hydrochloride, L-homoarginine, L-arginine methyl ester, Nα-benzoyl L-arginine ethyl ester, N^G-nitro L-arginine methyl ester (L-NAME), methylene blue (MB) and phenylephrine hydrochloride (Sigma Chemical Co., St Louis, Mo, U.S.A.), N^G-nitro L-arginine (L-NNA), N^G-nitro D-arginine (Peptide Institute, Osaka, Japan), N^G-monomethyl L-arginine (L-NMMA) (Peninsula Laboratories Inc., Belmont, CA, U.S.A.) and cilostamide (Otsuka Pharmaceutical Co., Tokushima, Japan). Haemoglobin (Hb, 1 mM from Sigma) was reacted with 10 mM sodium hydrosulphite and then the reducing agent was removed by dialysis at 4°C (Martin *et al.*, 1985).

Results

L-Arginine-induced relaxation

In all cases, in rat thoracic aorta the first application of L-arginine (10 μM) 60 min after isolation of the arteries caused scarcely any relaxation. However, during repeated application of 10 μM L-arginine at 60 min intervals, relaxation did develop, and increased progressively with time to a steady level in 6 h (Figure 1). The relaxation induced by L-arginine (0.3–100 μM) was concentration-dependent (Figures 1, 2, 3 and 4). A maximal relaxation of 89.6 ± 2.6% ($n = 9$) was observed at an L-arginine concentration of 100 μM, and its EC_{50} value was 2.76 ± 0.33 μM ($n = 9$).

During this treatment, phenylephrine-induced contractions of the aorta were not significantly altered (with endothelium: EC_{50} values, 96.4 ± 11.4 nM vs. 101.4 ± 16.5 nM, and maximal contraction 90.6 ± 10.6% of the initial, $n = 5$; without endothelium: EC_{50} values, initial 19.9 ± 5.8 nM vs. after 6 h 41.2 ± 9.7 nM, and maximal contraction 91.1 ± 5.3% of the initial, $n = 5$). In time-matched experiments, the arteries that had equilibrated for up to 6 h without repeated exposure to L-arginine, caused considerably less relaxation (Figure 1).

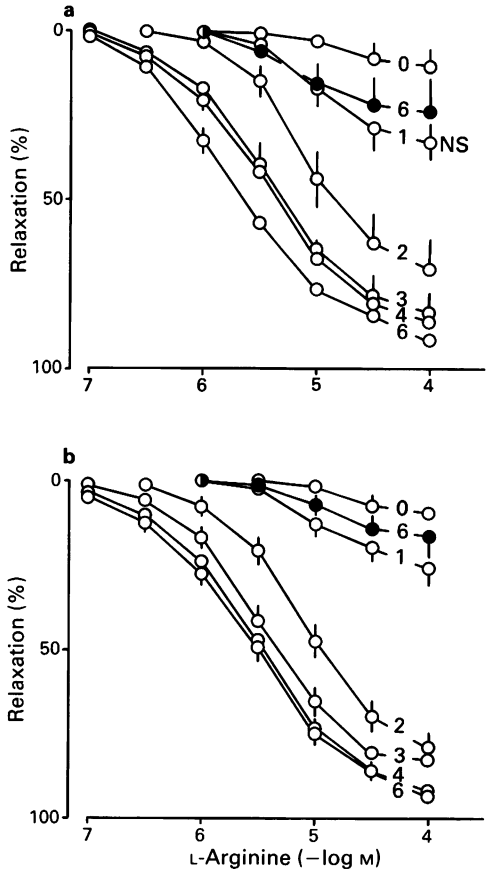

Figure 1 Time course of development of L-arginine-induced relaxation of rat thoracic aorta with (a) or without (b) endothelium. To produce tone, the arteries were first contracted with the EC_{80} concentrations of phenylephrine (0.3 μM for the arteries with endothelium and 0.1 μM for those without endothelium). L-Arginine was added cumulatively every 60 min. Relaxations are expressed as percentages of the respective contractions induced by the EC_{80} concentrations of phenylephrine. Numbers attached to curves represent time in hours after first application of L-arginine. (●) Relaxations induced by L-arginine at 6 h without repeated application of L-arginine. Each point is the mean of 6 separate experiments with s.e.mean shown by vertical lines. NS, not significant, compared with the value for the preparation without endothelium.

While L-arginine-induced relaxation was progressively increased with repeated exposure to L-arginine, acetylcholine (ACh)-induced relaxation remained constant during experiments; EC_{50} values of ACh were 18.0 ± 2.2 nM at the start of experiment and 21.8 ± 5.5 nM ($n = 5$) at 6 h after L-arginine-induced relaxation fully developed.

Removal of the endothelium, while abolishing ACh-induced relaxation, did not influence the relaxation induced by L-arginine (Figures 3 and 4), its EC_{50} value being 2.79 ± 0.32 μM ($n = 6$).

L-Arginine methyl ester, Nα-benzoyl L-arginine and L-homo-arginine had essentially similar relaxant effects (Figure 3). In contrast to L-arginine, D-arginine at concentrations of up to 300 μM did not induce relaxation (Figures 2 and 3). Pretreatment of the arteries with 300 μM D-arginine for 20 min had no significant effect on L-arginine-induced relaxation ($n = 4$, data not shown).

Effects of L-NAME, L-NNA, Hb, and MB on the L-arginine-induced relaxation

As shown in Figure 2, when the relaxation induced by 10 μM L-arginine reached maximum, application of L-NNA (10 μM) reversed the relaxation almost to the level before addition of L-arginine. This reversal was also observable in the

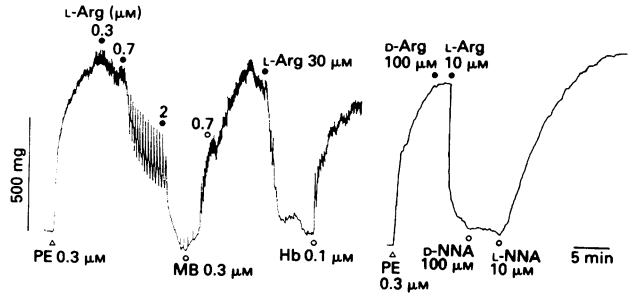

Figure 2 Relaxation induced by L-arginine, and reversal by N^G-nitro L-arginine (L-NNA), haemoglobin (Hb) or methylene blue (MB) on rat thoracic aorta with endothelium. The arteries were first contracted with the ED_{80} concentration of phenylephrine (PE, 0.3 μM) to produce tone. L-Arg, L-arginine; D-Arg, D-arginine; D-NNA, N^G-nitro D-arginine. Other experimental conditions are as for Figure 1.

endothelium-denuded preparations. In contrast, 100 μM N^G-nitro D-arginine was without effect (Figure 2).

L-NAME (100 μM), Hb (0.3 μM) and MB (1 μM) by themselves enhanced phenylephrine-induced maximal tension of the arteries with endothelium to 165.8 ± 21.7%, 130.5 ± 6.7%, and 150.1 ± 11.6% ($n = 5$), respectively, whereas in the endothelium-denuded arteries, they scarcely affected the contraction (L-NAME; to 116.8 ± 11.9%, Hb; 105.9 ± 1.7%, MB, 107.4 ± 3.3%, $n = 6$).

Pretreatment with L-NAME (1–100 μM) for 20 min concentration-dependently inhibited the L-arginine-induced relaxation, independently of the presence or absence of the endothelium (Figure 4). Essentially similar results were obtained with L-NNA (0.1–10 μM) ($n = 5$, data not shown).

Hb (10–300 nM) and MB (0.1–1 μM) also reversed or inhibited L-arginine-induced relaxation, irrespective of whether the endothelium was present or absent (Figures 2 and 5). In contrast, the relaxation induced by cilostamide used as a reference was not reversed by Hb or MB (Figure 5, right), independently of whether the endothelium was present or absent.

Cyclic GMP formation

In fresh preparations that had not yet relaxed in response to L-arginine, L-arginine failed to stimulate cyclic GMP formation. In the endothelium-intact preparations treated with 10 μM L-arginine for 1 min, the cyclic GMP level was 1.04 ± 0.22 pmol mg^{-1} protein, which was not different from the basal levels of 0.93 ± 0.24 pmol mg^{-1} protein ($n = 5$). Similarly, in the arteries without endothelium, the basal level

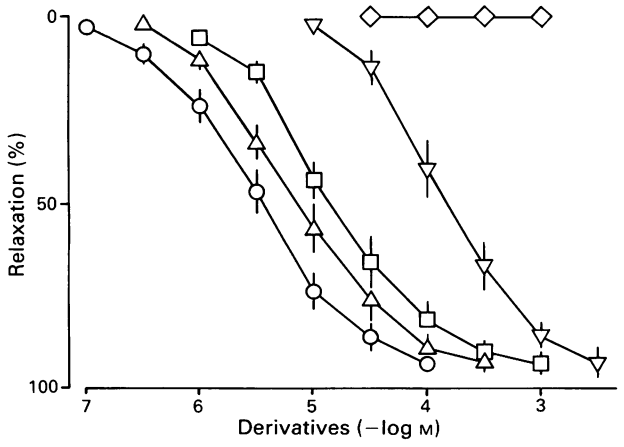

Figure 3 Concentration-response curves for L-arginine and its analogues in inducing relaxations of rat thoracic aorta without endothelium. (○) L-arginine; (◇) D-arginine; (△) L-homoarginine; (□) L-arginine methyl ester; (▽) Nα-benzoyl L-arginine methyl ester. Experimental conditions are as for Figure 1. Values are means for preparations from 6 to 8 rats; vertical lines show s.e.mean.

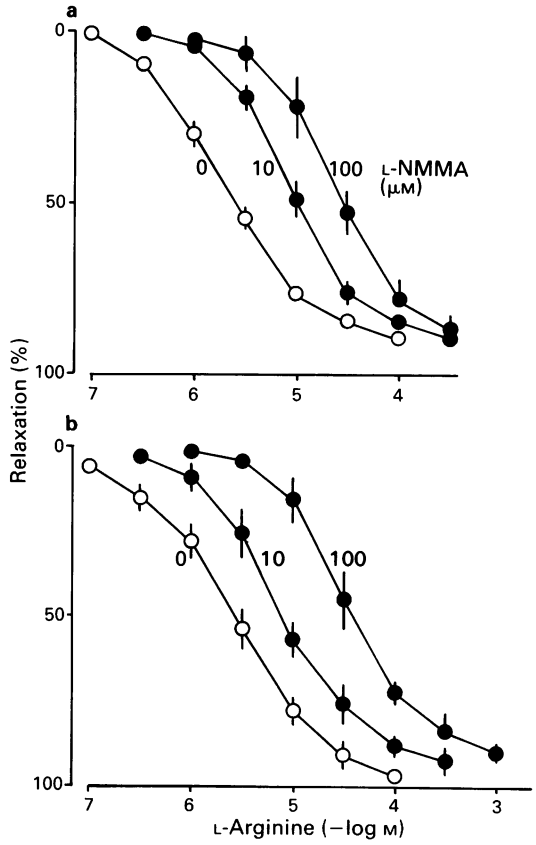

Figure 4 Inhibitory effect of N^G-monomethyl L-arginine (L-NMMA) on the L-arginine-induced relaxations of rat thoracic aorta with (a) and without (b) endothelium. Experimental conditions are as for Figure 1. The arteries were tested with the inhibitor for 20 min before application of L-arginine. Values are means for preparations from 6 rats; vertical lines shown s.e.mean.

of cyclic GMP (0.25 ± 0.04 pmol mg^{-1} protein, $n = 5$) was not elevated by 10 μM L-arginine (0.27 ± 0.02 pmol mg^{-1} protein). However, L-arginine was found to stimulate formation of cyclic GMP after the arteries had gained responsiveness to L-arginine by its repeated application every 60 min. The time

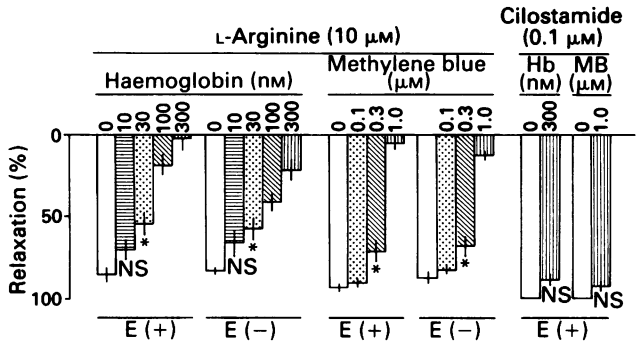

Figure 5 Inhibition by haemoglobin (Hb) and methylene blue (MB) of relaxations induced by L-arginine and cilostamide in rat thoracic aorta with and without endothelium. To examine the reversal, the arteries were first contracted with the EC_{80} concentration of phenylephrine (0.3 μM for endothelium intact arteries and 0.1 μM for arteries without endothelium). When the relaxations induced either by 10 μM L-arginine or 0.1 μM cilostamide reached a maximum, Hb or MB was added cumulatively (see Figure 2). Cilostamide was used as a reference. The ordinate scale indicates relaxations remaining after application of the inhibitors, as percentages of the contraction induced by the EC_{80} concentration of phenylephrine. E (+), preparations with endothelium; E (−), preparations without endothelium. Experimental conditions are as for Figure 1. Columns represent mean of values ($n = 5$–6) of preparations from 6 rats, and vertical lines indicate s.e.means. NS, not significant, $^*P < 0.05$, compared to respective control value obtained in the absence of the inhibitors.

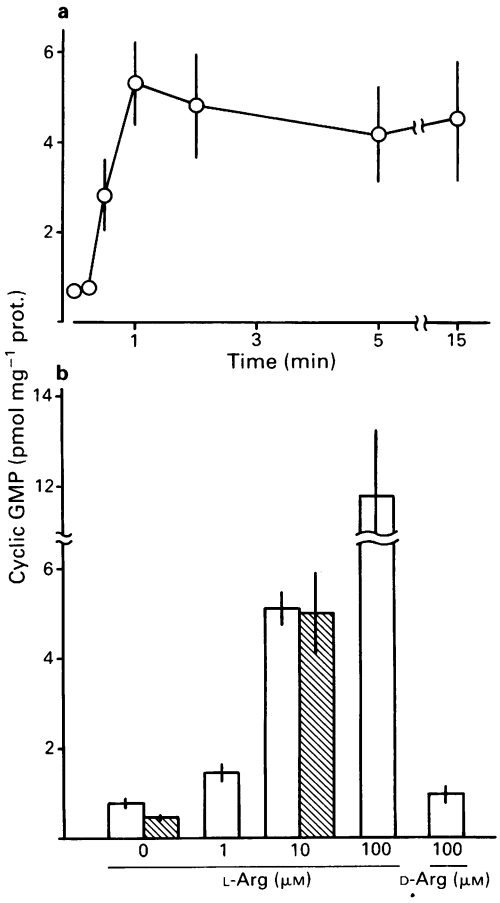

Figure 6 Effect of L-arginine on the production of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in rat thoracic aorta with endothelium. The levels of cyclic GMP were measured after confirming that the arteries had relaxed in response to 10 μM L-arginine. (a) Time course of L-arginine (10 μM)-stimulated cyclic GMP formation. The preparations were incubated with the EC_{80} concentration of phenylephrine (0.3 μM) for 2 min and then with 10 μM L-arginine. Each point represents means of values (n = 4–5) in preparations from 4 to 9 rats, and vertical lines indicate s.e.means. (b) Concentration-dependent production of cyclic GMP. Amounts of cyclic GMP were determined in 1 min. Open columns, with endothelium; hatched columns, without endothelium. Columns represent means of values (n = 5) in preparations from 4 to 9 rats, and vertical lines indicate s.e.means.

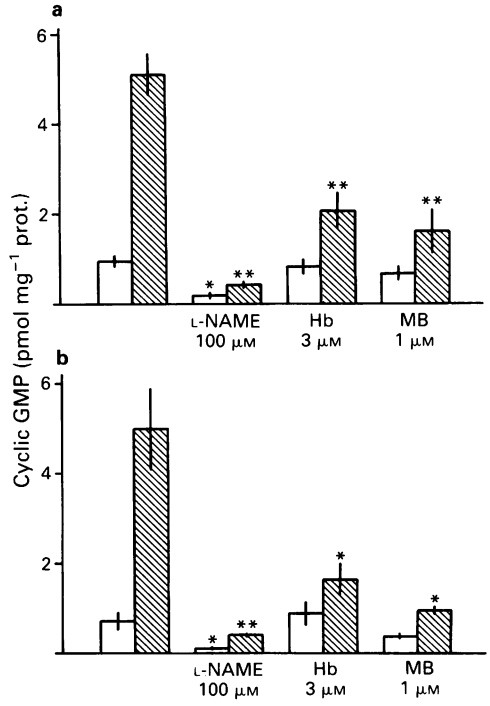

Figure 7 Effects of N^G-nitro L-arginine methyl ester (L-NAME), haemoglobin (Hb) and methylene blue (MB) on the L-arginine-induced production of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in the aorta with (a) and without (b) endothelium. L-NAME, pretreatment with 100 μM N^G-nitro L-arginine methyl ester for 20 min; Hb, pretreatment with 3 μM haemoglobin for 5 min; MB, pretreatment with 1 μM methylene blue for 20 min before addition of L-arginine. Amounts of cyclic GMP were determined after 1 min. Open columns, without L-arginine; hatched columns, with 10 μM L-arginine. Other experimental conditions are as for Figure 6. Columns represent the mean of values (n = 5–9) in preparations from 4 to 9 rats, vertical lines indicate s.e.mean. *P < 0.05, **P < 0.01, compared with respective control value obtained in the absence of the inhibitors.

course of L-arginine-induced cyclic GMP production is shown in Figure 6a. L-Arginine at a concentration of 10 μM, corresponding to the approximate EC_{80} concentration for inducing relaxation, increased formation of cyclic GMP in the arteries with endothelium from the control level of 0.72 ± 0.15 pmol mg^{-1} protein (n = 5) to steady level of 5.34 ± 0.95 pmol mg^{-1} protein in 1 min, and maintained up to at least 15 min. The concentration-dependent production of cyclic GMP induced by L-arginine is shown in Figure 6.

In preparations without endothelium, 10 μM L-arginine enhanced the cyclic GMP formation from the control value of 0.72 ± 0.20 pmol mg^{-1} protein to 5.01 ± 0.92 pmol mg^{-1} protein (n = 5), values not significantly different from those with intact endothelium (Figure 6). In contrast, D-arginine (100 μM) had no significant effect on production of cyclic GMP (n = 5).

Effects of MB, Hb and L-NAME on cyclic GMP formation

Pretreatment of the aortic segments with 100 μM L-NAME for 20 min, while slightly but significantly (P < 0.05) decreasing basal formation of cyclic GMP, abolished the increase in cyclic GMP production induced by 10 μM L-arginine, irrespective of the presence or absence of the endothelium (Figure 7). Similarly, 3 μM Hb (for 5 min) and 1 μM MB (for 20 min) significantly reduced L-arginine-induced cyclic GMP formation without affecting the basal level of cyclic GMP. The effects of Hb and MB were also independent of the endothelium.

Discussion

L-Arginine serves as the precursor for EDRF/NO (Palmer *et al.*, 1988a,b; Sakuma *et al.*, 1988; Schmidt *et al.*, 1988b). However, L-arginine did not relax rat thoracic aorta (Thomas & Rammell 1988) or porcine mammary artery (Thomas *et al.*, 1989b), and at very high concentrations in the millimolar range, it caused negligible relaxation in rabbit aorta (Palmer *et al.*, 1988; Rees *et al.*, 1989b) and rat aorta (Thomas *et al.*, 1989a). In the present study, we have demonstrated that L-arginine at micromolar concentrations induced relaxation and stimulated the formation of cyclic GMP in rat thoracic aorta. This discrepancy does not seem to be due to difference in the level of resting tension, or to agents used to maintain tone, because all the experiments have been performed under similar conditions. However, repeated exposure to L-arginine accelerated development of L-arginine-induced relaxation; in time matched control, L-arginine-induced relaxation was not observed or was considerably smaller in comparison with that observed in the arteries repeatedly exposed to L-arginine.

Development of L-arginine-induced relaxation was not due to reduction of tone, because the EC_{50} values of phenylephrine were virtually constant throughout the experiment. In addition, ACh-induced relaxation was not attenuated even after the arteries had gained responsiveness to L-arginine, indicating that development of L-arginine-induced relaxation was not due to nonspecific sensitization of the arteries to vascular

relaxants. L-Arginine-induced, endothelium-dependent relaxation has been demonstrated in bovine intrapulmonary artery after the artery had been rendered refractory to A23187 or ACh by incubation for 24 h in Krebs solution (Gold *et al.*, 1989a,b; Fukuto *et al.*, 1990). Our findings differ from these previous findings in that the relaxation was endothelium-independent. In addition, we found that repeated exposure to L-arginine accelerated induction of the relaxation to L-arginine. In contrast, it has been reported that the L-arginine-induced relaxation increased with time up to 6 h (Schini *et al.*, 1990) or that L-arginine caused relaxation by incubation for 24 h in Krebs solution without prior exposure to L-arginine (Wood *et al.*, 1990). These discrepancies may be due to differences in concentration of L-arginine applied to confirm the relaxation (10 μM in rat aorta vs. 100 μM in bovine aorta) or to species difference (rat vs. bovine).

L-Arginine increased cyclic GMP formation in the arteries that relaxed in response to L-arginine, but not in preparations that did not respond to L-arginine, indicating that the relaxation was mediated by cyclic GMP.

The L-arginine-induced relaxation and cyclic GMP formation of the aorta was antagonized by the inhibitor of NO biosynthesis L-NAME (Rees *et al.*, 1990), L-NMMA (Hibbs *et al.*, 1987; Palmer *et al.*, 1988b) and L-NNA (Moore *et al.*, 1990), which inhibit ACh-induced vasorelaxation (Moore *et al.*, 1990; Mülsch & Busse, 1990; Palmer *et al.*, 1988b; Rees *et al.*, 1989b; Sakuma *et al.*, 1988). In addition, as has been reported, the effects of L-arginine and the inhibitory arginine derivatives such as L-NNA were enantiomerically specific; the D-enantiomers, D-arginine (Amezcua *et al.*, 1989; Hibbs *et al.*, 1987; Palmer *et al.*, 1988; Sakuma *et al.*, 1988; Whittle *et al.*, 1989) and N^G-nitro D-arginine (Mülsh & Busse, 1990) were without effect. These results suggest that L-arginine-induced vascular relaxation and cyclic GMP formation are mediated through the L-arginine $\sim$ NO pathway.

Moreover, we found that Hb, which is an EDRF/NO inhibitor (Martin *et al.*, 1985), and the soluble guanylate cyclase inhibitor MB (Gruetter *et al.*, 1981; Martin *et al.*, 1985) attenuated the L-arginine-induced relaxation and formation of cyclic GMP. These results further support the idea that L-arginine-NO-soluble guanylate cyclase-cyclic GMP pathway is involved in the L-arginine-induced relaxation of rat thoracic aorta.

In the aorta with endothelium, Hb, MB and inhibitory arginine derivatives such as L-NMMA to some extent enhanced the phenylephrine-induced tone of the aorta with endothelium, possibly by inhibiting basal formation of NO (Martin *et al.*, 1986; Moore *et al.*, 1990; Mülsch & Busse, 1990; Rees *et al.*, 1989b). Nevertheless, the reduction of the L-arginine-induced relaxation by these inhibitors was not secondary to enhanced tone, because Hb, MB and L-NMMA reversed or inhibited L-arginine-induced relaxation of the endothelium-denuded preparations to similar extents to that of preparations with endothelium, although in endothelium-denuded arteries these inhibitors did not enhance phenylephrine-induced contraction. Furthermore, these inhibitors did not affect endothelium-independent relaxation induced by cilostamide, a cyclic AMP-phosphodiesterase inhibitor (Hidaka *et al.*, 1979).

Unlike our observations, the vascular effects of L-arginine and its derivatives, such as L-arginine methyl ester, N^a-benzoyl L-arginine, short chain peptides containing L-arginine and poly L-arginine, so far reported are endothelium-dependent (Fukuto *et al.*, 1990; Gold *et al.*, 1989a,b; Palmer *et al.*, 1988a,b; Rees *et al.*, 1989b; Thomas *et al.*, 1989a). However, the present findings that L-arginine-induced vasorelaxation and cyclic GMP production were not influenced by removal of the endothelium, and that even after removal of the endothelium, L-NAME, Hb and MB were effective in reversing L-arginine-induced relaxation and in inhibiting L-arginine-stimulated increase in cyclic GMP production, suggest that the site of NO formation is not the endothelium. Therefore, the site of NO formation is likely to be at the smooth muscle or other cells in the vessel wall, and thus an L-arginine-NO pathway (Moncada *et al.*, 1989) similar to that in the endothelium could exist in the vascular smooth muscle. Alternatively, it is possible that NO is derived from L-arginine enzymatically in the extracellular space, irrespective of whether the endothelium is present or absent. It is assumed that the same enzymic processes, analogous to deimination of L-arginine and subsequent oxidation to NO (Hibbs *et al.*, 1987; Marletta *et al.*, 1988), or a NADPH-dependent enzyme which has been reported to be present in the vascular endothelial cells (Palmer & Moncada, 1989), are involved in the relaxation of rat thoracic aorta to L-arginine. During repeated application of L-arginine, some enzymes or enzyme systems mediating the formation of NO from L-arginine may be induced for elimination of excess L-arginine. The enzyme(s) mediating this formation of NO and the mechanism(s) controlling this system remain to be studied.

This work was in part supported by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science and Culture of Japan (63571045).

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(Received August 28, 1990
Revised November 10, 1990
Accepted December 3, 1990)

Br. J. Pharmacol. (1991), **102**, 847–850

Human vascular smooth muscle cells inhibit platelet aggregation when incubated with glyceryl trinitrate: evidence for generation of nitric oxide

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1 The effect on platelet aggregation of glyceryl trinitrate in the presence of cultured vascular smooth muscle cells was determined turbidometrically. U46619 (a thromboxane mimetic) was used as agonist and experiments were performed in the presence of aspirin. Inorganic nitrite production from glyceryl trinitrate by vascular smooth muscle cells was also measured, to provide an indirect index of nitric oxide synthesis.

2 The combination of vascular smooth muscle cells together with glyceryl trinitrate, at concentrations that had little effect individually, profoundly inhibited platelet aggregation.

3 The inhibitory effect on platelet aggregation of vascular smooth muscle cells together with glyceryl trinitrate was markedly attenuated by haemoglobin, an inhibitor of nitric oxide.

4 These results show that vascular smooth muscle cells inhibit platelet aggregation when exposed to glyceryl trinitrate and suggest that this is due to generation of nitric oxide from glyceryl trinitrate by vascular smooth muscle.

Keywords: Nitric oxide; glyceryl trinitrate; vascular smooth muscle; platelets; aggregation

Introduction

Glyceryl trinitrate is used extensively to relieve angina pectoris, but its actions remain incompletely understood. It relaxes blood vessels by activating soluble guanylate cyclase (Katsuki *et al.*, 1977; Ignarro *et al.*, 1981), and is converted to nitrite ions when incubated with vascular tissue (Needleman & Johnson, 1973). Inorganic nitrite ions have only weak vasodilator activity, but are formed rapidly and non-enzymatically under physiological conditions from nitric oxide which is a potent vasodilator and activator of soluble guanylate cyclase (Palmer *et al.*, 1987; Ignarro *et al.*, 1981). Nitric oxide is bound avidly by haemoglobin (Haldane *et al.*, 1897), which is a potent inhibitor of the endothelium-derived relaxing factor described by Furchgott & Zawadski (1980). Endothelial cells synthesize nitric oxide from endogenous L-arginine, accounting quantitatively for relaxing factor activity (Palmer *et al.*, 1987; 1988). Vascular smooth muscle may metabolize glyceryl trinitrate to form nitric oxide which mediates its vasorelaxant effect, before conversion to inactive nitrite (Katsuki *et al.*, 1977). There is evidence that tolerance to glyceryl trinitrate occurs as a result of failure in the enzymic synthesis of nitric oxide, at the level either of an enzyme or a cofactor such as reduced glutathione, although the exact mechanism remains obscure (Needleman & Johnson, 1973; Newman *et al.*, 1990).

Glyceryl trinitrate is a very weak inhibitor of platelet aggregation *in vitro* (Loscalzo, 1985). However, it was shown recently that nitric oxide, in addition to its vasodilator action, potently inhibits platelet adhesion and aggregation (Radomski *et al.*, 1987). Consequently, if nitric oxide is indeed formed from glyceryl trinitrate by vascular smooth muscle, it could inhibit local thrombus formation. Such an effect could be important in the therapeutic action of glyceryl trinitrate in patients with ischaemic heart disease. In the present study we have therefore measured platelet aggregation in suspensions of platelet rich plasma mixed with cultured human vascular smooth muscle cells and glyceryl trinitrate in the presence and in the absence of haemoglobin. Aspirin was used to inhibit biosynthesis of anti-aggregatory prostaglandins. Glyceryl trinitrate can break down slowly to yield nitric oxide even in cell-free systems (Feelisch & Noack, 1987), so we also monitored inorganic nitrite as an index of nitric oxide production in the presence and absence of smooth muscle cells. In addition, we used vascular smooth muscle cells made tolerant to glyceryl trinitrate to determine whether anti-aggregatory activity is generated specifically by sensitive as opposed to tolerant cells.

Methods

Smooth muscle cells: culture and preparation

Human vascular smooth muscle cells were cultured by a modification of the method of Ives *et al.* (1978) from uterine artery obtained at hysterectomy and grown to confluence at passage 4–7 in 12-well plates (4.52 cm^2) in Medium E199 with penicillin/streptomycin 50 iu/50 $\mu g\,ml^{-1}$, fungizone 2.5 $\mu g\,ml^{-1}$, glutamine 10 $mg\,ml^{-1}$, foetal calf serum 10%, oestradiol 0.01 $\mu g\,ml^{-1}$ and ITS: insulin-transferrin-sodium selenite medium supplement. Cell morphology was monitored in unstained preparations by phase contrast microscopy with a Nikon TMD inverted stage microscope. Cells stained positively with anti-smooth muscle actin using monoclonal anti-α smooth muscle actin and fluorescein isothiocyanate-coupled rabbit anti-mouse immunoglobulin (Skalli *et al.*, 1986). Suspensions of cultured vascular smooth muscle cells were prepared from the 12-well plates as follows. After removal of medium, 2 ml of trypsinizing solution (0.05% trypsin and 0.02% EDTA in phosphate buffered saline) was added to each well for 2 min and then replaced by 0.5 ml of platelet-poor plasma and 1.5 ml of phosphate buffered saline. Clumps of cells were dispersed by gentle repeated aspiration with a fine tipped Pasteur pipette. This provided a cell suspension containing approximately 5×10^3 cells ml^{-1}. Tolerance was induced by 18-h incubation of cells with glyceryl trinitrate (220 μM).

Nitrite production

Medium from 12-well plates containing confluent vascular smooth muscle cells was replaced with 2 ml phosphate buffered saline at pH 7.4. Glyceryl trinitrate (final concentration

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220 μM) was added and incubated at 37°C. Supernatant was removed at 0, 5, 15, 60 and 120 min and assayed for nitrite by the Greiss reaction, as described by Rider & Melon (1946).

Platelet aggregation studies

Venous blood samples (20 ml) from healthy drug-free volunteers were collected with a 19-gauge needle and anticoagulated with trisodium citrate (0.31% w/v). Platelet-rich plasma and platelet-poor plasma were prepared by differential centrifugation at room temperature. Aspirin (final concentration 1 mM) was added to platelet-rich plasma to prevent thromboxane and prostaglandin biosynthesis. Platelet aggregation was studied at 37°C in a Payton aggregometer (Model 600 B) as described by Born & Cross (1963). The output from the aggregometer was processed by a Maclab™ system connected to an Apple Macintosh SE computer which allowed automated analysis. Platelet-rich plasma (0.5 ml) was added to each cuvette and the volume made up with phosphate buffered saline (final volume 650 μl). Aggregation was induced by a sub-maximal dose of U46619 (11α,9α-epoxymethano- prostaglandin H_2) (2 μM). Additions of glyceryl trinitrate (220 μM), cell suspension (100 μl), haemoglobin (5 μM), superoxide dismutase (15 units ml^{-1}) or combinations of these were made immediately before addition of U46619. Saturated nitric oxide solution was prepared by bubbling helium then nitric oxide (each for 45 min) through 0.9% sodium chloride solution. Measurements were made in duplicate. Aggregation was recorded as % change in light transmission 2 min after agonist; 100% aggregation was taken as the light transmission of platelet poor plasma.

Materials

All materials were obtained from Sigma, UK, except glyceryl trinitrate (Tridil, Du Pont, Stevenage, Herts), foetal calf serum, Medium E199 and phosphate buffered saline Dulbecco's Formula (modified) without calcium and magnesium (ICN Flow, High Wycombe, Bucks.) Fluorescein isothiocyanate coupled rabbit anti-mouse immunoglobulin was obtained from DAKO (High Wycombe, Bucks). Nitric oxide and helium were obtained from BOC, Brentford, Middlesex.

Analysis

Results are expressed as the mean ± s.e.mean. Differences were evaluated by the Wilcoxon signed rank test and considered significant when $P < 0.05$.

Results

Nitrite production

Nitrite was generated by cultured smooth muscle cells following addition of glyceryl trinitrate, 220 μM (Figure 1). In cells previously exposed to glyceryl trinitrate, production of nitrite was considerably reduced (Figure 1). Exposure of cells to glyceryl trinitrate did not affect their morphology as seen under light microscopy immediately after exposure and for up to six days subsequently. Nitrite was not detected in solutions of glyceryl trinitrate (220 μM) at 37°C in the absence of cells.

Platelet aggregation studies

U46619 (2 μM) caused platelet aggregation in the presence of aspirin (1 mM). Glyceryl trinitrate (220 μM) or 100 μl of vascular smooth muscle cell suspension added separately did not inhibit the response to U46619, whereas in combination they did (Figure 2). In 12 separate experiments this combination of glyceryl trinitrate with cell suspension inhibited aggregation to U46619 from 66.1 ± 1.7% in the presence of glyceryl trinitrate alone, and 66.1 ± 1.0% in the presence of cell suspension alone, to 4.0 ± 2.8% in the presence of both ($P < 0.001$).

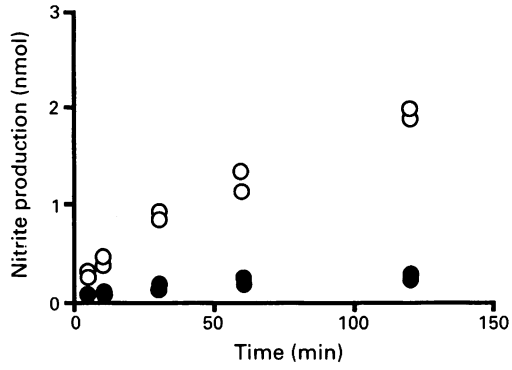

Figure 1 Generation of nitrite by cultured vascular smooth muscle cells incubated with glyceryl trinitrate (220 μM). Cells not previously exposed to glyceryl trinitrate (○) produced nitrite rapidly compared to cells preincubated for 18 h with this concentration of glyceryl trinitrate (●).

Suspensions of cells which had been made tolerant to glyceryl trinitrate, as shown by their impaired ability to metabolize glyceryl trinitrate to nitrite, lost the ability to inhibit platelet aggregation in combination with glyceryl trinitrate (Figure 3). In 8 further experiments, the combination of glyceryl trinitrate (220 μM) with 100 μl of a suspension of non-tolerant vascular smooth muscle cells inhibited platelet

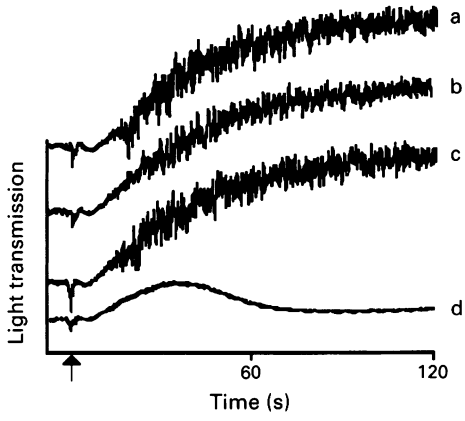

Figure 2 Representative traces showing platelet aggregation in response to U46619, alone (a), and in the presence of glyceryl trinitrate (b), smooth muscle cell suspension (c), and both glyceryl trinitrate and cell suspension (d). The arrow indicates additions of U46619.

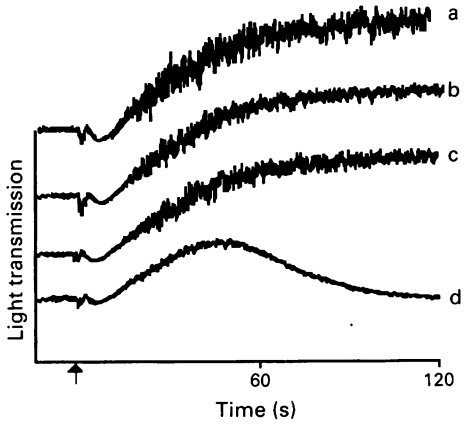

Figure 3 Representative traces showing platelet aggregation in response to U46619, alone (a), in the presence of glyceryl trinitrate (b), in the presence of glyceryl trinitrate and tolerant vascular smooth muscle cells pre-incubated with glyceryl trinitrate (c), and in the presence of glyceryl trinitrate and untreated vascular smooth muscle cells (d). The arrow indicates additions of U46619.

aggregation to U46619 (2 μM) from 68.4 $\pm$ 0.5% to 17.4 $\pm$ 9.0% whereas in paired measurements tolerant cells had significantly less effect, inhibiting aggregation from 66.8 $\pm$ 0.8% only to 58.4 $\pm$ 1.6%.

Addition of haemoglobin (5 μM) to platelet-rich plasma had no effect on aggregation caused by U46619, but antagonized the inhibitory effect of the combination of vascular smooth muscle cell suspension and glyceryl trinitrate. In further experiments ($n = 8$), U46619 (2 μM) in the presence of cells alone caused 77.3 $\pm$ 1.6% aggregation. Paired measurements in the presence of both vascular smooth muscle cells and glyceryl trinitrate confirmed that these inhibited aggregation, to 13.8 $\pm$ 3.2%, and showed that haemoglobin substantially restored the aggregation response to 56.3 $\pm$ 8.5%. Superoxide dismutase (15 units ml^{-1}), did not influence the inhibitory effect of a sub-maximally effective combination of cells with glyceryl trinitrate ($n = 8$; $P > 0.3$). However, the inhibition of aggregation observed with authentic nitric oxide solution (0.2–4 μl) was similarly unaffected by the same concentration of superoxide dismutase ($n = 4$).

Discussion

The main finding of this study is that platelet aggregation is inhibited by a combination of glyceryl trinitrate with a suspension of vascular smooth muscle cells, under conditions when neither glyceryl trinitrate nor cell suspension alone appreciably inhibits aggregation. Glyceryl trinitrate does have weak anti-aggregatory activity in its own right (Loscalzo, 1985), but in the present experiments we selected a dose that was approximately one tenth that needed to cause 25% inhibition of platelet aggregation. Vascular smooth muscle cells synthesize prostacyclin (Baenziger *et al.*, 1980) which is a potent inhibitor of platelet aggregation (Moncada *et al.*, 1976), so the present experiments were performed in the presence of aspirin to prevent prostacyclin synthesis, and cell suspension alone did not inhibit platelet aggregation. Vascular tissue is known to generate nitrite from glyceryl trinitrate (Needleman & Johnson, 1973) and more recently, in a cell-free system, Feelisch & Noack (1987) have demonstrated the formation of nitric oxide as an intermediate in the metabolism of organic nitrates. The most likely explanation for the present findings is therefore that vascular smooth muscle cells synthesize nitric oxide from glyceryl trinitrate, and it is this nitric oxide that inhibits platelet aggregation.

This explanation is supported by the findings that the inhibitory effect is prevented by low concentrations of haemoglobin, a potent inhibitor of nitric oxide (Martin *et al.*, 1984), and that the smooth muscle cells used in these experiments caused inorganic nitrite production from glyceryl trinitrate. Superoxide dismutase failed to enhance the inhibitory effect of the combination of glyceryl trinitrate with cells but it is possible that superoxide anion (which accelerates destruction of nitric oxide) is present only in low concentrations under these experimental conditions. This is consistent with our finding that superoxide dismutase was similarly ineffective in altering the sensitivity of platelet aggregation to authentic nitric oxide.

The finding that vascular smooth muscle cells previously exposed to a high concentration of glyceryl trinitrate were unable either to synthesize inorganic nitrite or to inhibit platelet aggregation when re-exposed to glyceryl trinitrate demonstrates that generation of platelet inhibitory activity is not a non-specific effect, and is consistent with the proposal (Needleman & Johnson, 1973; Newman *et al.*, 1990) that tolerance to glyceryl trinitrate is due to loss of the ability of smooth muscle cells to convert organic nitrate to nitric oxide.

The concentration of glyceryl trinitrate used in these experiments with cell suspensions *in vitro* was much higher than concentrations achieved therapeutically. We were limited as to the density of suspended vascular smooth muscle cells that we could use, because of the optical method used to measure platelet aggregation. It is possible that in the presence of an intact layer of smooth muscle in the media of small and medium sized arteries that sufficient nitric oxide could be produced from glyceryl trinitrate to have clinically important effects on platelet activation *in vivo*. Such an action could contribute to the therapeutic effect of glyceryl trinitrate, for instance in acute myocardial infarction where intravenous nitrates have a substantial beneficial effect on mortality (Yusuf *et al.*, 1988).

We would like to thank Miss J. Rymer and Miss M. Thom for providing uterine arteries, Hilary S. Doktor for expert technical assistance, the Guy's Special Trustees for support, and Sally Todd for preparing the illustrations.

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(Received September 7, 1990
Revised November 15, 1990
Accepted December 6, 1990)

Br. J. Pharmacol. (1991), **102**, 851–854

Antagonism by reactive blue 2 but not by brilliant blue G of extracellular ATP-evoked responses in PC12 phaeochromocytoma cells

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1 The effects of reactive blue 2 and brilliant blue G, which have been shown to block extracellular ATP-evoked responses, were investigated to discover whether these compounds act as P_2-purinoceptor antagonists in PC12 phaeochromocytoma cells.

2 Reactive blue 2 (10 to 100 μM) suppressed the ATP-stimulated dopamine secretion from PC12 cells in a dose-dependent manner. The concentration-response curve for ATP was shifted to the right and the maximal response was decreased by reactive blue (30 and 100 μM). Brilliant blue G (up to 100 μM) did not significantly affect the secretion.

3 Reactive blue 2 (10 to 100 μM) suppressed the ATP-activated inward current recorded from the voltage-clamped cells in a concentration-dependent manner. Brilliant blue G (up to 100 μM) did not affect the current.

4 The results suggest that reactive blue 2 but not brilliant blue G is a P_2-purinoceptor antagonist in PC12 cells. The purinoceptors in these cells may be the same type as those involved in ATP-evoked smooth muscle relaxation, judging from the antagonism by reactive blue 2.

Keywords: Phaeochromocytoma PC12 cell; dopamine secretion; membrane current; purinoceptors; antagonist

Introduction

Adenosine triphosphate (ATP) released from nerve terminals exerts transmitter-like activity on neuronal cells and on non-neuronal cells including smooth muscle cells (Burnstock & Kennedy, 1985; Gordon, 1986; Bean & Friel, 1990). Although these effects are thought to be mediated through receptors specific for ATP (P_2-purinoceptors; Burnstock & Kennedy, 1985), studies concerning specific antagonists have been insufficient, especially in neuronal cells. We previously showed that suramin antagonizes an ATP-activated ionic current (Nakazawa *et al.*, 1990b) and ATP-stimulated catecholamine-secretion in phaeochromocytoma PC12 cells (Inoue *et al.*, 1991), and suggested that suramin is an antagonist of P_2-purinoceptors in these cells as well as smooth muscle cells (Dunn & Blakeley, 1988; Den Hertog *et al.*, 1989; Hoiting *et al.*, 1990). In this paper, we have investigated effects of two blue dyes, reactive blue 2 and brilliant blue G, on the ATP-evoked responses in PC12 cells. Reactive blue 2 is known to inhibit ATP-evoked responses in smooth muscle cells (e.g., Kerr & Krantis, 1979; Choo, 1981; Crema *et al.*, 1983). Brilliant blue G is a more potent blocker than reactive blue 2 in blocking ATP-induced ^{45}Ca entry in rat parotid acinar cells (Soltoff *et al.*, 1989).

Methods

Cell culture

PC12 cells were prepared as previously described by Inoue & Kenimer (1988). Briefly, PC12 cells were cultured in Dulbecco's modified Eagle's medium containing 7% foetal bovine serum, 7% heat-inactivated horse serum, 2 mM L-glutamine, and 50 μg ml^{-1} gentamicin sulphate at 37°C in an atmosphere of 10% CO_2, and then plated onto collagen-coated polystyrene dishes at 1×10^6 cells per dish. After an additional 2 to 3 days in culture, the cells were used for experimentation.

Dopamine-secretion

Secretion of dopamine from PC12 cells was measured by a modification of a procedure described by Inoue & Kenimer (1988). The dishes were washed and incubated with 1 ml of balanced salt solution (BS) containing (in mM): NaCl 150, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1.2, NaH_2PO_4 1.2, glucose 10, EDTA 0.1, and HEPES 25 (pH adjusted to 7.4 with NaOH) for 1 h at room temperature. After washing once with BS (1 ml), the cells were stimulated by ATP in the absence or presence of reactive blue 2 or brilliant blue G. The incubation solution was removed 1 min after addition, and mixed with 0.2 ml of 1 N perchloric acid. The cells remaining in the dishes were incubated with 1 ml of 0.2 N perchloric acid for 5 min, removed from the dish, and disrupted by sonication. After centrifugation (1 min, 1000 *g*), supernatants of both the incubation solution and the cell suspension were collected for measurement of dopamine content.

Dopamine content was determined with a high-performance liquid chromatography-electrochemical detector (h.p.l.c.-e.c.d.) system (Bioanalytical Systems). The electrode potential of the detector was set at +0.7 V. A part of the supernatant (10 μl) was applied to the h.p.l.c. system which consisted of a pre-column (4.6 mm × 30 mm, ODS 5 μm, Bioanalytical Systems) and an analytical column (4.6 mm × 150 mm, ODS 5 μm, Bioanalytical Systems). The mobile phase consisted of a monochloroacetate buffer (140 mM, pH 3.1) containing 10% methanol, 0.003% sodium octanesulphonate and 0.15 mM EDTA (flow rate, 1.0 ml min^{-1}). The chromatography was performed at 30°C. The data were analysed with a chromatographic data processor (Shimadzu C-R3A).

Percentage secretion was calculated from the values obtained for dopamine content in the incubation solution (A) and dopamine content remaining in the cells (B) by the following equation: % of total dopamine min^{-1} = 100 × A/(A + B).

Electrophysiological study

Membrane currents were measured by whole-cell patch-clamp techniques (Hamill *et al.*, 1981) under the conditions described elsewhere (Nakazawa *et al.*, 1990a). The cells were superfused

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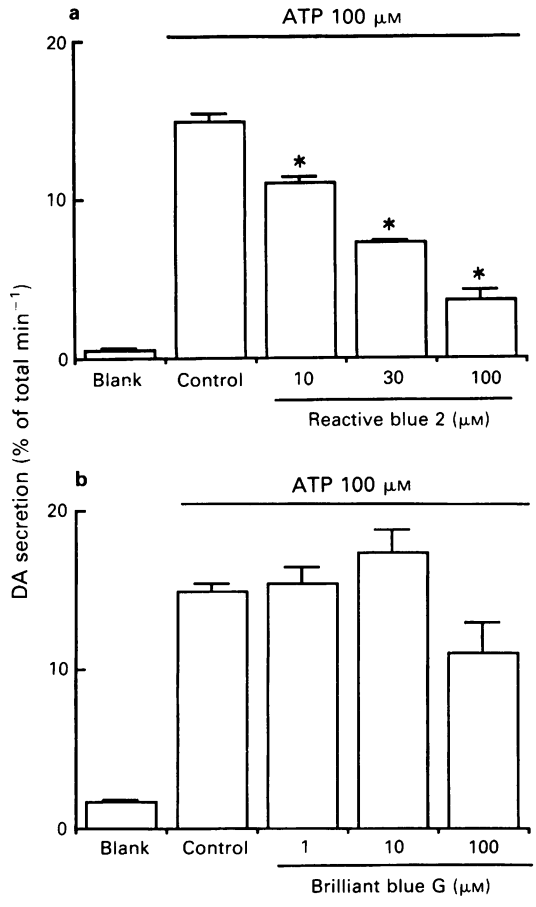

Figure 1 Effects of reactive blue 2 (a) and brilliant blue G (b) on ATP (100 μM)-stimulated dopamine (DA) secretion from PC12 cells. Each column and bar represent mean and s.e.mean from 3 dishes of the same batch. Dopamine-secretion after stimulation with ATP-free buffer is shown as blank in each panel. Data shown were representative of 3(a) or 2(b) experiments. Asterisks indicate significant difference from control (Student's t test, $P < 0.05$).

with an extracellular solution containing (in mM) NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 11.1 and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10.0 (adjusted with NaOH to pH 7.4). Patch pipettes were

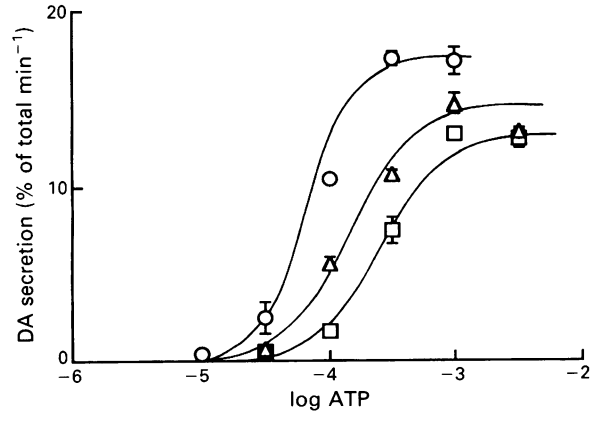

Figure 2 Concentration-response curves for ATP-stimulated dopamine (DA)-secretion in the absence (○) and presence of 30 μM (△) or 100 μM (□) reactive blue 2. Each point represents the mean from 3 dishes tested. Vertical bars are s.e.mean. Smooth curves were fitted to the data assuming an equation (Tallarida & Jacob, 1979). $E/(E_{max} - E) = ([ATP]/EC_{50})^n$. In this equation, E is the effect induced by each concentration of ATP. The values used for fitting were n = 2.5 (control), 1.8 (30 μM reactive blue 2) or 1.8 (100 μM reactive blue 2), and EC_{50} = 72 μM (control), 148 μM (30 μM reactive blue 2) or 275 μM (100 μM reactive blue 2).

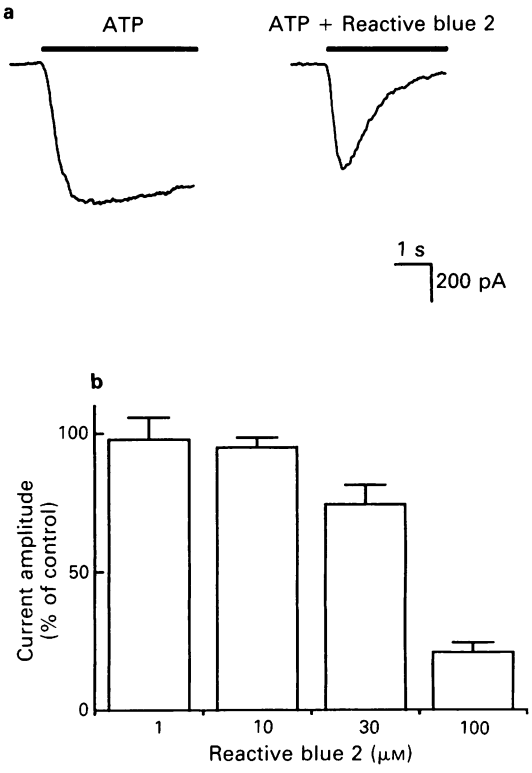

Figure 3 Inhibition by reactive blue 2 of an ATP-activated inward current in PC12 cells. The cells were held at −60 mV. (a) Current traces obtained from one cell. The cell was first exposed to ATP (100 μM) alone and, after a 5 min rinse, exposed to ATP plus reactive blue 2 (30 μM). (b) Concentration-dependence of inhibition of the ATP (100 μM)-activated current by reactive blue 2. The peak current amplitude with each concentration of reactive blue 2 was normalized to that without reactive blue 2 in each cell. Each column and bar represent mean and s.e.mean from 4 to 6 cells tested.

filled with an intracellular solution containing (in mM) CsCl 150, HEPES 10.0 and glycoletherdiamine-N,N,N',N'-tetra-acetic acid (EGTA) 5.0. The membrane current measurements were done at room temperature (about 25°C).

Drugs

Drugs used were ATP (Sigma), reactive blue 2 (Fluka), brilliant blue G (Sigma), and nicotine (Sigma).

Statistical analysis

Statistical analyses were made by Student's t test. Comparison was made within same batches (i.e., data in a series of experiments obtained in a day).

Results

ATP (100 μM) evoked dopamine-secretion from PC12 cells (Figure 1). Reactive blue 2 inhibited the ATP-evoked dopamine-secretion in a concentration-dependent manner (Figure 1a). Brilliant blue G (up to 100 μM) did not affect significantly the ATP-evoked secretion (Figure 1b). Reactive blue 2 or brilliant blue G (up to 100 μM) did not affect basal dopamine-secretion in 3 dishes tested. Figure 2 shows the concentration-response curves for ATP in evoking dopamine-secretion. The curve was shifted to the right and the maximal response was decreased by reactive blue 2 (30 to 100 μM). The slope of the concentration-response curve was decreased by reactive blue 2 in a manner similar to that previously reported for the ATP-induced muscle relaxation of rabbit portal vein (Reilly *et al.*, 1987) or rabbit mesenteric artery (Burnstock & Warland, 1987).

The ATP-evoked catecholamine-secretion from PC12 cells was closely correlated with an ATP-activated inward current (Inoue *et al.*, 1989). This ATP-activated inward current is produced by ion channels which are rather tightly coupled to ATP-receptors, judging from kinetics and behaviour of the single-channel currents in cell-free membrane patches (Nakazawa *et al.*, 1990a,c). As shown in Figure 3, reactive blue 2 inhibited the ATP-activated current over a concentration-range that was similar to that which inhibited ATP-evoked dopamine secretion (Figures 1 and 2). The ATP-activated current fully recovered after washout of reactive blue 2 (not shown). These results suggest that the decrease in dopamine secretion by reactive blue 2 is due to a change in the receptor-operated channel activity in these cells. Brilliant blue G (up to 100 μM) did not affect the ATP-activated current (not shown).

We previously showed that suramin (30 to 300 μM) inhibited the dopamine-secretion and the current evoked by ATP but did not affect these responses evoked by nicotine in PC12 cells (Nakazawa *et al.*, 1990b; Inoue *et al.*, 1991). We investigated effects of reactive blue 2 on the nicotine-evoked responses to compare its selectivity with suramin. Reactive blue 2 (100 μM) decreased the dopamine-secretion induced by 10 μM nicotine from $9.4 \pm 0.1\%$ to $6.3 \pm 0.3\%$ of total min^{-1} (mean $\pm$ s.e.mean, $n = 3$). Reactive blue 2 also inhibited the current activated by 10 μM nicotine; the current in the presence of 100 μM reactive blue 2 was $66.9 \pm 0.6\%$ ($n = 3$) of control. The results suggest that reactive blue 2 weakly discriminates between ATP- and nicotine-evoked responses as compared with suramin.

Discussion

Reactive blue 2 inhibited ATP-evoked dopamine-secretion and the ATP-activated inward current in PC12 cells. These results suggest that receptors mediating the effects of ATP in these cells are the same type as those mediating ATP-induced relaxation of rabbit mesenteric artery (Burnstock & Warland, 1987), or rabbit portal vein (Reilly *et al.*, 1987). It was reported that reactive blue 2 selectively inhibited the response via P_{2y}-purinoceptors, for which ATP is a more potent agonist than α,β methylene ATP or β,γ-methylene ATP, but did not affect the response via P_{2x}-purinoceptors, for which α,β-methylene ATP or β,γ-methylene ATP is a more potent agonist than ATP, in vascular smooth muscles (Burnstock & Warland, 1987; Reilly *et al.*, 1987). In contrast, reactive blue 2 inhibited ATP-induced smooth muscle contraction of rat and guinea-pig urinary bladder (Choo, 1981), which may be mediated through P_{2x}-purinoceptors (Brown *et al.*, 1979; Burnstock *et al.*, 1983). The effects of reactive blue 2 in this report may be due to its antagonism to P_{2y}-purinoceptors because ATP but neither α,β-methylene ATP (Nakazawa *et al.*, 1990a) nor β,γ-methylene ATP (Inoue *et al.*, 1990) was a potent agonist in these cells.

The maximal response and the slope of the concentration-response curve of ATP-evoked dopamine secretion were decreased by reactive blue 2 (Figure 2). These results suggest that the antagonism of ATP-evoked dopamine-secretion by reactive blue 2 is non-competitive as has been reported previously for antagonism of the ATP-evoked relaxation of smooth muscles (Burnstock & Warland, 1987; Reilly *et al.*, 1987). The non-competitive nature is not due to irreversible blockade of the purinoceptors because the inhibition of the ATP-activated current was fully recovered after washout of reactive blue 2. This non-competitive antagonism suggests that reactive blue 2 affects a site other than the ATP-binding site.

Unlike reactive blue 2, brilliant blue G did not inhibit these ATP-evoked responses in PC12 cells. This result was much different from that seen in rat parotid acinar cells, where brilliant blue G is about 100 times more potent than reactive blue 2 in antagonizing the ATP-induced Ca influx (Soltoff *et al.*, 1989). This discrepancy is indicative of different populations of purinoceptors in PC12 cells and rat parotid acinar cells. The purinoceptors in rat parotid acinar cells were characterized as P_{2z}-type (Soltoff *et al.*, 1989) because they mediated the effects of the free-acid form of ATP (ATP^{4-}) (Gordon, 1986). Honoré *et al.* (1989) have suggested that the effective form of ATP for the activation of P_{2y}-purinoceptors is also the free-acid form, judging from the inhibitory effects of divalent cations which bind to ATP^{4-} (Fabiato & Fabiato, 1979). We recently reported a similar result in PC12 cells (Nakazawa *et al.*, 1990c). Therefore, it may be more informative to distinguish the populations of purinoceptors in PC12 cells (P_{2y}-type) and rat parotid acinar cells (other than P_{2y}-type) on the basis of different sensitivity to brilliant blue G than on the basis of the effective form of ATP.

In conclusion, we have shown that reactive blue 2, but not brilliant blue G, antagonizes ATP-evoked dopamine-secretion and an ATP-activated inward current in PC12 cells. The purinoceptors involved in these responses may be the same type (P_{2y}-type) as those involved in ATP-evoked vascular smooth muscle relaxation.

We thank Dr J.G. Kenimer for improving the manuscript, and N. Sekine and H. Sato for technical assistance.

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(Received August 28, 1990
Revised November 28, 1990
Accepted December 10, 1990)

Br. J. Pharmacol. (1991), **102**, 855–860

Evidence for facilitatory and inhibitory muscarinic receptors on postganglionic sympathetic nerves in mouse isolated atria

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1 McNeil A 343 (10 μM–30 μM) enhanced the fractional stimulation-induced (S-I) outflow of radioactivity from mouse isolated atria which had been incubated with [^{3}H]-noradrenaline. The enhancing effect of McNeil A 343 was not altered by hexamethonium (300 μM) suggesting that it was not due to an action at nicotinic receptors. It is also unlikely that McNeil A 343 enhanced the S-I outflow of radioactivity in mouse atria by blocking neuronal reuptake of noradrenaline since the effect persisted in the presence of cocaine (30 μM).

2 The facilitatory effect of McNeil A 343 on the S-I outflow of radioactivity was attenuated by atropine (0.3 μM), pirenzepine (0.2 μM or 1.0 μM), dicyclomine (1.0 μM) and methoctramine (1.0 μM) and was thus due to activation of muscarinic receptors.

3 In contrast to the effect of McNeil A 343, another muscarinic receptor agonist, carbachol (3.0 μM) significantly decreased the S-I outflow of radioactivity. The receptors through which McNeil A 343 acts to enhance the S-I outflow of radioactivity appear to be distinct from inhibitory prejunctional muscarinic receptors. The relatively M_1-selective antagonist, pirenzepine (0.2 μM), attenuated the facilitatory effect of McNeil A 343 whereas a higher concentration (1.0 μM) was required to block the inhibitory effect of carbachol. Conversely, the relatively M_2-selective antagonist, methoctramine (0.1 μM), blocked the inhibitory effect of carbachol but a higher concentration of methoctramine (1.0 μM) was required to block the facilitatory effects of McNeil A 343. These results tentatively ascribe facilitatory muscarinic receptors as belonging to the M_1 subtype and inhibitory muscarinic receptors as belonging to the M_2 subtype.

4 The non-selective muscarinic receptor antagonist, atropine, enhanced the S-I outflow of radioactivity, suggesting that there was tonic activation of inhibitory prejunctional muscarinic receptors by endogenous acetylcholine released from parasympathetic nerves. However, pirenzepine (0.03 μM–1.0 μM) did not decrease the S-I outflow of radioactivity, suggesting that under the conditions of the present study, facilitatory muscarinic receptors are not tonically activated by endogenous acetylcholine.

Keywords: McNeil A 343; muscarinic receptors; methoctramine; noradrenaline release; pirenzepine

Introduction

Activation of prejunctional muscarinic receptors on postganglionic sympathetic nerve terminals inhibits noradrenaline release from a range of tissues (see Starke, 1977; Muscholl, 1980). Muscarinic receptor antagonists enhance noradrenaline release in the heart, suggesting that there is tonic activation of inhibitory prejunctional muscarinic receptors by endogenous acetylcholine released from nearby parasympathetic nerves (Levy & Blattberg, 1976; Muscholl & Muth, 1982; Habermeier-Muth & Muscholl, 1988). In rat heart (Fuder, 1982; Fuder *et al.*, 1982) and rabbit heart (Fuder *et al.*, 1981; Fuder, 1982), inhibitory prejunctional muscarinic receptors are similar to postjunctional muscarinic receptors in these tissues, and thus resemble the M_2 subtype (Fuder, 1982; Fuder *et al.*, 1981; 1982; see also Mitchelson, 1988).

The muscarinic receptor agonist, McNeil A 343 inhibited noradrenergic transmission in rabbit ear artery (Rand & Varma, 1971; Allen *et al.*, 1974; Choo *et al.*, 1985) but enhanced noradrenaline release from guinea-pig atria (Allen *et al.*, 1972; 1974; Vizi *et al.*, 1989), rabbit heart (Fozard & Muscholl, 1974), rabbit pulmonary artery (Nedergaard, 1980; 1981) and rat atria (Arbilla *et al.*, 1986). In these tissues where McNeil A 343 enhanced noradrenaline release, other muscarinic receptor agonists such as acetylcholine, carbachol and methacholine consistently inhibited noradrenaline release (see Starke, 1977). McNeil A 343 is not a typical muscarinic receptor agonist and appears to be functionally selective for M_1 receptors (see Birdsall *et al.*, 1988; Mitchelson, 1988). Thus it is possible that the enhancing effect of McNeil A 343 on noradrenergic transmission may be due to activation of a specific prejunctional facilitatory M_1 muscarinic mechanism. Indeed there is recent evidence for a prejunctional M_1 facilitatory muscarinic receptor site that is activated by endogenous acetylcholine in rabbit atria (Muscholl *et al.*, 1989; Altes *et al.*, 1990).

The aim of the present study was to determine if the enhancing effect of McNeil A 343 on noradrenaline release could be blocked by antagonists which are able to discriminate between various muscarinic receptor subtypes, and thus provide evidence for the existence of facilitatory muscarinic receptors on sympathetic nerve terminals. Our hypothesis was that sympathetic nerve terminals are endowed with inhibitory M_2 muscarinic receptors and facilitatory M_1 muscarinic receptors.

Methods

Preparation of mouse atria

Female mice (15–25 g) were decapitated and the heart was rapidly removed and placed in Krebs-Henseleit solution which was bubbled with a mixture of 5% CO_2 and 95% O_2. The atria were dissected free of the surrounding tissue and then placed in an organ bath with Krebs-Henseleit solution which was maintained at 37°C and constantly gassed with 5% CO_2, 95% O_2. The atria were incubated with [^{3}H]-noradrenaline (15 μCi ml^{-1}; 0.37 μM), for 20 min to label transmitter noradrenaline stores. Following incubation, the atria were tied loosely to a disc and transferred to individual flow cells. The tissues were washed by superfusing them at a rate of 2 ml min^{-1} for 60 min with Krebs-Henseleit solution which was maintained at 37°C and constantly gassed with 5% CO_2, 95% O_2. A priming stimulation was delivered to the atria 45 min after the beginning of the washing period. The priming stimulation consisted of square wave pulses, 1 ms duration, 15 V cm^{-1} field gradient, and was delivered at 3 Hz for 60 s

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through two platinum electrodes which were fixed into the flow cell and situated either side of the tissue. The washing procedure and the priming stimulation were designed to remove unbound and loosely bound radioactive compounds from the atria. After the washing period, 3 min fractions of the solution superfusing the atria were collected for the next 54 min. There were two test stimulation periods (S_1 and S_2), delivered at 6 and 42 min from the onset of sample collection. The stimulation parameters for the test stimulations were the same as those for the priming stimulation (15 V cm^{-1}, 3 Hz, 60 s). The effects of drugs were assessed by superfusing them only during the second stimulation period. In most experiments antagonists or blocking drugs were present from 24 min prior to the second stimulation period, whilst agonists were present from 15 min prior to the second stimulation period. In some experiments antagonists were present in the superfusion solution from just after the priming stimulation until the end of the experiment (designated 'throughout'). In one series of experiments, S_1 and S_2 were 69 min apart and pirenzepine was present from 57 min before the second stimulation period. At the end of all the experiments the atria were dissolved in Soluene (Packard Instruments, U.S.A.).

The samples of the superfusion solution and the atria were added to Picofluor 40 (Packard Instruments, U.S.A.), and the radioactive content of both the samples and the dissolved atria was measured with a liquid scintillation counter. Corrections for counting efficiency were made by automatic external standardization and all measurements were expressed as disintegrations per min (d.p.m.).

Calculation of radioactive outflow

The fractional radioactive outflow of a sample was calculated as the radioactive content of the sample expressed as a percentage of the total tissue radioactivity at the time the sample was collected. The spontaneous (resting) outflow of radioactivity was calculated as the mean value of the fractional radioactive content of the sample taken immediately before the stimulation and of the sample taken 9 min following the onset of stimulation. The fractional stimulation-induced outflow of radioactivity was calculated by adding the fractional radioactive content of the sample during which stimulation occurred and the sample immediately following, and then subtracting twice the mean fractional resting outflow of radioactivity.

Statistical analysis of results

All data are expressed as mean and standard error of the mean (s.e.mean). The data were analysed by Student's *t* tests. Where multiple comparisons against a single control were made, one way analysis of variance (ANOVA) followed by Dunnett's tests were used. Where appropriate, two-way ANOVA were also carried out. Probability levels (P) of less than 0.05 were taken to indicate statistical significance in all cases.

Materials

The following drugs were used: (−)-2,5,6-[^{3}H]-noradrenaline (specific activity 40.8 Ci mmol^{-1}, N.E.N., U.S.A.), McNeil A 343 (4-(*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride, McNeil Laboratories, Australia), atropine sulphate (Sigma, U.S.A.), carbachol (carbamylcholine chloride, Sigma, U.S.A.), hexamethonium bromide (May and Baker, Australia), cocaine HCl (Macfarlan-Smith, Australia), methoctramine HCl (Research Biochemicals Inc., U.S.A.). dicyclomine HCl was a generous gift from Dr Fred Mitchelson, Victorian College of Pharmacy, Australia. Pirenzepine HCl was a gift from Boehringer Ingelheim, Australia. All of the drugs except for dicyclomine HCl and methoctramine HCl were dissolved in Krebs-Henseleit solution. Dicyclomine and methoctramine were initially dissolved in distilled H_2O before being diluted in Krebs-Henseleit solution. The modified Krebs-Henseleit solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.03, $MgSO_4$ 0.45, D-(+)-glucose 11.1, disodium edetate 0.067 and ascorbic acid 0.14.

Results

The effect of McNeil A 343 on the stimulation-induced outflow of radioactivity and the influence of hexamethonium and cocaine

Mouse atria were incubated with [^{3}H]-noradrenaline, two stimulations were delivered (S_1 and S_2) and the absolute fractional stimulation-induced (S-I) outflow of radioactivity in the first stimulation period (FR_1) is given in Table 1. McNeil A 343, present only in the second stimulation period concentration-dependently enhanced the fractional S-I outflow of radioactivity (Figure 1). When they were present only in the second stimulation period, cocaine (30 μM) but not hexamethonium (300 μM), significantly enhanced the fractional S-I outflow of radioactivity (Figure 2). The enhancing effect of McNeil A 343 (10 μM) on the S-I outflow of radioactivity was not altered in the presence of either cocaine or hexamethonium (Figure 2).

Table 1 Fractional stimulation-induced outflow of radioactivity in the first stimulation period (S_1) from mouse isolated atria

Drug	S_1	n
Control	0.45 ± 0.01	157
Pirenzepine (0.2 μM)	0.44 ± 0.04	12
Atropine (0.3 μM)	0.43 ± 0.02	17
Dicyclomine (0.1 μM)	0.34 ± 0.04	12
Dicyclomine (1.0 μM)	0.41 ± 0.03	10
Methoctramine (0.1 μM)	0.51 ± 0.03	14
Methoctramine (1.0 μM)	1.09 ± 0.09*	8

Mouse atria were incubated with [^{3}H]-noradrenaline and then continually superfused. There were two test stimulation periods, and the fractional S-I outflow of radioactivity during the first stimulation period is shown. Where they were present, drugs were added from 20 min prior to the first stimulation period. Figures are a percentage of the total tissue radioactivity. Mean ± s.e.mean values are given. *n* is the number of experiments. * represents a significant difference from control ($P < 0.05$, Dunnett's test after one-way ANOVA).

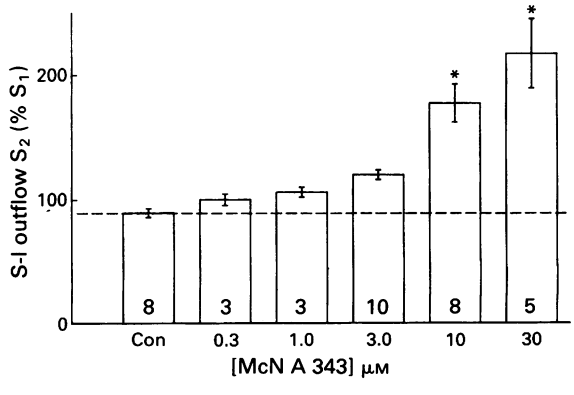

Figure 1 The effect of McNeil A 343 (McN A 343, 0.3 μM–30 μM) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria which had been incubated with [^{3}H]-noradrenaline. There were two stimulation periods (3 Hz, 60 s), and McNeil A 343 was present only for the second stimulation period. The mean fractional S-I outflow of radioactivity in the second stimulation period (S_2), expressed as a percentage of that in the first (S_1) is shown. The vertical lines represent s.e.mean, and the number of experiments is at the base of each column. * represents a significant difference from control ($P < 0.05$, Dunnett's test, after one way ANOVA).

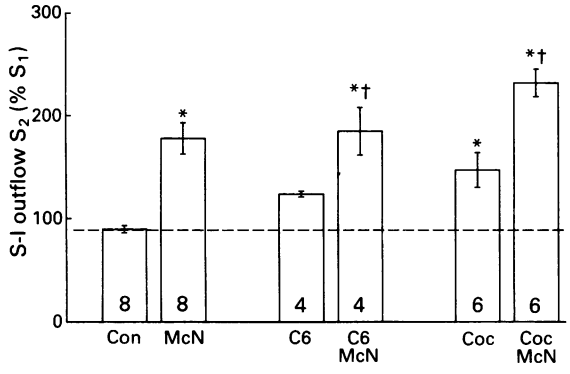

Figure 2 The enhancing effect of McNeil A 343 (McN, 10 μM) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria, and the influence of hexamethonium (C6, 300 μM) or cocaine (Coc, 30 μM) on the enhancing effect of McN A 343. There were two stimulation periods, and each of the drugs was present only for the second stimulation period. The mean fractional S-I outflow in the second stimulation period (S_2) expressed as a percentage of that in the first (S_1) is shown. * represents a significant difference from control ($P < 0.05$, Dunnett's test after one way ANOVA); † represents a significant difference from the appropriate McNeil A 343-free experiment ($P < 0.05$, Student's t test). When hexamethonium or cocaine were present, the effect of McNeil A 343 on the S-I outflow of radioactivity was not different from that in the absence of the drugs ($P > 0.05$, two-way ANOVA).

The effect of muscarinic receptor antagonists on the enhancement of S-I outflow of radioactivity by McNeil A 343

Pirenzepine Pirenzepine (0.2 μM or 1.0 μM), present only in the second stimulation period, had no significant effect on the fractional S-I outflow of radioactivity (Figure 3). However, pirenzepine (0.2 μM or 1.0 μM) significantly reduced the enhancing effect of McNeil A 343 on the S-I outflow of radioactivity (Figure 3). In the experiments shown in Figure 3

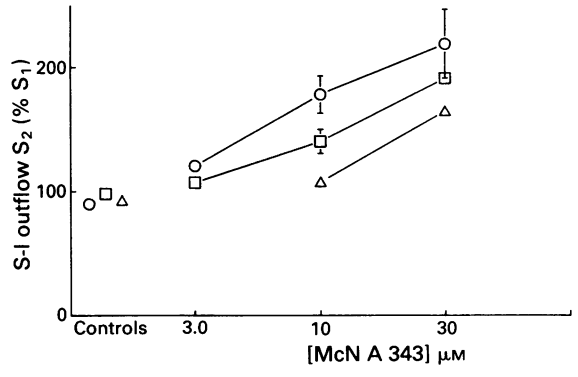

Figure 3 The influence of pirenzepine (0.2 μM or 1.0 μM) on the enhancing effect of McNeil A 343 (McN A 343, 3.0 μM, 10 μM or 30 μM) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria which had been pre-incubated with [^{3}H]-noradrenaline. There were two stimulation periods, and drugs were present only for the second stimulation period. The mean fractional S-I outflow in the second stimulation period (S_2) expressed as a percentage of that in the first (S_1) is shown. On some points, the s.e.mean is not seen since the symbol size is larger than the standard error line. (○) represents experiments in the absence of pirenzepine; (□) represents experiments in the presence of pirenzepine (0.2 μM), and (△) represents experiments in the presence of pirenzepine (1.0 μM) (n = 4–10 for each point). Experiments in the absence of McNeil A 343 are shown on the left. The concentration-response relationship of McNeil A 343 was analysed by least squares regression. The lines for McNeil A 343 in the absence of pirenzepine and in the presence of pirenzepine (0.2 μM or 1.0 μM) did not differ significantly from parallelism ($P > 0.05$, two-way ANOVA), but did differ significantly in elevation ($P < 0.05$, two-way ANOVA), indicating a significant reduction in the effect of McNeil A 343 at both concentrations of pirenzepine.

pirenzepine was present for only 9 min before McNeil A 343 was introduced. Experiments were also done with pirenzepine present for 41 min (throughout) before the addition of McNeil A 343 (10 μM). Under these conditions the facilitatory effect of McNeil A 343 (10 μM) on the S-I outflow of radioactivity was also significantly reduced (Figure 4a). Pirenzepine (0.2 μM) had no effect on absolute fractional S-I outflow of radioactivity in the first stimulation period (Table 1).

Atropine When atropine (0.3 μM) was present during both stimulation periods, the enhancing effect of McNeil A 343 (10 μM) on the fractional S-I outflow of radioactivity was significantly reduced (Figure 4a). Atropine had no effect on the absolute fractional S-I outflow of radioactivity in the first stimulation period (Table 1).

Dicyclomine Dicyclomine (0.1 μM), present throughout the experiment, did not alter the enhancing effect of McNeil A 343 (10 μM) on the fractional S-I outflow of radioactivity (Figure 5). In the presence of a higher concentration of dicyclomine (1.0 μM) however, the effect of McNeil A 343 (10 μM) was almost totally abolished (Figure 5). Dicyclomine (0.1 μM or 1.0 μM) had no effect on the absolute fractional S-I outflow of radioactivity in the first stimulation period (Table 1).

Methoctramine When methoctramine (0.1 μM) was present during both stimulation periods, the enhancing effect of McNeil A 343 (10 μM) on the fractional S-I outflow of radioactivity was not attenuated (Figure 4a). In the presence of a higher concentration of methoctramine (1.0 μM) however, the

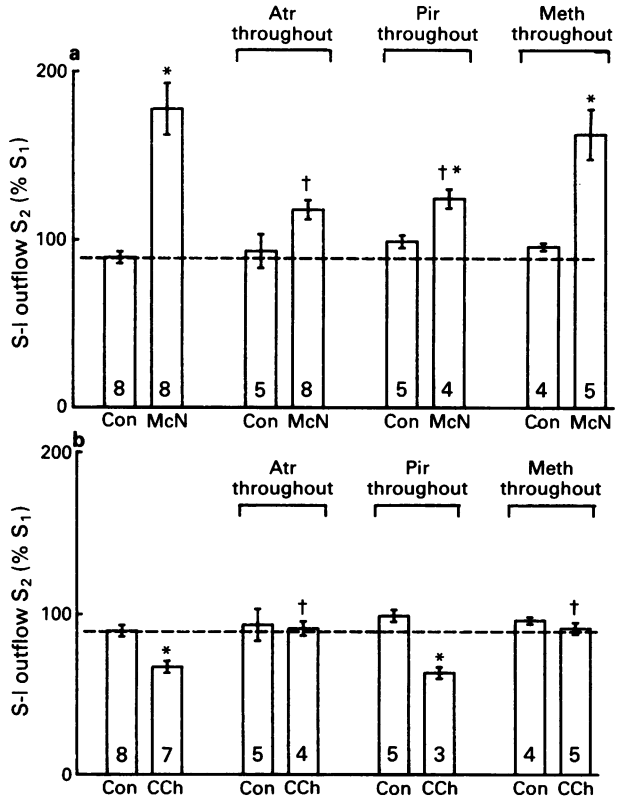

Figure 4 The effect of pirenzepine (Pir, 0.2 μM), atropine (Atr, 0.3 μM) and methoctramine (Meth, 0.1 μM) on the modulation of the fractional stimulation-induced (S-I) outflow of radioactivity by McNeil A 343 (McN, 10 μM) (a), or by carbachol (CCh, 3.0 μM) (b). Mouse atria were pre-incubated with [^{3}H]-noradrenaline and stimulated twice. McNeil A 343 or carbachol were present only during the second stimulation period. Other drugs were present during both stimulation periods. The mean fractional S-I outflow in the second stimulation period (S_2) expressed as a percentage of that in the first (S_1) is shown. * represents a significant difference from the respective control ($P < 0.05$, Student's t test); † represents a significantly reduced effect of McNeil A 343 or carbachol in the presence of the antagonist compared to the effect of McNeil A 343 or carbachol alone ($P < 0.05$, two-way ANOVA).

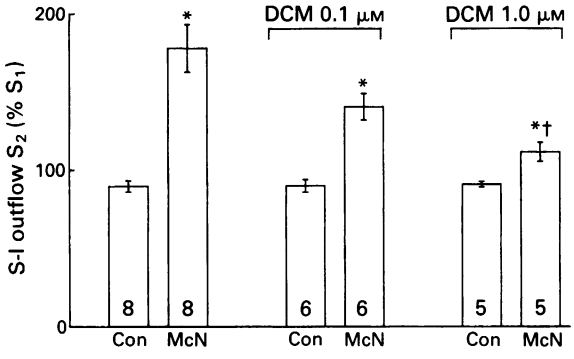

Figure 5 The effect of McNeil A 343 (McN, 10 μM) in the presence of dicyclomine (DCM, 0.1 μM or 1.0 μM), on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria which had been preincubated with [^{3}H]-noradrenaline. The atria were stimulated twice, and McN A 343 was present only for the second stimulation period. Dicyclomine was present during both stimulation periods. The mean fractional S-I outflow in the second stimulation period (S_2) expressed as a percentage of that in the first (S_1) is shown. * represents a significant difference from the appropriate control ($P < 0.05$, Student's t test). † represents a significantly reduced effect of McNeil A 343 in the presence of dicyclomine compared to the effect of McN A 343 alone ($P < 0.05$, two-way ANOVA).

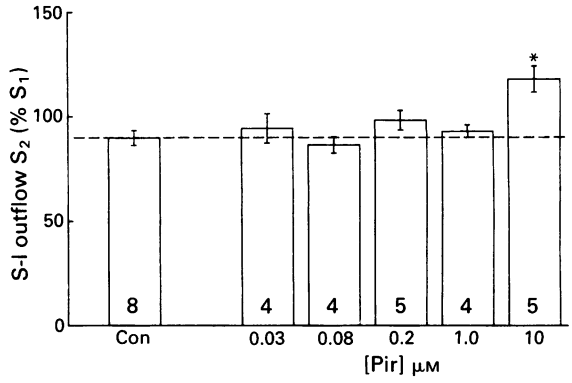

Figure 6 The effect of pirenzepine (Pir, 0.03 μM–10 μM) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria which had been pre-incubated with [^{3}H]-noradrenaline and stimulated twice. Pirenzepine was present only for the second stimulation period. The mean fractional S-I outflow in the second stimulation period (S_2) as a percentage of that in the first (S_1) is shown. * represents a significant difference from control ($P < 0.05$, Dunnett's test after one way ANOVA).

enhancing effect of McNeil A 343 (10 μM) was totally abolished. In this case the fractional S-I outflow of radioactivity in the second stimulation period as a percentage of that in the first was 92.6 ± 5.3% ($n = 4$) for methoctramine (1.0 μM) throughout, and 103.9 ± 3.4% ($n = 4$) for McNeil A 343 (10 μM) in the presence of methoctramine (1.0 μM) (compare with Figure 1). The absolute fractional S-I outflow of radioactivity in the first stimulation period was significantly increased when methoctramine (1.0 μM, but not 0.1 μM) was present throughout the experiment (Table 1).

The effect of muscarinic receptor antagonists on the inhibition of S-I outflow of radioactivity by carbachol

Carbachol (3.0 μM), present only in the second stimulation period, significantly reduced the fractional S-I outflow of radioactivity (Figure 4b). When atropine (0.3 μM) was present throughout the experiment, the inhibitory effect of carbachol was abolished (Figure 4b). Pirenzepine (0.2 μM), present throughout the experiment, did not alter the inhibitory effect of carbachol on the S-I outflow of radioactivity (Figure 4b). At the higher concentration, pirenzepine (1.0 μM), blocked the inhibitory effect of carbachol. In this case the fractional S-I outflow of radioactivity in the second stimulation period as a percentage of that in the first was 92.6 ± 3.0% ($n = 4$) for pirenzepine (1.0 μM) alone, and 85.6 ± 6.7% ($n = 4$) for carbachol in the presence of pirenzepine (compare with Figure 4b). When methoctramine (0.1 μM) was present throughout the experiment, the inhibitory effect of carbachol (3.0 μM) on the S-I outflow of radioactivity was totally abolished (Figure 4b).

The effect of muscarinic receptor antagonists on the S-I outflow of radioactivity

Atropine (0.3 μM), present only in the second stimulation period, significantly increased the S-I outflow of radioactivity. The fractional S-I outflow of radioactivity in the second stimulation period as a percentage of that in the first was 89.6 ± 3.5% ($n = 8$) for control, and 133.5 ± 12.6% ($n = 8$) for atropine.

Pirenzepine (0.03 μM–1.0 μM) did not alter the S-I outflow of radioactivity when it was present only in the second stimulation period (Figure 6). At the higher concentration of pirenzepine (10 μM), there was a significant increase in the S-I outflow of radioactivity (Figure 6). When the interval between the two stimulation periods was increased to 69 min to allow a longer contact time with the antagonist, pirenzepine (0.2 μM) present from 57 min before the second stimulation period, did not alter the S-I outflow of radioactivity. The fractional S-I outflow of radioactivity in the second stimulation period as a percentage of that in the first was 79.0 ± 2.1% for control and 81.7 ± 2.1% for pirenzepine ($n = 5$ in both cases).

When methoctramine (0.1 μM) was present in the second stimulation period, there was a significant increase in the S-I outflow of radioactivity. The fractional S-I outflow of radioactivity in the second stimulation period as a percentage of that in the first was 89.6 ± 3.5% ($n = 8$) for control, and 143.7 ± 7.1% ($n = 5$) for methoctramine.

The effect of McNeil A 343 on the spontaneous outflow of radioactivity from mouse atria

McNeil A 343 (10 μM or 30 μM), either by itself or in the presence of other drugs, inhibited the spontaneous outflow of radioactive compounds by about 10% from mouse isolated atria which had been incubated with [^{3}H]-noradrenaline (not shown). In the presence of cocaine (30 μM), which by itself increased the spontaneous outflow of radioactive compounds by about 15%, McNeil A 343 did not affect the spontaneous outflow of radioactive compounds (not shown). None of the other drugs used in the present study altered the spontaneous outflow of radioactivity from mouse atria (not shown).

Discussion

Stimulation-induced (S-I) outflow of radioactivity from tissues which had been previously incubated with [^{3}H]-noradrenaline has been determined to be an appropriate assessment of transmitter noradrenaline release (see Langer, 1974). In the present study in mouse atria which had been preincubated with [^{3}H]-noradrenaline, the M_1-selective muscarinic receptor agonist McNeil A 343 (see Mitchelson, 1988; Birdsall *et al.*, 1988), enhanced S-I release of noradrenaline in a concentration-dependent manner. Facilitation of noradrenaline release by McNeil A 343 has been known for some time (see Introduction), however an adequate explanation for this effect is lacking. In mouse atria the enhancing effect of McNeil A 343 on the S-I release of noradrenaline was not prevented by the antinicotinic drug hexamethonium, thus it is unlikely that the effect was due to activating facilitatory nicotinic receptors. This is in accord with previous reports that McNeil A 343 has little or no activity at nicotinic receptors (Roszkowski, 1961; Nedergaard, 1980).

Previous studies have suggested that McNeil A 343 enhances noradrenaline release to some extent by blocking

neuronal reuptake of noradrenaline (Fozard & Muscholl, 1974; Allen *et al.*, 1972). In mouse atria, this mechanism probably does not contribute to a substantial proportion of the enhancing effect of McNeil A 343, since previously blocking neuronal uptake with a high concentration of cocaine (30 μM) did not alter the ability of McNeil A 343 to enhance noradrenaline release. Furthermore, McNeil A 343 (10 μM) increased noradrenaline release by almost twice as much as cocaine (30 μM) itself. It has been reported (Nedergaard, 1980) that McNeil A 343 is a much weaker neuronal uptake blocker than cocaine, therefore it is possible that blockade of neuronal uptake is only apparent at higher concentrations of McNeil A 343 (100 μM used in the study of Allen *et al.*, 1972; 20 μM used in the study of Fozard & Muscholl, 1974).

The possibility that McNeil A 343 enhances S-I noradrenaline release by a receptor-mediated process has been dismissed previously because the effect was found to be resistant to blockade by atropine (Fozard & Muscholl, 1974; Allen *et al.*, 1974; Nedergaard, 1980; 1981; Arbilla *et al.*, 1986). However in the present study in mouse atria, atropine markedly reduced the enhancing effect of McNeil A 343 on noradrenaline release. A similar finding in guinea-pig atria has also been reported by Vizi *et al.* (1989). It is possible that the effect of McNeil A 343 was resistant to blockade by atropine in previous studies because very high concentrations of McNeil A 343 were used and non-receptor-mediated actions may have contributed to the effect of McNeil A 343 on noradrenaline release.

In the present study in addition to atropine, the relatively M_1-selective antagonists pirenzepine (Hammer & Giachetti, 1982), and dicyclomine (Giachetti *et al.*, 1986; Giachetti & Micheletti, 1988), and a high concentration of the relatively M_2-selective antagonist methoctramine (Melchiorre *et al.*, 1987; Wess *et al.*, 1988), were also able to reduce the enhancing effect of McNeil A 343 on noradrenaline release. This further supports the proposal that the facilitatory effect of McNeil A 343 is by a receptor-mediated process.

The question arises as to why McNeil A 343 enhances noradrenaline release yet other muscarinic receptor agonists such as carbachol inhibit release of the transmitter. A likely explanation is that muscarinic receptors through which McNeil A 343 acts to facilitate noradrenaline release are different from inhibitory prejunctional muscarinic receptors. In the present study we were able to differentiate the effect of McNeil A 343 and that of carbachol using selective muscarinic receptor antagonists. Firstly 0.2 μM pirenzepine, a relatively M_1-selective muscarinic receptor antagonist (Hammer & Giachetti, 1982; see also Eglen & Whiting, 1986), reduced the facilitatory effect of McNeil A 343 without affecting the inhibitory action of carbachol on the S-I noradrenaline release. A higher concentration of pirenzepine (1.0 μM) however, blocked the effects of both drugs. An opposite order of antagonism was found with methoctramine, a relatively M_2-selective muscarinic receptor antagonist (Melchiorre *et al.*, 1987; Wess *et al.*, 1988). At a low concentration methoctramine (0.1 μM) blocked the inhibitory effect of carbachol but did not reduce the facilitatory effect of McNeil A 343. However, a higher concentration of methoctramine (1.0 μM), blocked the effect of carbachol as well as that of McNeil A 343. Together these results suggest that facilitatory muscarinic receptors resemble the M_1 subtype. It should be noted that the concentrations of pirenzepine used to block the effects of McNeil A 343 were higher than its reported pA_2 values at M_1 receptors (about 8–8.4, see Mitchelson, 1988). Therefore, some caution must be used when assigning a definite muscarinic receptor subtype in spite of the obvious difference between the order of antagonist effectiveness for inhibitory and facilitatory muscarinic mechanisms.

It is widely accepted that acetylcholine released from parasympathetic nerves can activate inhibitory prejunctional muscarinic receptors on sympathetic nerve endings (see Muscholl, 1980). In mouse atria, it is likely that endogenous acetylcholine modulates noradrenaline release through inhibitory prejunctional muscarinic receptors since atropine was found to enhance noradrenaline release. Methoctramine, as well as a high concentration of pirenzepine (10 μM), also enhanced noradrenaline release which further indicates that in this field-stimulated preparation, inhibitory prejunctional muscarinic receptors were tonically activated by endogenous acetylcholine. Facilitatory prejunctional muscarinic receptors were probably not tonically activated since, as would have been expected, lower concentrations of pirenzepine (0.03 μM–1.0 μM) did not inhibit noradrenaline release. This lack of effect was probably not due to insufficient equilibration with the antagonist, since an even longer contact time with pirenzepine also did not reduce noradrenaline release.

In a recent study on a nerve-stimulated rabbit atria preparation (Habermeier-Muth & Muscholl, 1988), it was found that pirenzepine inhibited noradrenaline release under certain conditions of dual parasympathetic-sympathetic nerve stimulation, suggesting that endogenous acetylcholine may activate facilitatory prejunctional M_1 receptors (Muscholl *et al.*, 1989; Altes *et al.*, 1990). However in these studies it was found that the timing of the vagal nerve stimulation in relation to the sympathetic nerve stimulation was crucial (Muscholl *et al.*, 1989; Altes *et al.*, 1990). Pirenzepine did not affect noradrenaline release if the vagal pulses preceded the sympathetic nerve pulses by a short interval (3 ms) or a long interval (233 ms). However if the pulses arrived at an interval of 100 ms pirenzepine inhibited noradrenaline release (Muscholl *et al.*, 1989; Altes *et al.*, 1990). The reasons for these temporal effects are unclear. However, the lack of effect of pirenzepine on S-I noradrenaline release in the present study may be because with field stimulation there is concomitant parasympathetic and sympathetic nerve activation.

In conclusion, postganglionic sympathetic nerve terminals are endowed with prejunctional facilitatory muscarinic receptors in addition to typically observed inhibitory muscarinic receptors. The selectivity of pirenzepine and methoctramine for each of these receptors suggests that facilitatory prejunctional muscarinic receptors resemble the M_1 subtype, and inhibitory prejunctional muscarinic receptors resemble the M_2 subtype. While inhibitory prejunctional muscarinic receptors are probably activated by endogenous acetylcholine, this does not appear to be the case for facilitatory prejunctional muscarinic receptors in field-stimulated mouse atria.

M.C. holds an Australian Government Postgraduate Research Award. H.M. is an Australian Wellcome Senior Research Fellow. The work was supported by grants from the National Health and Medical Research Council of Australia.

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(Received September 25, 1990
Accepted December 10, 1990)

Br. J. Pharmacol. (1991), **102**, 861–864

A method for studying the pharmacodynamic profile of neuromuscular blocking agents on vocal cord movements in anaesthetized cats

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1 A new *in vivo* experimental method is described whereby the neuromuscular blocking effects of muscle relaxants can be investigated on the intrinsic laryngeal muscles of anaesthetized cats. The peripheral tibialis anterior muscle preparation is employed in the same animal to compare the blocking effect on both preparations.

2 The intrinsic laryngeal muscles react with different sensitivities to the neuromuscular blocking agents when compared to the tibialis anterior muscle.

3 The neuromuscular response in both muscle preparations is similar with steroidal agents but appeared to be different after suxamethonium or isoquinoline analogues.

4 It is concluded that this preparation may become a useful tool for studying new muscle relaxants developed to facilitate rapid intubation conditions.

Keywords: Larynx; muscle relaxants; skeletal muscle; vocal cords

Introduction

Following the introduction of (+)-tubocurarine in 1942 (Griffith & Johnson, 1942), several new neuromuscular blocking agents have been developed and are commonly used in clinical anaesthetic practice. Knowledge of the neuromuscular blocking effects of these agents in man is primarily based on measurements of twitch tension of the indirectly stimulated adductor pollicis muscle. However, it has been known for many years that different muscles in animals (Taylor *et al.*, 1964; Day *et al.*, 1982) and in man (Johansen *et al.*, 1964; Ali *et al.*, 1975; Derrington & Hindocha, 1988; Laycock *et al.*, 1988) differ in sensitivity towards the neuromuscular blocking effects of muscle relaxants. In addition, it has been reported that the magnitude and time-course of action of muscle relaxants differ significantly between the intrinsic laryngeal muscles and peripheral skeletal muscles (Gabriel, 1975; Baer, 1984). This may explain intubation difficulties and complications encountered during upper airway surgery (Baer & Pukander, 1982; Sia *et al.*, 1982). One of the objectives in the development of new neuromuscular blocking agents is that the 'ideal' relaxant should have a fast onset to facilitate rapid intubation (Savarese & Kitz, 1975). Therefore, we have developed a method in the cat, which allows the study of the neuromuscular blocking effects of muscle relaxants simultaneously on the (peripheral) tibialis anterior muscle and on vocal cord movement, the latter evoked by indirect stimulation of the intrinsic laryngeal muscles.

Methods

General

Adult cats (male and female, 2 to 4 kg body weight) were anaesthetized with pentobarbitone sodium (Nembutal) 40 mg kg^{-1} intraperitoneally and, after cannulating the cephalic vein, an infusion with pentobarbitone sodium was started at a rate of 4.8 mg kg^{-1} h^{-1}. Heart frequency was monitored with a BT-1200 Biotachometer and arterial blood pressure was measured by a Gould Statham P23Db pressure transducer via a cannula (Angiocath 18G) inserted in a femoral artery. Another cannula was placed in the saphenous vein and was used for the administration of the muscle relaxant under study. The lungs were ventilated by means of a V5KG ventilator (Narco-Biosystems Inc.) using a tidal volume of room air of 15 ml kg^{-1} at a frequency of 20 min^{-1}. End tidal P_{CO_2} was measured by a Capnograph Mark II and kept at 3.5–4.5 kPa. Rectal temperature was continuously measured and maintained at 37 ± 0.5 °C with a heated water mattress placed underneath the supine cat.

Peroneal nerve-tibialis anterior muscle preparation

The twitch tension of the tibialis anterior muscle, elicited by supramaximal square wave stimuli of 0.2 ms duration applied to the common peroneal nerve at a frequency of 0.1 Hz using a Grass S88 stimulator, was recorded with a Gould Statham UTC3 force displacement transducer and a physiopolygraph Varioscript 443 (Schwarzer) recorder.

Recurrent laryngeal nerve-vocal cord preparation

A median skin incision was made just caudally from the cricoideal cartilage towards the fossa jugularis. The sternomastoideal muscle was bisected along the media raphe to allow access to the underlying sternothyroideal muscles. These muscles were divided longitudinally along the junction of both muscle bellies and were pulled laterally, together with the underlying sternothyroideal muscles, exposing the trachea. A tracheotomy was performed approximately 3 to 4 cartilageal rings caudal of the cricoideus, and a Portex tracheal tube (4.5 or 5.0 mm internal diameter) was inserted and positioned with two ligatures. The right recurrent laryngeal nerve was carefully loosened from its surrounding tissues and attached to the stimulus electrodes, made of two U-shaped silver wires (diameter 0.6 mm), 8 mm apart. A sterile, inert non-conducting silicon oil was applied to the wound in order to prevent desiccation. Direct stimulation of the nuchal and laryngeal muscles was also prevented in this way. The nerve was stimulated supramaximally with square wave pulses of 0.2 ms duration and a frequency of 0.1 Hz using the apparatus described above.

The head of the cat was fixed and the jaws stretched by placing an oval metal ring (2.5 × 4 cm) behind the eyeteeth. A curved, pointed metal blade was introduced into the mouth,

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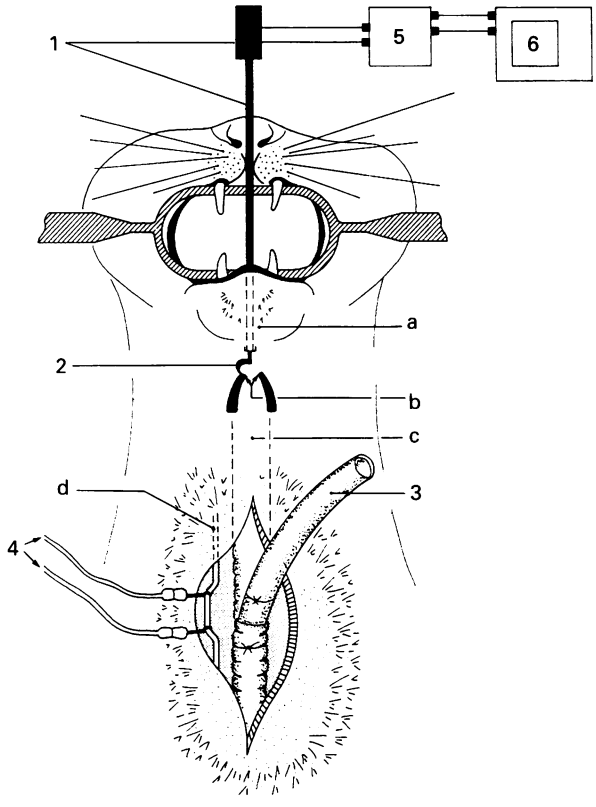

Figure 1 Schematic presentation of the laryngeal nerve-vocal cord preparation: (1) force transducer; (2) little hook (see text); (3) endotracheal tube; (4) stimulation electrodes; (5) muscle relaxation monitor; (6) recorders; (a) supraglottal area; (b) vocal cords; (c) trachea; (d) recurrent laryngeal nerve.

gently elevating the epiglottis ventrally and, hence, visualising the vocal cords. A force displacement transducer (Strain Measurement Devices Ltd., series 100; 0.5 N range) was modified by fixing a small, sharp, pointed hook at its end. This transducer was introduced through the mouth and the pharynx, and the hook was connected to the right vocal cord to quantify the abduction force evoked by right recurrent laryngeal nerve stimulation. A schematic presentation of the preparation is shown in Figure 1.

Experimental procedure

Both vocal cord and tibialis preparations were employed in the same animal to allow simultaneous measurement of the neuromuscular blocking effects of the administered muscle relaxants. In preliminary studies the doses of drugs producing approximately 75% depression of the single twitch height of the tibialis anterior muscle were ascertained (Table 1). After intravenous injection of suxamethonium, (+)-tubocurarine, atracurium, vecuronium or pancuronium, the maximum block was measured, defined as the percentage of maximal depression of the single twitch height compared to the control twitches. Also, the onset time (the time from the end of injection to the attainment of maximum block) and the total duration (the time from the end of injection to the time when 90% recovery of the twitch height was achieved) were measured in both muscles after each administration of a muscle relaxant. Each muscle relaxant was given 3 times and all administered to a different cat.

Results

In these animals, heart rate was approximately 200 beats per minute. The mean arterial blood pressure was always greater than 120 mmHg throughout the experiments. In addition, the end-tidal P_{CO_2} and central temperature were stable. Both the peroneal nerve-tibialis anterior muscle and laryngeal nerve-vocal cord preparations appeared to be stable for at least 6 h in pilot studies. No signs of fatigue were observed in either muscle during that period.

The maximum blockade, the onset time and the total duration time for suxamethonium, (+)-tubocurarine, atracurium, pancuronium and vecuronium are presented in Table 1. Figure 2 shows results obtained with (+)-tubocurarine and pancuronium.

Following the administration of suxamethonium, (+)-tubocurarine or atracurium, the maximum blockade of the intrinsic largyngeal muscle was significantly less when compared to the tibialis anterior muscle. In addition, both the onset time and the total duration were shorter for the vocal cord paralysis. Because in one cat the maximal degree of muscle paralysis in the vocal cord preparation amounted to 10% after administration of suxamethonium, the duration could not be calculated and, therefore, only two duration times are listed in Table 1.

No differences were found between the preparations for the maximum blockade following injection of pancuronium and vecuronium. Both the onset time and duration were shorter in the vocal cord preparation for both drugs.

Discussion

Semiquantitative measurements of the neuromuscular blocking effects of the depolarizing muscle relaxant suxamethonium in man (Gabriel, 1975; Baer, 1984) has shown that the laryngeal muscles recover earlier than peripheral arm muscles. In addition, Gilly *et al.* (1986) and Streinzer *et al.* (1986) have attempted to quantify the laryngeal muscle relaxation in patients by electromyographic (EMG) recording of evoked responses elicited by surface electrodes stimulating the laryngeal nerve. They showed that, following a bolus dose of

Table 1 Neuromuscular blocking effects on the tibialis anterior and intrinsic laryngeal muscles in the cat, following approximately equi-effective doses of five routinely used muscle relaxants

Drug	*Dose* ($\mu g\,kg^{-1}$)	*Max. block* (mean) (%)				*Onset* (mean) (min)				*Duration* (mean) (min)			
Suxamethonium	30 T	66	66	74	(69)	1.3	1.5	1.9	(1.6)	3.5	3.7	4.2	(3.8)
	V	10	17	22	(16)	0.4	0.9	1.0	(0.8)	1.0	1.3		(1.2)
(+)-Tubocurarine	200 T	83	83	89	(85)	1.7	1.9	2.7	(2.1)	9.6	11.9	15.6	(12.4)
	V	23	23	25	(24)	1.5	1.7	2.4	(1.9)	5.2	5.5	7.6	(6.1)
Atracurium	75 T	74	80	91	(82)	2.4	2.7	3.8	(3.0)	8.9	8.9	9.0	(8.9)
	V	11	34	40	(29)	1.6	2.9	4.3	(2.9)	4.7	4.8	9.1	(6.2)
Vecuronium	20 T	69	74	91	(78)	3.7	4.0	4.7	(4.1)	9.7	10.4	13.7	(11.3)
	V	55	71	86	(71)	2.5	3.1	3.3	(3.0)	6.8	7.7	8.0	(7.5)
Pancuronium	15 T	43	65	85	(64)	6.8	4.8	4.1	(5.2)	15.4	12.9	26.9	(18.4)
	V	86	57	63	(69)	2.9	2.7	4.1	(3.2)	10.1	5.9	9.6	(8.5)

T = peroneal nerve-tibialis anterior muscle preparation. V = recurrent laryngeal nerve-vocal cord preparation.

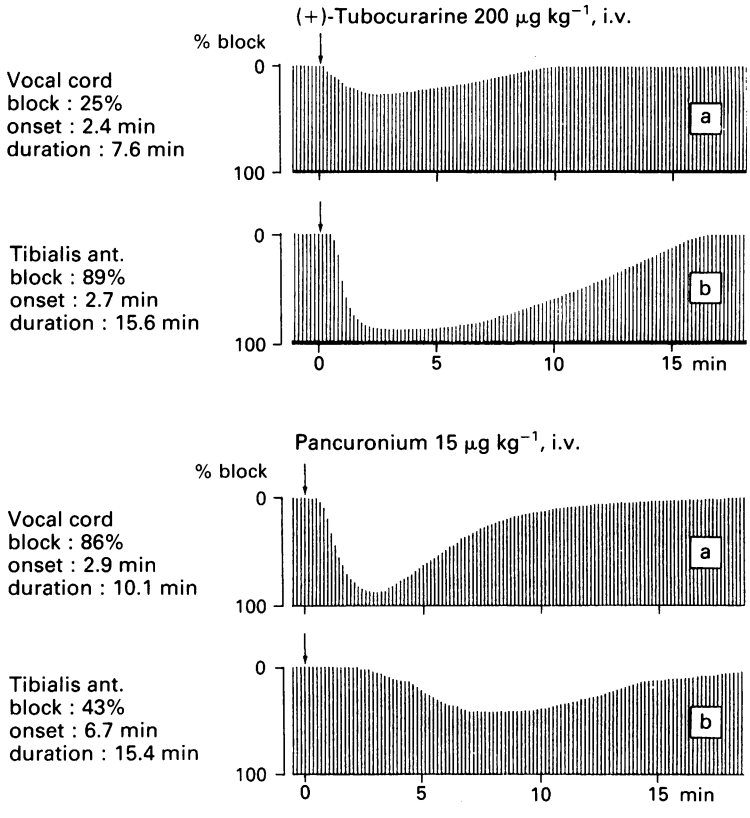

Figure 2 Experimental recordings of contractions of cat laryngeal nerve-vocal cord preparation (a) and peroneal nerve-tibialis anterior muscle preparation (b) in response to 0.1 Hz stimulation following intravenous administration of (+)-tubocurarine (200 $\mu g\,kg^{-1}$) or pancuronium (15 $\mu g\,kg^{-1}$).

0.06 $mg\,kg^{-1}$ vecuronium, the vocal cords gave varying degrees of blockade, ranging from 61–92%. In contrast, the skeletal adductor pollicis muscle was nearly completely (97–100%) blocked. These findings support clinical observations (Sia *et al.*, 1982).

Movement of the vocal cords involves a complex combination of action of the intrinsic laryngeal muscles, controlled by, in part, the superior but predominantly, by the inferior laryngeal nerves. The evoked movement of the homolateral vocal cord, after stimulation of the inferior or recurrent laryngeal nerve, is the resultant of the summation of contractions of the various activated muscles. Inhibition of vocal cord movement, necessary during intubation, can only be successful if all muscles involved are paralysed. Simple extrapolation of neuromuscular blocking effects of muscle relaxants at the adductor pollicis muscle to the vocal cords, as is done empirically in clinical practice, should be done with great caution. Contraction of the adductor pollicis muscle is simple and differs markedly from the abduction of the vocal cords. In addition, there are different microanatomical and histological structures and types of innervation (Rossi & Cortesina, 1965; Johnson *et al.*, 1973), which result in different neurophysiological manifestations (Buchthal & Rosenfalck, 1955; Buchthal, 1959) between muscles. Furthermore, differences in muscle temperature and blood supply, presumably responsible for different pharmacokinetic and pharmacodynamic effects at the site of action, i.e. the neuromuscular junction, must be taken into account.

Due to the small number of experiments in the various groups, the data of this study have not been statistically analysed. These should only be viewed as an illustration of the technical feasibility of the execution of these experiments.

In our experiments we have shown that, using single twitch measurements, intrinsic laryngeal muscles and the tibialis anterior muscle of the cat respond differentially to the neuromuscular blocking effects of muscle relaxants *in vivo*. Suxamethonium causes a more pronounced blockade of peripheral tibialis anterior muscle. Baer (1984) and Gabriel (1975) had comparable findings in man. In clinical practice, however, suxamethonium is primarily used for the rapid facilitation of intubation and should block pharyngeal and intrinsic laryngeal neuromuscular transmission totally. The isoquinoline derivatives, (+)-tubocurarine and atracurium, produce more pronounced blockade of peripheral skeletal muscle. The steroidal compounds, pancuronium and vecuronium, produced a more comparable degree of blockade in both types of muscle, more or less comparable with those found by Gilly *et al.* (1986) and Streinzer *et al.* (1986) for man. Both the onset time and duration were shorter at the vocal cords for both compounds.

Kharkevich & Fisenko (1981) investigated the sensitivity of acetylcholine receptors of six different skeletal muscles to muscle relaxants in cats, and showed that the sequence of relaxation of different muscles was variable with drugs of different chemical structures, but was similar after administration of analogous compounds of the same chemical group. The results of their studies are in agreement with those obtained in our *in vivo* preparations, although they did not investigate the laryngeal muscles. An explanation for this phenomenon has not yet been presented. Apparently, one can assume the existence of differences in types, organisation, or sensitivities of acetylcholine receptors in various groups of muscles. Changes in temperature among muscles or differences in local blood flow may contribute to these pharmacodynamic alterations, but they cannot be fully responsible.

This laryngeal nerve-vocal cord preparation, in combination with the tibialis anterior (skeletal) muscle, may become a useful tool for investigation of new compounds with neuromuscular blocking activities, which should have a high affinity for acetylcholine receptors in the intrinsic laryngeal muscles, in order to develop new neuromuscular blocking agents for rapid facilitation of optimal intubation conditions.

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(Received August 27, 1990
Revised October 18, 1990
Accepted December 17, 1990)

Br. J. Pharmacol. (1991), **102**, 865–870

Effect of the leukotriene receptor antagonists FPL 55712, LY 163443, and MK-571 on the elimination of cysteinyl leukotrienes in the rat

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1 Leukotriene elimination via bile and urine is an important mechanism of inactivation for these potent lipid mediators. We investigated whether the elimination of cysteinyl leukotrienes is a target for the action of leukotriene receptor antagonists.

2 Experiments were performed in male rats under deep thiopentone anaesthesia. The bile duct and the urinary bladder were cannulated. Tritium labelled leukotrienes and leukotriene receptor antagonists were given via central venous catheters. Elimination of leukotrienes produced *in vivo* was studied following stimulation of endogenous leukotriene biosynthesis by operative trauma. ^{3}H-leukotriene metabolites were identified by h.p.l.c. analysis. Leukotrienes produced *in vivo* were measured by combined use of h.p.l.c. and RIA.

3 Under control conditions, 49 ± 12% of the injected ^{3}H-leukotriene radioactivity was recovered in bile and 1 ± 0.8% in urine within 90 min. Operative trauma resulted in initial hepatobiliary secretion of 887 ± 206 pmol kg^{-1} h^{-1} of the endogenous leukotriene metabolite N-acetyl leukotriene E_4 (LTE_4NAc).

4 FPL 55712 strongly inhibited hepatobiliary elimination of ^{3}H-leukotriene radioactivity in a dose-dependent manner after i.v. injection of [^{3}H]-LTC_4, [^{3}H]-LTD_4 or [^{3}H]-LTE_4, respectively. Biliary [^{3}H]-LTD_4 was reduced most effectively. The leukotriene antagonist potently prevented biliary elimination of LTE_4NAc produced *in vivo*. Bile flow and elimination from blood into bile of [^{3}H]-ouabain were also impaired by FPL 55712, but to a lesser extent.

5 LY 163443 reduced biliary [^{3}H]-LTD_4 after i.v. administration of [^{3}H]-LTD_4. However, the total elimination of ^{3}H-leukotriene metabolites into bile was not significantly inhibited by the drug.

6 MK-571 reduced the biliary concentration of tracer after administration of ^{3}H-leukotrienes most potently with respect to [^{3}H]-LTD_4. In contrast, the total recovery of ^{3}H-leukotrienes in bile tended to increase. This is explained by a drug-induced increase in bile flow.

7 Urinary elimination of ^{3}H-leukotrienes, quantitatively less important in the rat, was not significantly influenced by the leukotriene receptor antagonists. Recovery of ^{3}H-leukotriene radioactivity in liver and kidneys was quantitatively insignificant.

8 From our data, we conclude that leukotriene receptor antagonists have the potential to affect leukotriene elimination by a mechanism not necessarily related to receptor blockade. Inhibition of elimination by the receptor antagonists may prolong the biological half life of leukotrienes. This effect may counteract the antagonistic properties of these drugs.

Keywords: Leukotrienes; leukotriene receptor antagonists; leukotriene inactivation

Introduction

Cysteinyl leukotrienes are potent putative mediators of allergy and inflammation (Parker, 1987; Piper, 1989; Brain & Williams, 1990). Receptor blockade is regarded as a promising approach to antagonize leukotriene action. A variety of potent leukotriene receptor antagonists have been designed (Musser *et al.*, 1986; Snyder & Fleisch, 1989). There is no doubt that many leukotriene effects are mediated via receptors (Crooke *et al.*, 1989; Halushka *et al.*, 1989), but it is not known to date whether leukotriene inactivation also proceeds via receptor-mediated events. The effectiveness of leukotriene inactivation may be expected to be a significant determinant in the biological activity of these mediators. An important mechanism of leukotriene inactivation in rodents and primates is rapid elimination of cysteinyl leukotrienes from blood into bile and urine (Hagmann *et al.*, 1984; Denzlinger *et al.*, 1986; Foster *et al.*, 1987; Maltby *et al.*, 1990).

In the present study, we investigated the effect of the leukotriene receptor antagonists FPL 55712 (Sheard *et al.*, 1977), LY 163443 (Fleisch *et al.*, 1986), and MK-571 (Jones *et al.*, 1988) on the elimination of ^{3}H-labelled cysteinyl leukotrienes into bile and urine of anaesthetized rats. In addition, the effect of FPL 55712 on the elimination of leukotrienes generated *in vivo* was studied.

A preliminary report on minor parts of this work has been presented earlier (Denzlinger *et al.*, 1990a).

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Methods

Experimental protocol

Experiments were performed in male Wistar rats (180–220 g) under deep thiopentone anaesthesia (initial dose, 75 mg kg^{-1}, i.p.). Both jugular veins were cannulated followed immediately by the infusion of physiological saline at 700 µl h^{-1}. The bile duct and urinary bladder were cannulated. Body temperature was kept above 35°C by infra-red radiation.

Bile and urine were collected continuously into ice cold aqueous methanolic (90 vol.%) solution containing HTMP, 1 mmol l^{-1}, and EDTA, 0.5 mmol l^{-1}, pH adjusted to 7.4. Sampling periods were 10 min for bile and 90 min for urine. Bile flow and urine production were determined by weight.

In the tracer experiments, leukotriene receptor antagonists and their vehicles were given into one jugular vein as a continuous infusion after a bolus injection starting 3 min before administration of ^{3}H-leukotrienes. ^{3}H-labelled leukotrienes

were adjusted to give a final specific activity of 12 TBq mol^{-1}, diluted in physiological saline and injected as a bolus via central venous catheter at a dose of 37 kBq per kg body weight, corresponding to 3 nmol per kg body weight. ^{3}H-leukotrienes were given twice: at 0 min together with the appropriate vehicle for the leukotriene receptor antagonists and at 90 min together with the drug to be tested, or again with the vehicle alone (control experiments). At the end of the experiments, 2 ml of blood were withdrawn by puncture of the aorta. The liver and kidneys were removed and immediately frozen in liquid nitrogen. Bile, urine and blood plasma in methanolic solution, as well as the frozen organs were stored at −40°C. Aliquots of the biological samples were counted for tritium radioactivity. In addition, bile was analysed by high performance liquid chromatography (h.p.l.c.) for ^{3}H-leukotriene metabolites.

In the experiments on elimination of leukotrienes generated in anaesthetized rats, the operative trauma necessary for sample collection (i.e. laparotomy and cannulation of the bile duct) was used as a stimulus for endogenous leukotriene generation. FPL 55712 (20 μmol kg^{-1} h^{-1}) or the vehicle were given as a continuous infusion starting 10 min before the laparatomy and continuing until 30 min after the operation.

High-performance liquid chromatography

Isocratic h.p.l.c. was performed as described earlier (Denzlinger *et al.*, 1986; 1990b) on a C18 Hypersil column (4.6 × 250 mm, 5 μm particles, Shandon, Runcorn, Cheshire) with a C18 precolumn (Waters, Milford, MA, U.S.A.). The mobile phase consisted of methanol, water, acetic acid (65:35:0.1 by volume), EDTA, 1 mmol l^{-1}, pH 5.6 adjusted with ammonium hydroxide. The flow rate was 1 ml min^{-1}. Bile was deproteinized for h.p.l.c. by storage in 80% aqueous methanol at −40°C for at least 12 h and centrifugation at 2500 *g* for 20 min at −10°C. Storage and centrifugation were repeated, if necessary. The supernatant was concentrated to dryness in a centrifuge under low pressure (Savant Speed Vac, Framingdale, NY, U.S.A.) and redissolved in 30% aqueous methanol. In the studies on tracer elimination, ^{3}H-leukotriene metabolites were identified by comigration with authentic leukotriene standards. In the studies on elimination of leukotrienes generated *in vivo*, an aliquot of h.p.l.c. fractions was counted for tritium radioactivity to determine retention times of ^{3}H-leukotriene standards added in trace amounts to the samples prior to h.p.l.c. Another aliquot was stored at −20°C under argon for further analysis by radioimmunoassay (RIA).

Radioimmunoassay

RIA analysis was performed in aliquots of h.p.l.c. fractions as described earlier (Denzlinger *et al.*, 1986; 1990b). The antiserum was diluted 1:5700. [^{3}H]-LTC_4 (130 Bq) was used as radioligand. The lower detection limit of the assay system was below 10 fmol for LTC_4, LTD_4, LTE_4 and LTE_4NAc; the relative percentage cross reactivities on a molar basis at 50% binding were 100, 68, 53 and 82, respectively. LTB_4 does not cross react in this system at amounts of up to 1 μmol.

Materials

Leukotriene receptor antagonists were kindly provided by Fisons, Loughborough, Leicestershire (FPL 5571), Eli Lilly, Indianapolis, IN, U.S.A. (LY 163443), and Merck Frosst, Quebec, Canada (MK-771). FPL 55712 (sodium 7-[3-{4-acetyl-3-hydroxy-2-propylphenoxy}-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylate) was dissolved in 5% glucose. LY 163443 (1-[2-hydroxy-3-propyl-4-{[4-(1H-tetrazol-5-ylmethyl) phenoxy]methyl}phenyl]ethanone) was dissoved in sodium carbonate/bicarbonate buffer, 50 mmol l^{-1}, pH 9.0 and diluted in physiological saline. MK-571 (former name L-660, 711, (3-[3-{2-(7-chloro-2-quinolinyl)ethenyl}phenyl]{(3 - [dimethylamino - 3 - oxo propyl] thio)methyl}thio)propanoic acid) was dissolved in physiological saline. In a guinea-pig ileum assay system 0.1 μmol l^{-1} of FPL 55712, LY 163443 or MK-571 reduced contraction induced by 8 nmol l^{-1} LTD_4 to 65, 57, or 12% of control values, respectively.

Unlabelled LTC_4, LTD_4 and LTE_4 were purchased from Paesel, Frankfurt, F.R.G. 14,15-[^{3}H]-LTC_4, 14,15-[^{3}H]-LTD_4 and [^{3}H]-ouabain were obtained from Amersham International, Buckinghamshire. 14,15-[^{3}H]-LTE_4 was bought from DuPont, Boston, MA, U.S.A. N-acetyl LTE_4 (LTE_4NAc) and [^{3}H]-LTE_4NAc were synthesized from LTE_4 and [^{3}H]- LTE_4 as described by Hagmann *et al.* (1986). The concentration of unlabelled leukotrienes was determined by absorbance measurements at 280 nm. 4-Hydroxy-2,2,6,6,-tetramethyl- piperidine-1-oxyl (HTMP) was bought from Sigma, St. Louis, MO, U.S.A.

Thiopentone (Trapanal) was purchased from Byk Gulden, Konstanz, F.R.G. The rabbit cysteinyl leukotriene antiserum was kindly donated by Professor B.A. Peskar, Ruhr-Universität, Bochum, F.R.G.

Statistics

The *t* test for paired observations was used to analyse significance of differences between ^{3}H-leukotriene elimination or bile flow respectively during vehicle and leukotriene antagonist administration. The *t* test for unpaired observations was used to calculate significance between elimination of leukotrienes generated *in vivo* in the treated and untreated animals. The null hypothesis was rejected in $P < 0.05$.

Results

Effects of leukotriene receptor antagonists on elimination of ^{3}H-leukotriene radioactivity

After i.v. injection of ^{3}H-labelled cysteinyl leukotrienes in anaesthetized rats, 49 ± 12% (mean ± s.d., $n = 31$) of the tritium radioactivity was recovered in bile within 90 min. Only 1.0 ± 0.8% (mean ± s.d., $n = 31$) of injected tritium radioactivity was found in urine, and radioactivity in blood was below the detection limit after 90 min.

Elimination characteristics of ^{3}H-leukotrienes were highly reproducible if the injection was repeated in the same rat (Figure 1, vehicle). In the pharmacological studies ^{3}H-leukotrienes were injected first (at 0 min) together with the vehicle for the drug to be tested, second (at 90 min) together with the drug to enable intra-individual comparison of ^{3}H-leukotriene secretion. Elimination of ^{3}H-leukotrienes during administration of leukotriene antagonist was related to elimination of ^{3}H-leukotrienes during administration of vehicle.

Using this experimental design we obtained the following results. FPL 55712 strongly reduced hepatobiliary elimination of ^{3}H-leukotrienes in a dose-dependent manner (Figure 1, Table 1). Infusion of 20 μmol kg^{-1} h^{-1} of FPL 55712 decreased elimination of [^{3}H]-LTC_4 to 23.1 ± 9.8% of control (mean ± s.d.). This corresponds to less than 13% of the injected radioactivity recovered in bile. Reduction of ^{3}H-leukotriene elimination by FPL 55712 was more pronounced with respect to the total amount of radioactivity secreted (Table 1A) than with respect to the tritium concentration in bile (Table 1C) because FPL 55712 also reduced bile flow (Table 1B).

Hepatobiliary elimination of [^{3}H]-ouabain was also inhibited by FPL 55712, but to a lesser extent. Using the same experimental design as for ^{3}H-leukotrienes, 20 μmol kg^{-1} h^{-1} of the leukotriene antagonist reduced elimination of [^{3}H]-ouabain to 60.8 ± 8.8% (mean ± s.d.) of control.

LY 163443, 25 μmol kg^{-1} h^{-1}, caused a slight decrease in total ^{3}H-leukotriene elimination into bile that was neither sta-

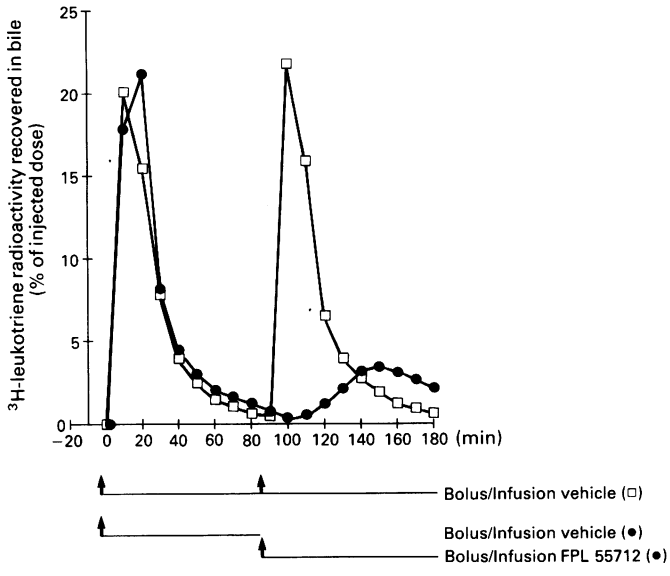

Figure 1 Effect of FPL 55712 on the recovery of ^{3}H-leukotriene radioactivity in bile. Representative results obtained in two rats are shown. One rat (□) was treated twice with the vehicle (5% glucose). In the other rat (●) FPL 55712 was administered during and after the second injection of [^{3}H]-LTC$_4$. The tracer was injected at 0 and at 90 min. Intravenous bolus injections of the vehicle or FPL 55712 (10 μmol kg^{-1}) were given 3 min before the tracer, as indicated by arrows. The bolus was followed by infusion of the vehicle or FPL 55712 (20 μmol kg^{-1} h^{-1}) as indicated by bars. Bile was sampled continuously. Percentage recoveries of the injected dose detected in the respective bile fractions are indicated.

tistically significant on the basis of the total ^{3}H-leukotriene recovery in bile (Table 1A) nor on the basis of total biliary ^{3}H-leukotriene concentrations (Table 1C). The drug also had no significant effect on bile flow (Table 1B).

MK-571, 15 μmol kg^{-1} h^{-1}, strongly reduced biliary concentrations of tritium radioactivity after i.v. injection of ^{3}H-leukotrienes (Table 1C). In contrast, the total amount of ^{3}H-leukotrienes recovered in bile tended to increase. The latter effect was statistically significant after application of ^{3}H-LTC$_4$ (Table 1A). The opposing actions of MK-571 on biliary ^{3}H-leukotriene concentrations and total elimination of tracer may in part be explained by a choleretic effect of MK-571 resulting in an increase in bile flow averaging 141% of control (Table 1B).

Cysteinyl leukotriene elimination into urine, quantitatively less important in the rat, was not influenced to a statistically significant extent by the leukotriene receptor antagonists.

Under control conditions, 90 min after injection of ^{3}H-leukotrienes, radioactivity in blood was below the detection limit and radioactivity in liver and kidneys amounted to 0.011 or 0.003% of injected dose, respectively. At that time, none of the leukotriene receptor antagonists significantly increased the ^{3}H-leukotriene concentration in blood or the ^{3}H-leukotriene content of the kidneys. There was only a small but significant increase in ^{3}H-leukotriene content in the liver (0.015%) following 20 μmol kg^{-1} h^{-1} of FPL 55712.

Effects of leukotriene receptor antagonists on the pattern of biliary ^{3}H-leukotriene metabolites

Bile samples obtained during the experiments described in the previous section were analysed by h.p.l.c. to evaluate the effects of the leukotriene receptor antagonists on the proportions of biliary ^{3}H-leukotriene metabolites i.e. [^{3}H]-LTC$_4$, [^{3}H]-LTD$_4$, [^{3}H]-LTE$_4$, [^{3}H]-LTE$_4$NAc and polar ^{3}H-leukotriene metabolites (i.e. metabolites more polar than [^{3}H]-LTC$_4$).

Under control conditions, the metabolite pattern was similar after i.v. [^{3}H]-LTC$_4$ or [^{3}H]-LTD$_4$ apart from a greater proportion of [^{3}H]-LTC$_4$ in the first bile fraction after i.v. [^{3}H]-LTC$_4$. Proportions in bile of [^{3}H]-LTC$_4$ and [^{3}H]-LTD$_4$ decreased in a time-dependent manner as proportions

Table 1 Effects of FPL 55712, LY 163443, and MK-571 on (A) the recovery of ^{3}H-leukotriene radioactivity in bile, (B) bile flow, (C) the biliary concentration of ^{3}H-leukotriene radioactivity, and (D) the contribution of [^{3}H]-LTD$_4$ to biliary ^{3}H-leukotriene radioactivity

		FPL 55712		*LY 163443*	*MK-571*	
Bolus (μmol kg^{-1})			10	2.5	75	10
Infusion [μmol kg^{-1} h^{-1}]		20	5	25	15	
(A) ^{3}H-leukotriene-recovery in bile						
(% of control,	[^{3}H]-LTC$_4$	23.1 ± 9.8^c	ND	ND	110.3 ± 0.2^c	
mean ± s.d.)	[^{3}H]-LTD$_4$	35.3 ± 14.9^a	66.9 ± 1.7^c	95.1 ± 7.1	102.4 ± 3.0	
	[^{3}H]-LTE$_4$	40.7 ± 16.8^a	ND	91.5 ± 18.4	ND	
(B) Bile flow						
(% of control, mean ± s.d.)		75.8 ± 15.8^c	81.8 ± 10.5	97.1 ± 19.5	140.9 ± 9.9^c	
(C) ^{3}H-leukotriene-concentration in bile						
(% of control,	[^{3}H]-LTC$_4$	35.1 ± 16.7^b	ND	ND	52.0 ± 6.7^b	
mean ± s.d.)	[^{3}H]-LTD$_4$	57.2 ± 14.2^a	78.2 ± 10.6	84.2 ± 9.9	47.3 ± 13.9^a	
	[^{3}H]-LTE$_4$	56.0 ± 10.9^a	ND	105.1 ± 35.3	ND	
(D) Contribution of [^{3}H]-LTD$_4$ to biliary ^{3}H-leukotrienes (% of control, mean ± s.d.]		27.3 ± 14.6^a	ND	55.7 ± 12.2^a	51.7 ± 15.2^a	

Experiments were performed as outlined in Figure 1. Leukotriene receptor antagonists were given as continuous infusion following bolus injection.

(A) The total amount of ^{3}H-leukotriene radioactivity recovered in bile within 90 min after the second injection of [^{3}H]-LTC$_4$, D$_4$ or E$_4$ during infusion with leukotriene antagonist was compared to the total amount of ^{3}H-leukotriene radioactivity recovered within 90 min after the first injection of tracer during infusion of the vehicle (control, 100%).

(B) Bile flow during infusion with leukotriene antagonist was compared to bile flow during the preceding control period with vehicle given alone (control, 100%). Under control conditions bile flow was 4.2 ± 0.4 μm kg^{-1} h^{-1} (mean ± s.d.).

(C) The biliary concentration of ^{3}H-leukotriene radioactivity after the second injection of ^{3}H-leukotriene during infusion with leukotriene antagonist was compared to the biliary concentration of ^{3}H-leukotriene radioactivity after the first injection of tracer during infusion of vehicle (control, 100%).

(D) The contribution of [^{3}H]-LTD$_4$ to the total of biliary ^{3}H-leukotriene metabolites obtained 0–10 min after the second injection of [^{3}H]-LTD$_4$ during infusion of leukotriene antagonist was compared to the proportions within the 0–10 min interval after the first injection of tracer during infusion of vehicle (control, 100%).

Mean values ± s.d. from at least 3 animals are indicated. $^{a, b, c}$ indicate a statistically significant difference from control by $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively, as determined by the t test for paired events. ND: not determined.

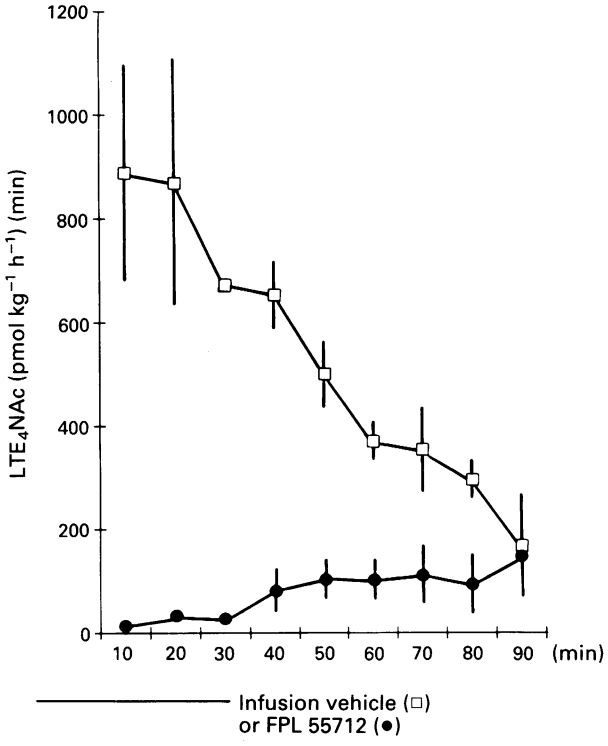

Figure 2 Effect of FPL 55712 on the elimination of cysteinyl leukotrienes generated *in vivo*. Endogenous leukotriene generation was elicited by the operative trauma necessary for sample collection (laparatomy and cannulation of the bile duct) at 0 min. Intravenous infusion of FPL 55712 (20 μmol kg^{-1} h^{-1}, ●) or the vehicle (5% glucose, □) was started 10 min before laparotomy and continued until 30 min after the operation. Mean values ± s.d. of biliary elimination of LTE_4NAc from 3 rats are shown. The difference between the treated and untreated animals was statistically significant from 0 to 80 min (i.e. until 40 min after end of FPL 55712 infusion) as determined by *t* test for unpaired events.

of [^{3}H]-LTE_4NAc and of the polar ^{3}H-leukotriene metabolites increased. Only [^{3}H]-LTE_4NAc, polar ^{3}H-leukotriene metabolites, and small amounts of [^{3}H]-LTE_4 were detected after i.v. [^{3}H]-LTE_4. Contribution of [^{3}H]-LTE_4 to biliary ^{3}H-leukotrienes never exceeded 5%. Cumulative secretion of [^{3}H]-LTE_4NAc was predominant under all conditions tested.

Leukotriene receptor antagonists had a significant effect on the biliary ^{3}H-leukotriene metabolite pattern only with respect to biliary [^{3}H]-LTD_4. [^{3}H]-LTD_4 was the predominant biliary ^{3}H-leukotriene metabolite only within the first 10 min bile fraction after i.v. [^{3}H]-LTC_4 or [^{3}H]-LTD_4. After i.v. [^{3}H]-LTD_4 proportions in this fraction were 6.1 ± 1.6 [^{3}H]-LTC_4: 44.7 ± 11.8 [^{3}H]-LTD_4: 1.1 ± 2.3 [^{3}H]-LTE_4: 24.4 ± 7.5 [^{3}H]-LTE_4NAc: 23.8 ± 7.6 polar ^{3}H- leukotriene metabolites (%, mean ± s.d., $n = 9$). In this initial phase of ^{3}H-leukotriene elimination, the relative contribution of [^{3}H]-LTD_4 to the total biliary ^{3}H-leukotriene metabolites was strongly reduced by each of the leukotriene antagonists tested (Table 1D). During infusion with the leukotriene antagonists, the proportions of [^{3}H]-LTC_4 and of the polar ^{3}H-leukotriene metabolites also tended to decrease and the proportion of [^{3}H]-LTE_4NAc tended to increase. However, these changes were not statistically significant.

Effects of FPL 55712 on elimination of leukotrienes produced in vivo

Stimulation of endogenous leukotriene generation in anaesthetized rats by the operative trauma necessary for sample collection (laparotomy and cannulation of the bile duct) resulted in the initial hepatobiliary secretion of 887 ± 206 pmol kg^{-1} h^{-1} (mean ± s.d.) of LTE_4NAc as detected by h.p.l.c. and RIA. The secretion rate gradually decreased within 90 min following the operative trauma (Figure 2). FPL 55712 (20 μmol kg^{-1} h^{-1}) reduced the initial secretion of LTE_4NAc to 13 ± 6 pmol kg^{-1} h^{-1} (mean ± s.d.). Secretion of LTE_4NAc slowly increased after discontinuation of FPL 55712 infusion (Figure 2). The difference between the treated and the untreated animals was statistically significant until 40 min after the antagonist infusion had been terminated. No endogenous leukotriene metabolites other than LTE_4NAc were detected in bile by h.p.l.c. and RIA analysis.

Discussion

Our results demonstrate that leukotriene receptor antagonists may affect the biological inactivation of cysteinyl leukotrienes by inhibition of their hepatobiliary elimination. Elimination characteristics of ^{3}H-leukotrienes (Figure 1), ^{3}H-leukotriene metabolites, and of leukotrienes produced *in vivo* following operative tissue trauma (Figure 2) were in agreement with results from previous investigations (Hagmann *et al.*, 1984; Denzlinger *et al.*, 1985; Hagmann *et al.*, 1986; Foster *et al.*, 1987). The efficiency of hepatobiliary clearance of leukotrienes from circulating blood allows their determination in bile as a measure for leukotrienes generated *in vivo*. This has been demonstrated in the rat following administration of lipopolysaccharide (Hagmann *et al.*, 1984), tumor necrosis factor (Huber *et al.*, 1988), platelet-activating factor (Huber & Keppler, 1987), frog virus (Hagmann *et al.*, 1987), after pulmonary anaphylaxis (Foster *et al.*, 1988) and after different kinds of tissue trauma (Denzlinger *et al.*, 1985). Urinary leukotrienes are much less indicative of endogenous leukotriene production in the rat as only a very small portion of leukotrienes is cleared into urine in this species.

In the tracer studies, leukotriene receptor antagonists were given as a continuous infusion following a bolus injection to achieve a rapid and constant saturation of binding sites. In the studies on elimination of leukotrienes generated *in vivo* a 10 min period of leukotriene antagonist infusion preceded the operative trauma used as a stimulus for endogenous leukotriene production. At the doses applied, effective leukotriene antagonistic actions of the leukotriene receptor antagonists may be expected to occur *in vivo*. FPL 55712, 20 μmol kg^{-1} repeatedly i.p. has been used to antagonize leukotriene actions in a mouse model of endotoxin shock (Hagmann *et al.*, 1984). LY 163443, 77 μmol kg^{-1} p.o. (Fleisch *et al.*, 1986) and MK-571, 1.9 μmol kg^{-1} p.o. (Jones *et al.*, 1988) have been shown to improve significantly pulmonary parameters impaired by LTD_4 *in vivo* in guinea-pigs and monkeys, respectively.

FPL 55712 was a strong inhibitor of hepatobiliary elimination of ^{3}H-leukotrienes (Figure 1, Table 1). This effect was partially due to reduction in bile flow induced by FPL 55712 (Table 1B) and also reflected in impaired secretion of [^{3}H]-ouabain. Inhibition of ^{3}H-leukotriene elimination, however, markedly exceeded inhibition of bile flow. The effect of FPL 55712 was most pronounced with respect to reduction of biliary [^{3}H]-LTD_4. The small portion of ^{3}H-leukotriene radioactivity detected after 90 min in liver and kidneys does not significantly account for the radioactivity not recovered in bile and urine. The small increase in ^{3}H-leukotriene radioactivity in the liver after administration of FPL 55712 also does not account for the reduced biliary recovery of tracer. Most likely, leukotrienes not eliminated via bile are subjected to oxidative degradation (Örning, 1987). This process may be expected to play a major role under conditions where hepatobiliary elimination is impaired.

Our finding that FPL 55712 dramatically reduces the hepatobiliary elimination of leukotrienes produced *in vivo* (Figure 2) expands our findings from the tracer studies to a pathophysiological situation *in vivo*.

In contrast to FPL 55712, LY 163443 was only a weak inhibitor of total ^{3}H-leukotriene elimination in our rat

model. Only secretion of [^{3}H]-LTD_4 was significantly reduced by the drug (Table 1).

MK-571 inhibited biliary ^{3}H-leukotriene secretion with respect to biliary ^{3}H-leukotriene concentrations. [^{3}H]-LTD_4 in bile was most strongly reduced. The drug did not inhibit but rather enhanced the total biliary ^{3}H-leukotriene elimination (Table 1). This was apparently due to a strong choleretic effect of MK-571 compensating for reduced biliary ^{3}H-leukotriene concentrations induced by the drug.

Elimination of cysteinyl leukotrienes via urine was not significantly influenced by the leukotriene receptor antagonists. This question should be re-evaluated in primates where renal clearance of leukotrienes is quantitatively more important than in the rat (Denzlinger *et al.*, 1986; Maltby *et al.*, 1990).

From our data, we cannot define the mechanism of action of leukotriene receptor antagonists responsible for the effect on hepatobiliary leukotriene elimination. Knowledge concerning leukotriene elimination is very limited. The rapid time course of biliary clearance of leukotrienes from blood suggests that receptor-mediated events might be involved. If receptors were involved, one might speculate whether they were similar or different from leukotriene receptors mediating leukotriene effects. The pharmacological activity of FPL 55712, LY 163443 and MK-571 has been described to be selectively directed against LTD_4/LTE_4 receptors (Fleisch *et al.*, 1986; Jones *et al.*, 1988; Snyder & Fleisch, 1989). Rapid conversion in circulating blood of LTC_4 to LTD_4 and LTE_4 (Huber & Keppler, 1987) may explain why the principal elimination characteristics of i.v. injected [^{3}H]-LTC_4, [^{3}H]-LTD_4, and [^{3}H]-LTE_4 were similar under control conditions. Only the contribution of [^{3}H]-LTC_4 and [^{3}H]-LTD_4 to the ^{3}H-leukotriene metabolite pattern in the early bile fractions depended on the administered ^{3}H-leukotriene metabolite. The similarity in elimination characteristics of ^{3}H-leukotriene metabolites appeared to be largely preserved in the presence of the leukotriene receptor antagonists except for [^{3}H]-LTD_4, the concentration of which in bile was preferentially reduced by the drugs (Table 1D). Preferential or even exclusive reduction of biliary [^{3}H]-LTD_4 by FPL 55712, MK-571, or LY 163443 respectively, could be in line with a specific receptor component in leukotriene elimination impaired by LTD_4 antagonism. The relative potency of the leukotriene receptor antagonists used in inhibiting leukotriene actions in organ preparations *in vitro* (e.g. guinea-pig ileum assay, MK-571 > LY 163443 > FPL 55712) is, however, not related to their relative impact on ^{3}H-leukotriene elimination (Table 1). This argues in favour of the interpretation that receptors mediating leukotriene actions and, if existent, receptors mediating leukotriene elimination may be different.

Recently, almost complete biliary secretion and pronounced enterohepatic circulation of a LTD_4/E_4 antagonist has been demonstrated (Christensen *et al.*, 1990). This raises the possibility that leukotrienes and leukotriene receptor antagonists might compete for a common transport system.

In conclusion, our data show that leukotriene inactivation by hepatobiliary elimination is a potential target for leukotriene receptor antagonists. Inhibition of leukotriene elimination by receptor antagonists may prolong the biological half life of leukotrienes. This effect may counteract the leukotriene antagonistic properties of these drugs *in vivo*. Our data do not predict that the demonstrated effects of the leukotriene receptor antagonists are of relevance for the therapeutic situation *in vivo*. Doses required for therapeutic use may be lower than those used in the present study. It is possible that in primates alternative mechanisms of leukotriene inactivation (renal elimination and oxidative degradation) are sufficiently active to compensate for impaired hepatobiliary elimination. Even if enhanced local or systemic leukotriene concentrations should occur, they might be too small to cause any significant effect, especially in the presence of a leukotriene receptor antagonist. It is suggested, however, that leukotriene antagonists should be screened for their effect on leukotriene inactivation. Leukotriene receptor antagonists with minor effects on leukotriene elimination (LY 163443) or inducing effects compensatory to their inhibitory action of leukotriene elimination (MK-571) seem more suitable to antagonize leukotriene effects *in vivo* than receptor antagonists with a strong inhibitory effect on leukotriene inactivation (FPL 55712 in the rat model used). On the other hand, elucidation of the way in which leukotriene receptor antagonists affect elimination of leukotrienes from blood into bile will help define the mechanisms underlying hepatobiliary secretions of these eicosanoids. This may in turn be of relevance for the future development of leukotriene antagonistic drugs.

We are indebted to Prof. B.A. Peskar, Bochum, F.R.G., for providing the leukotriene antibody. We wish to thank Prof. R. Schulz, München, under whose guidance the guinea-pig ileum assays with the leukotriene receptor antagonists were performed. We are grateful for the leukotriene receptor antagonists generously provided by Fisons, Eli Lilly and Merck Frosst. This work was supported by the Deutsche Forschungsgemeinschaft (De 397/1-2).

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(Received September 17, 1990
Revised December 3, 1990
Accepted December 17, 1990)

Br. J. Pharmacol. (1991), **102**, 871–878

A patch-clamp study of K^+-channel activity in bovine isolated tracheal smooth muscle cells

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1 Single smooth muscle cells were isolated from bovine trachealis by enzymic digestion. The properties of large conductance plasmalemmal K^+-channels in these cells were studied by the patch-clamp recording technique.

2 Recordings were made from inside-out plasmalemmal patches when $[K^+]$ was symmetrically high (140 mM) and when $[Ca^{2+}]$ on the cytosolic side of the patch was varied from nominally zero to 10 μM. Large unitary currents of both Ca^{2+}-dependent and -independent types were observed. Measured between +20 and +40 mV, the slope conductances of the channels carrying these currents were 249 ± 18 pS and 268 ± 14 pS respectively.

3 Lowering $[K^+]$ on the cytosolic side of the patches from 140 to 6 mM, shifted the reversal potentials of the two types of unitary current from approximately zero to $\gg$ +40 mV, suggesting that both currents were carried by K^+-channels.

4 The Ca^{2+}-dependent and -independent K^+-channels detected in inside-out plasmalemmal patches could also be distinguished on the basis of their sensitivity to inhibitors (tetraethylammonium (TEA), 1–10 mM; Cs^+, 10 mM; Ba^{2+}, 1–10 mM; quinidine, 100 μM) applied to the cytosolic surface of the patches.

5 Recordings were made from outside-out plasmalemmal patches when $[K^+]$ was symmetrically high (140 mM) and when $[Ca^{2+}]$ on the cytosolic side of the patch was varied from nominally zero to 1 μM. Ca^{2+}-dependent unitary currents were observed and the slope conductance of the channel carrying these currents was 229 ± 5 pS.

6 Activity of the Ca^{2+}-dependent K^+-channel detected in outside-out patches could be inhibited by application of TEA (1 mM), Cs^+ (10 mM), Ba^{2+} (10 mM) or quinidine (100 μM) to the external surface of the patch. 4-Aminopyridine (4-AP; 1 mM) was ineffective as an inhibitor.

7 The activity of the Ca^{2+}-dependent K^+-channel recorded from outside-out patches was reversibly inhibited by charybdotoxin (100 nM).

8 When whole-cell recording was performed, the application of a depolarizing voltage ramp evoked outward current which was dependent on the $[Ca^{2+}]$ in the recording pipette and which could be reversibly inhibited by charybdotoxin (50 nM–1 μM) applied to the external surface of the cell.

9 We conclude that bovine trachealis cells are richly endowed with charybdotoxin-sensitive, large conductance, Ca^{2+}-dependent K^+-channels. These channels carry most of the outward current evoked by a depolarizing ramp and could play a major role in determining the outward rectifying properties of the trachealis cells. The role of the large Ca^{2+}-independent K^+-channels remains unclear.

Keywords: Bovine trachealis; patch-clamp recording; Ca^{2+}-dependent and -independent K^+-channels; whole-cell currents; 4-aminopyridine; Cs^+; charybdotoxin; Ba^{2+}; quinidine; tetraethylammonium

Introduction

The plasmalemma of airways smooth muscle cells exhibits such strong outward rectification that regenerative action potentials do not arise spontaneously and cannot be evoked by the transmembrane passage of cathodal current (Small & Foster, 1988; Small *et al.*, 1990). Patch-clamp studies have shown that the plasmalemma of canine and porcine trachealis is richly endowed with Ca^{2+}-dependent K^+-channels of high (266–290 pS) specific conductance (McCann & Welsh, 1986; Huang *et al.*, 1987). The plasmalemma of trachealis muscle from the guinea-pig (Hisada *et al.*, 1990; Small *et al.*, 1990) and rabbit (Kume *et al.*, 1990) also contains such channels.

The K^+-channel inhibitor tetraethylammonium (TEA) has been shown to reduce rectification in airways smooth muscle and therefore to allow spike-like action potentials to be discharged in response to cathodal current pulses (Kroeger & Stephens, 1975; Suzuki *et al.*, 1976; Kannan *et al.*, 1983). The susceptibility of the large Ca^{2+}-dependent K^+-channels to blockade by TEA, their high specific conductance and their relatively high frequency of occurrence in plasmalemmal patches were among factors that led McCann & Welsh (1986) to propose that such channels were responsible for the pronounced outward rectification exhibited by trachealis muscle.

However, there are two reasons why it may be premature, at this stage, to ascribe the outward rectifying behaviour of trachealis muscle solely to the activity of these K^+-channels. Firstly, TEA is non-selective as an inhibitor among the different types of K^+-channel (Cook, 1988). Secondly, the outward current evoked by depolarizing voltage steps in trachealis muscle seems to have several components and these can possibly be attributed to the presence of different types of K^+-channel. For example, in canine and rabbit trachealis cells arranged for whole-cell recording, depolarization to potentials positive to zero evokes a large transient outward current (I_T) followed by a more sustained outward current (I_S) of lower amplitude (Hisada *et al.*, 1990; Muraki *et al.*, 1990). In canine cells, I_T, but not I_S, was abolished by substituting Cd^{2+} for Ca^{2+} in the extracellular medium. In rabbit cells, I_T was inhibited by nifedipine while I_S was resistant to this agent (Hisada *et al.*, 1990). Kotlikoff (1989) has reported that, in canine tracheal myocytes, currents similar to I_T and I_S were both resistant to charybdotoxin, an agent reported specifically to block large Ca^{2+}-dependent K^+-channels (Smith *et al.*,

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1986). These observations collectively suggest that several different types of K^+-channel exist in the plasmalemma of trachealis muscle and that channels other than the large Ca^{2+}-dependent K^+-channel could contribute to its outward rectifying behaviour.

The experiments of the present study were designed to characterize the K^+-channels found in the plasmalemma of bovine trachealis cells and to estimate the contribution of large conductance Ca^{2+}-dependent K^+-channels to the carriage of outward current evoked by depolarizing stimuli.

Methods

Cell separation

Bovine tracheae were transported from the Manchester abattoir in cold Krebs solution. Strips of trachealis muscle were dissected from the organ and incubated for 15 min in a Ca^{2+}-free physiological salt solution (Ca^{2+}-free PSS; composition (mM): NaCl 126, KCl 6, Na_2HPO_4 1.2, $MgCl_2$ 1.2, glucose 11, HEPES 10, adjusted to pH 7.4 with NaOH). The tissue was then finely chopped and subjected to two periods of incubation (30 min at 35°C) in 2 ml Ca^{2+}-free PSS to which had been added 4 mg bovine serum albumin, 1400 μg collagenase (Worthington type I) and 60 μg elastase (Sigma type IIa). After its removal from the enzymic digestion medium, the tissue was resuspended in 2 ml Ca^{2+}-free PSS. The tissue was then repeatedly aspirated (sucked in and out of a blunt Pasteur pipette) to promote cell shedding. The supernatant fluid was drawn off and examined for its content of isolated smooth muscle cells. The tissue was resuspended in a fresh aliquot of Ca^{2+}-free PSS and aspiration was repeated. Working in this way, several samples of supernatant fluid were collected. Samples containing the greatest cell numbers were pooled and the cells were spun down. The cells were resuspended in Ca^{2+}-free PSS and plated onto cover slips coated with poly-L-lysine (molecular wt. 500,000). The cells were kept for up to 8 h at 4°C before use.

Recording media

For recording from inside-out plasmalemmal patches the recording pipette contained 140 mM KCl solution. Cells from which patches were to be prepared were stored in the main body of the recording chamber. This contained the PSS described above but with the addition of 1.2 mM $CaCl_2$. Patches prepared from the stock of cells were taken to a side arm of the recording chamber which was independently perfused with a K^+-rich PSS of composition (mM): KCl 126, NaCl 6, $MgCl_2$ 1.2, EGTA 2, $CaCl_2$ 1.7, glucose 11 and HEPES 10. The pH of this solution was adjusted to 7.4 with KOH solution. Normally the free $[Ca^{2+}]$ in this solution was 1 μM but, when required, this was varied in the range zero to 10 μM, by adjusting the amount of $CaCl_2$ included in the formulation. When inhibitors were added to this solution the concentration of KCl was correspondingly reduced to maintain osmolarity constant.

For recording from outside-out patches the recording pipette contained the K^+-rich PSS described as superfusing the inside-out patches with similar adjustment of free $[Ca^{2+}]$ when required. The extracellular surface of the patches was superfused with the same solution and free $[Ca^{2+}]$ was 1 μM.

For whole-cell recording the solution inside the recording pipette was similar to that used for outside-out patches. The extracellular surface of the cells was superfused with the same solution but free $[Ca^{2+}]$ was 1.2 mM.

Patch-clamp recording

Recordings were made at 22°C using an Axopatch 1B amplifier (Axon Instruments Inc.). Data was stored on magnetic tape and analysed using a microcomputer and pCLAMP Software (Axon Instruments Inc.). Unitary current amplitudes were measured using a Gould DSO 400 digital storage oscilloscope.

The probability of an individual ion channel being open (P_{open}) was calculated as

$$\sum_{j=1}^{N} t_j j/TN$$

where t_j is the time spent with $j = 1,2,\ldots, N$ channels open, N is the maximum number of channels seen and T is the duration of the experiment (Standen *et al.*, 1989). The maximum number of channels present in each patch was estimated from the maximum number of channels simultaneously open at any potential from −60 to +60 mV. In the case of outside-out patches where the pipette solution was nominally Ca^{2+}-free, the activity of individual channels was very low. Accordingly the maximum number of channels present was estimated during additional depolarization to +80 mV.

Whole-cell currents were activated by voltage ramps from −100 to +50 mV over 4 s. These currents were activated and recorded on line with pCLAMP software. Passive ohmic currents across the membrane were subtracted on line by the Axopatch amplifier.

Drugs and solutions

Drug concentrations are expressed in terms of the molar concentration of the active species. The following drugs were used: 4-aminopyridine (Sigma), barium chloride (May & Baker), caesium chloride (Sigma), purified charybdotoxin (Latoxan), quinidine hydrochloride (Sigma), tetraethylammonium bromide (Sigma).

The Krebs solution used for tissue transport to the laboratory had the composition (mM): Na^+ 143.5, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 127.6, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.1.

Results

Recording from inside-out plasmalemmal patches

Patches set up for recording under conditions where K^+ concentrations on either side of the patch were symmetrically high (140 mM) exhibited large, outwardly-directed unitary currents when held at potentials in the range +20 to +60 mV. In the majority of patches these large, unitary currents were all of equal amplitude. However, in some patches, large, unitary currents of two slightly different amplitudes could be observed (Figure 1a). Some patches exhibited much smaller unitary currents but these smaller events were not further investigated in the present study.

The Ca^{2+}-dependency of the ion channels carrying the large unitary currents was examined by altering free $[Ca^{2+}]$ on the cytosolic side of the patches. In 44 out of 70 patches examined, the relationship between P_{open} of the ion channel and membrane potential was strongly Ca^{2+}-dependent (Figure 2a). In these patches containing Ca^{2+}-dependent ion channels, P_{open} assumed a high value (>0.3) and was independent of membrane potential when cytosolic free $[Ca^{2+}]$ was in the range 3–10 μM. When cytosolic free $[Ca^{2+}]$ was 1 μM, P_{open} was close to zero at negative membrane potentials. However, P_{open} increased progressively as membrane potential assumed values more positive than zero. This relationship between P_{open} and membrane potential was shifted to more positive potentials and was depressed when cytosolic free $[Ca^{2+}]$ was reduced below 1 μM (Figure 2a).

In 26 out of the 70 patches examined, the relationship between P_{open} of the ion channel and membrane potential was virtually independent of cytosolic free $[Ca^{2+}]$. In patches containing Ca^{2+}-independent ion channels, P_{open} assumed a very low value at a holding potential of −60 mV but increased progressively as the membrane potential was moved towards

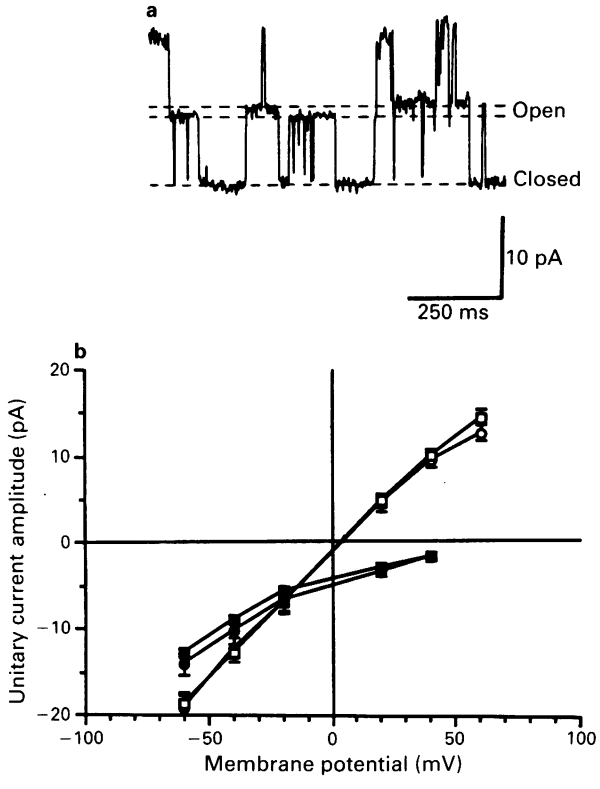

Figure 1 K^+-channel activity recorded from inside-out plasmalemmal patches from bovine trachealis cells. Except where stated otherwise, $[K^+]$ on each side of the patch was 140 mM and $[Ca^{2+}]$ on the cytosolic side of the patch was 1 μM. (a) Recording from patch held at +40 mV. Note that unitary currents of two distinct (albeit large) amplitudes can be discerned. The lowest broken line indicates the position where no channels are open. The upper two broken lines indicate currents corresponding to the opening of a single channel of each type. (b) Unitary current amplitude/voltage relationship for large conductance K^+-channels: (○) Ca^{2+}-dependent channel: symmetrical $[K^+]$; (●) Ca^{2+}-dependent channel: cytosolic $[K^+]$ 6 mM; (□) Ca^{2+}-independent channel: symmetrical $[K^+]$; (■) Ca^{2+}-independent channel: cytosolic $[K^+]$ 6 mM. Points indicate mean of values from at least 11 (symmetrical $[K^+]$) or 3 (6 mM cytosolic $[K^+]$) patches; vertical bars show s.e.mean.

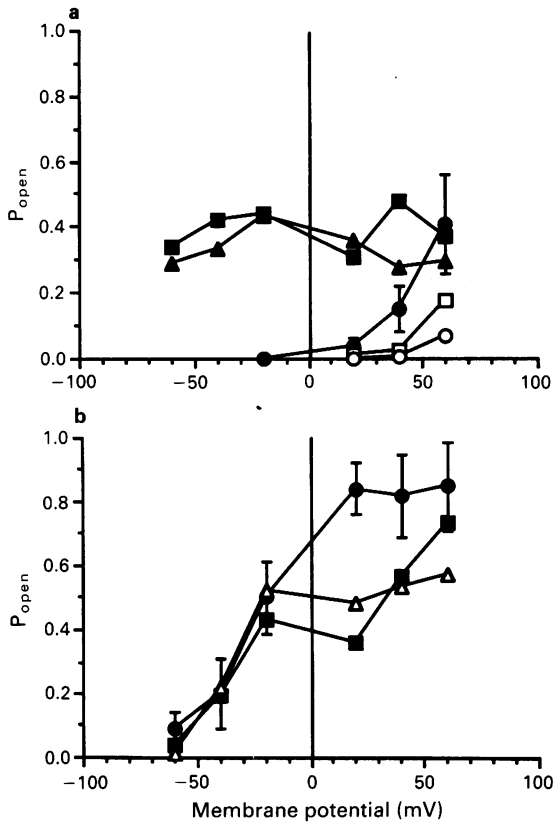

Figure 2 K^+-channel activity recorded from inside-out plasmalemmal patches from bovine isolated trachealis cells: the influence on P_{open} of $[Ca^{2+}]$ on the cytosolic side of the patch. (a) Large, Ca^{2+}-dependent K^+-channels; (b) large, Ca^{2+}-independent K^+-channels. The $[Ca^{2+}]$ on the cytosolic side of the patch was zero (△), 0.01 μM (○), 0.1 μM (□), 1 μM (●), 3 μM (▲) or 10 μM (■). Data for 1 μM Ca^{2+} are means (with s.e.mean) of values from at least 7 patches. Other data are from a single representative patch in each case.

0 mV. At positive holding potentials the channel showed a consistently high P_{open} (frequently in excess of 0.8). This relationship between P_{open} and membrane potential was little altered by increasing cytosolic free $[Ca^{2+}]$ from 1 μM to 10 μM or by reducing cytosolic free $[Ca^{2+}]$ to zero (Figure 2b).

The effects of holding potential on unitary current amplitude were examined both in patches containing Ca^{2+}-dependent channels and in patches containing $[Ca^{2+}]$-independent channels. Cytosolic free $[Ca^{2+}]$ was 1 μM in each case and K^+ concentrations on either side of the patches were symmetrically high (140 mM). The current/voltage relationships for the two types of channel are shown in Figure 1b and were used to calculate the slope conductances of each channel. At negative membrane potentials the slope conductances of the two channels were indistinguishable (306 ± 13 pS). At positive membrane potentials some inward rectification of the unitary currents was manifest as a reduction in the slope of the current/voltage curve. This rectification was more marked in the case of the Ca^{2+}-dependent channels. Hence, when slope conductance was measured over the range +20 to +40 mV, the conductance of the Ca^{2+}-dependent channel (249 ± 18 pS) was slightly lower than that of the Ca^{2+}-independent channel (268 ± 14 pS).

When K^+ concentrations on either side of the patch were symmetrically high (140 mM) the reversal potential of both types of unitary current was approximately zero. However, when $[K^+]$ on the cytosolic side of the patches was reduced to 6 mM the reversal potential of both types of unitary current was shifted to a value in excess of +40 mV (Figure 1b). Extrapolation of the unitary current amplitude/voltage curves in this circumstance indicated a reversal potential close to that expected for K^+ i.e. +80 mV. This suggested that the large, Ca^{2+}-dependent and -independent unitary currents observed in the inside-out plasmalemmal patches were carried by K^+-channels.

The inhibitor sensitivity of the large Ca^{2+}-dependent and large Ca^{2+}-independent K^+-channels recorded in inside-out patches was assessed in experiments where the inhibitors were added to the PSS bathing the cytosolic surface of the patches. TEA (3 mM) had no significant effect on the activity of the large Ca^{2+}-dependent K^+-channel. However, when the concentration of TEA was raised to 10 mM it caused some reduction in unitary current amplitude (Figure 3). The mean reduction in unitary current amplitude measured in 6 patches was 16%.

The Ca^{2+}-independent K^+-channel was much more sensitive to blockade by TEA. At a concentration of 100 μM, TEA caused flickering block of the Ca^{2+}-independent K^+-channel. TEA (1 mM) reduced unitary current amplitude to less than 25% of control values (Figure 4). Block of these channels by TEA (10 mM) was virtually complete (Figure 3).

Cs^+ (10 mM) had no effect on the large Ca^{2+}-dependent K^+-channel, affecting neither the amplitude of unitary currents (Figures 3 and 4) nor P_{open} (5 patches). In contrast Cs^+ (10 mM) caused voltage-dependent blockade of the Ca^{2+}-independent K^+-channel. Cs^+ (10 mM) did not affect the inward unitary currents seen at negative membrane potentials but reduced outward currents observed at positive membrane potentials. At a holding potential of +60 mV, Cs^+ (10 mM)

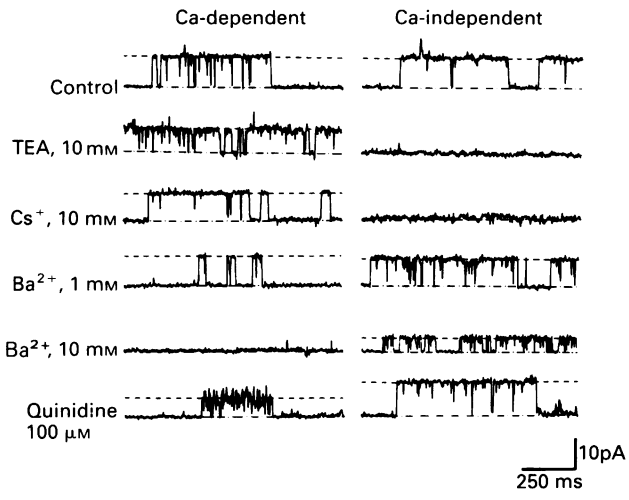

Figure 3 The effects of some inhibitors on the activity of large Ca^{2+}-dependent (left hand panel) and Ca^{2+}-independent (right hand panel) K^+-channels recorded from inside-out plasmalemmal patches from bovine isolated trachealis cells. In each case $[K^+]$ on each side of the patch was 140 mM, $[Ca^{2+}]$ on the cytosolic side of the patch was 1 μM and the holding potential was +40 mV. In each recording the lower broken line indicates the position where no channels are open and the upper broken line indicates the unitary current corresponding to the opening of a single Ca^{2+}-dependent or -independent K^+-channel. All inhibitors were applied to the cytosolic surface of the patches. Note that, compared with the Ca^{2+}-independent channel, the Ca^{2+}-dependent channel is relatively resistant to blockade by tetraethylammonium (TEA, 10 mM) or Cs^+ (10 mM). In contrast, the Ca^{2+}-dependent channel is relatively more susceptible to inhibition by Ba^{2+} (1–10 mM). Quinidine (100 μM) causes flickering block of the Ca^{2+}-dependent channel but has relatively little effect against the Ca^{2+}-independent channel.

reduced the outward unitary currents to 14% (mean value from 5 patches) of control values (Figure 4b). In some patches held at +40 mV, Cs^+ (10 mM) caused total blockade of unitary current activity (Figure 3).

In patches containing large, Ca^{2+}-dependent K^+-channels, Ba^{2+} (1 mM) did not affect unitary current amplitude (Figures 3 and 4). However, this agent decreased P_{open} at positive potentials and increased P_{open} at negative potentials, thereby inverting the relationship between P_{open} and membrane potential for the large Ca^{2+}-dependent K^+-channel (4 patches; Figure 5). Ba^{2+} (10 mM) fully inhibited the opening of the large Ca^{2+}-dependent K^+-channels in patches held at +40 mV (Figure 3).

In the case of the large, Ca^{2+}-independent K^+-channel, Ba^{2+} (1 mM) caused some depression of the relationship between P_{open} and membrane potential (4 patches; Figure 5). This was the result of fast, flickering channel block at positive membrane potentials (Figure 3). At positive, but not at negative membrane potentials, Ba^{2+} (1–10 mM) reduced the amplitude of unitary currents carried by the large Ca^{2+}-independent K^+-channel (Figures 3 and 4).

Quinidine (100 μM) caused flickering block of the large Ca^{2+}-dependent K^+-channels in patches held at +40 mV but did not affect the activity of the large Ca^{2+}-independent K^+-channels (Figure 3). 4-Aminopyridine (1 mM) had little effect on either of the two large K^+-channels. This agent did not modify the unitary current/voltage relationship for either the large Ca^{2+}-dependent K^+-channel or the large Ca^{2+}-independent K^+-channel (Figure 4).

The inside-out patches each contained several large conductance K^+-channels. In most cases these channels were all of the Ca^{2+}-dependent or all of the Ca^{2+}-independent type. As mentioned above, very few of the patches contained both types of channel. This suggests that the two types of large conductance K^+-channel had a clustered distribution in the plasmalemma. We attempted to estimate the relative numbers of the two types of channel in the plasmalemma by counting

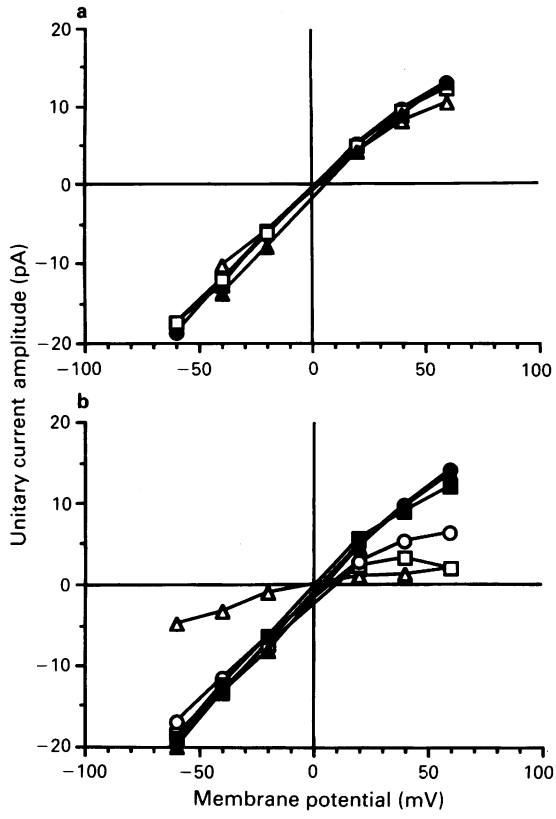

Figure 4 K^+-channel activity recorded from inside-out plasmalemmal patches from bovine isolated trachealis cells: the effects of some inhibitors on the unitary current amplitude/voltage relationship for the large Ca^{2+}-dependent K^+-channel (a) and the large Ca^{2+}-independent K^+-channel (b). All inhibitors were applied to the cytosolic surface of the patch. In each case $[K^+]$ on each side of the patch was 140 mM and $[Ca^{2+}]$ on the cytosolic side of the patch was 1 μM. Data points are means of values from at least 3 patches: (●) control (no inhibitor), (△) TEA, 10 mM in (a), 1 mM in (b); (□) 10 mM Cs^+; (■) 1 mM Ba^{2+}; (○) 10 mM Ba^{2+} and (▲) 1 mM 4-aminopyridine.

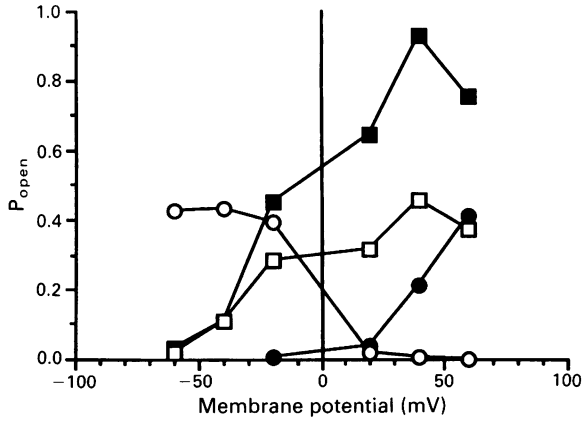

Figure 5 K^+-channel activity recorded from inside-out plasmalemmal patches from bovine isolated trachealis cells: the effect of Ba^{2+} (1 mM applied to the cytosolic surface) on P_{open} for the large Ca^{2+}-dependent and large Ca^{2+}-independent K^+-channels. In each case $[K^+]$ on each side of the patch was 140 mM and $[Ca^{2+}]$ on the cytosolic side of the patch was 1 μM. The data plotted are from two separate patches, one containing Ca^{2+}-dependent channels and one containing Ca^{2+}-independent channels. (●) Ca^{2+}-dependent channel; control; (○) Ca^{2+}-dependent channel; in presence of Ba^{2+} (1 mM); (■) Ca^{2+}-independent channel; control; (□) Ca^{2+}-independent channel; in presence of Ba^{2+} (1 mM). Note the ability of Ba^{2+} to invert the P_{open}/voltage relationship for the Ca^{2+}-dependent K^+-channel. Ba^{2+} depresses but does not invert the corresponding relationship for the Ca^{2+}-independent channel.

the numbers of channels present in the patches. In 21 patches containing Ca^{2+}-dependent channels, the mean (±s.e.mean) number of channels present per patch was 6.1 ± 3.3 while for 18 patches containing Ca^{2+}-independent channels the mean number of channels present per patch was 3.1 ± 1.0. Using these mean values to calculate the total numbers of Ca^{2+}-dependent and Ca^{2+}-independent channels present in the 70 patches examined yielded a ratio of 3.4:1 for the numbers of Ca^{2+}-dependent channels present in the plasmalemma relative to the number of Ca^{2+}-independent channels.

Recording from outside-out plasmalemmal patches

Patches set up for recording under conditions where K^+ concentrations on either side of the patch were symmetrically high (140 mM) exhibited large, outwardly-directed unitary currents when held at potentials in the range +20 to +60 mV.

The Ca^{2+}-dependency of the ion channels carrying the unitary currents was examined by altering the free [Ca^{2+}] inside the recording pipette. When the pipette solution contained 1 μM free Ca^{2+}, P_{open} of the channels carrying the currents was close to zero at a holding potential of −40 mV. However, P_{open} increased to a relatively high value (0.4) as membrane potential was moved through zero to +60 mV (Figure 6a). This relationship between P_{open} and membrane potential was depressed and moved to more positive potentials when the free [Ca^{2+}] inside the pipette was reduced to 0.1 μM or to zero (Figure 6a). The channels carrying the large unitary currents were therefore Ca^{2+}-dependent. Figure 6b shows the unitary current/voltage relationship obtained for the Ca^{2+}-dependent unitary currents of outside-out patches. The slope conductance of the channel carrying these currents was calculated from this relationship and, measured between +20 and +40 mV, had a value of 229 ± 5 pS.

We had anticipated that, when the recording pipette contained 2 mM EGTA but no Ca^{2+}, we would observe unitary currents with a voltage-dependence similar to that of the currents carried by the large Ca^{2+}-independent K^+-channels detected in the inside-out patches. However, currents of this kind were not observed under these conditions in any of 14 patches examined, nor were they seen in a further 6 patches with a solution at the extracellular surface identical to that which was used in the patch pipette for recording from inside-out patches (140 mM KCl). In a few patches large unitary currents of slightly different amplitude were seen but these did not show the same voltage-dependence as the Ca^{2+}-independent channels observed in inside-out patches. Little evidence for the existence of these channels was therefore obtained in the outside-out patches.

The effects of various inhibitors applied to the external surface of outside-out patches were tested against the unitary currents carried by the large Ca^{2+}-dependent K^+-channels. TEA applied to the external surface was a more potent inhibitor of such channels than TEA applied to the cytosolic surface. TEA (1 mM) applied to the external surface of outside-out patches markedly reduced unitary current amplitude at both positive and negative holding potentials (Figure 6b). Cs^+ (10 mM) applied to the external surface of the outside-out patches produced voltage-dependent channel block, reducing unitary current amplitude at negative but not positive potentials. Ba^{2+} (10 mM) had an effect similar to that of Cs^+. In contrast, 4-aminopyridine (2 mM) had little or no effect against unitary current amplitude at any holding potential (Figure 6b).

Charybdotoxin (100 nM) reduced the number of openings of the large Ca^{2+}-dependent K^+-channel without affecting unitary current amplitude (Figure 7). This reduction in channel activity was observed at all potentials tested from 0 to +60 mV.

Whole-cell recording

Recording pipettes of low (3–5 MΩ) resistance were used when recording in whole-cell mode. Current ramps were applied to the cells to achieve depolarization from a holding potential of −100 mV to +50 mV over a 4 s period. In these experiments the solution inside the recording pipette contained 2 mM EGTA and the free [Ca^{2+}] was nominally zero, 0.1 μM or 1 μM. When the recording pipette contained nominally Ca^{2+}-

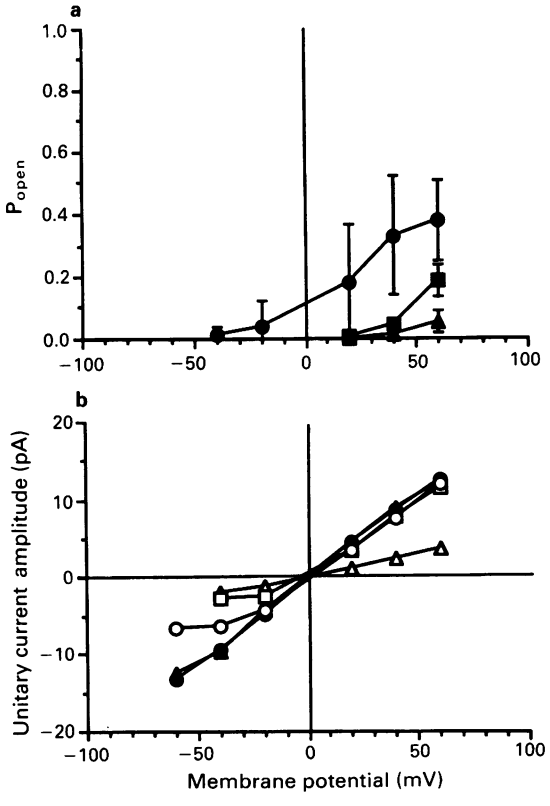

Figure 6 Ca^{2+}-dependent K^+-channel activity recorded from outside-out plasmalemmal patches from isolated bovine trachealis cells. (a) The relationship between P_{open} and [Ca^{2+}] on the cytosolic side of the patch. Data are means of values from 5 patches; s.e.mean shown by vertical bars. The free [Ca^{2+}] on the cytosolic side of the patch was nominally zero (▲), 0.1 μM (■) or 1 μM (●). The [K^+] on each side of the patch was 140 mM. (b) The unitary current amplitude/voltage relationship and its modulation by some inhibitors applied to the external surface of the patch. The free [Ca^{2+}] on the cytosolic and external sides of the patch was 1 μM. The [K^+] on each side of the patch was 14 mM. (●) Control (no inhibitor); (△) 1 mM tetraethylammonium, (□) 10 mM Cs^+, (○) 10 mM Ba^{2+} and (▲) 2 mM 4-aminopyridine. Data are means of values from 5 patches.

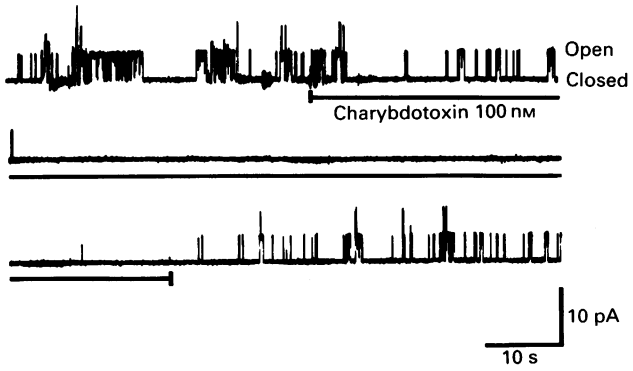

Figure 7 Continuous unitary current recording from an outside-out plasmalemmal patch from bovine trachaelis. The holding potential was 0 mV. The solution bathing the external surface of the patch contained 1.2 mM Ca^{2+} and 6 mM K^+ while that bathing the cytosolic surface contained 1 μM Ca^{2+} and 140 mM K^+. The bar underneath the trace indicates local perfusion of charybdotoxin, 100 nM, by positioning a separate pipette near the patch. Recovery occurred as the charybdotoxin diffused away after the removal of the application pipette.

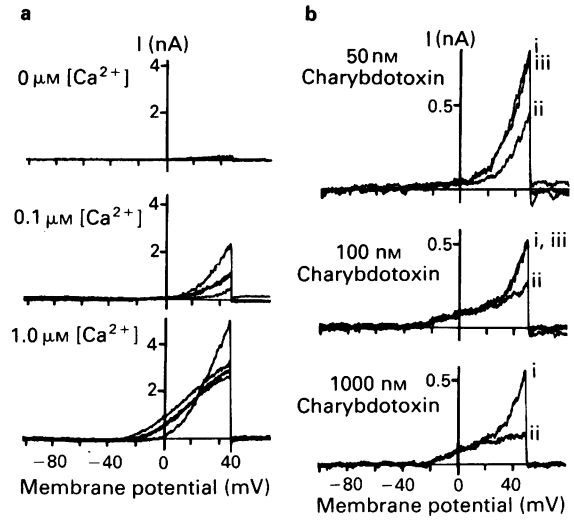

Figure 8 Whole-cell recording from bovine trachealis: outward currents evoked by a ramp depolarization from a holding potential of −100 mV to +50 mV over a period of 4 s. (a) Effects of varying $[Ca^{2+}]$ in the recording pipette solution. Note that the outward current increases in amplitude and shifts leftwards along the voltage axis as $[Ca^{2+}]$ is raised from nominally zero to 1 μM. Each of the plotted currents is from a different cell. (b) Effects of applying charybdotoxin (50 nM–1 μM) to the external surface of three different cells. Note the activation of the second, larger component of current at +10 to +20 mV and the suppression of this current by charybdotoxin. In each case current (i) is the initial control, (ii) was observed after exposure of the cell to charybdotoxin and (iii) was observed after toxin washout. Each superimposed trace is from the same cell. The cell exposed to 1 μM charybdotoxin was lost before full recovery from the toxin was achieved. Note the different vertical scales used in (a) and (b).

free solution, the application of a current ramp induced an outward current. This current was of low (<0.5 nA) amplitude and activated at a threshold of approximately 0 mV. When the free $[Ca^{2+}]$ inside the pipette was raised to 0.1 μM or 1 μM the effect was to increase the amplitude of the evoked outward current and to move the threshold for its activation to more negative potentials (Figure 8a and b). In some recordings it was evident that the outward current could be resolved into two components. The first component activated at approximately −40 mV and current amplitude increased slowly as the transpatch potential was moved to more positive values. The second component activated between −20 and +30 mV and current amplitude increased rapidly as the transpatch potential was moved to more positive potentials.

The effects of externally-applied charybdotoxin were examined in experiments where the free $[Ca^{2+}]$ inside the pipette was 0.1 μM. Charybdotoxin (50 nM–1 μM) selectively inhibited the second of the two components of outward current evoked by the conditioning ramp. The effects of charybdotoxin were concentration-dependent and reversible on washout (Figure 8b).

Discussion

Characteristics of the large, Ca^{2+}-dependent K^+-channel

In many ways the large Ca^{2+}-dependent K^+-channel which we have detected in bovine trachealis resembles that observed in trachealis muscle of other species and in smooth muscle from outside the respiratory tract. For example, the specific conductance of the large, Ca^{2+}-dependent K^+-channel observed in the present study (249 pS) was similar to that of equivalent channels in canine (266 pS; McCann & Welsh, 1986) and porcine (214 pS; Huang *et al.*, 1987) trachealis.

McCann & Welsh (1986) found that the opening of the large, Ca^{2+}-dependent K^+-channel in canine trachealis was dependent both upon $[Ca^{2+}]$ on the cytosolic side of the patch and on the membrane potential. The activity of the large Ca^{2+}-dependent K^+-channels seen in bovine trachealis was similarly sensitive to $[Ca^{2+}]$ and potential (present study). Furthermore, the influence of the cytosolic free $[Ca^{2+}]$ on the relationship between P_{open} and membrane potential (Figure 2) was strikingly similar to that observed for large Ca^{2+}-dependent K^+-channels in the plasmalemma of smooth muscle cells from rabbit jejunum and guinea-pig mesenteric arteries (Benham *et al.*, 1986).

The shape of the P_{open}/membrane potential curve for the large Ca^{2+}-dependent K^+-channels in bovine trachealis suggests that, provided cytosolic free $[Ca^{2+}]$ is 1 μM or less, very few of the large Ca^{2+}-dependent K^+-channels will be open at the normal resting membrane potential of the cell. However, should cytosolic free $[Ca^{2+}]$ rise above 1 μM, then the channels become quite active even at the resting membrane potential.

Fluorescence measurements have indicated that the cytosolic free $[Ca^{2+}]$ in resting airways smooth muscle cells is in the range 0.05–0.35 μM. During cellular excitation this rises to 0.3–1.0 μM (for review see Rodger & Small, 1991). At first sight the cytosolic free $[Ca^{2+}]$ achieved during excitation seems barely adequate to activate the large Ca^{2+}-dependent K^+-channels at potentials close to the resting membrane potential of the cell. However, Benham *et al.* (1986) have pointed out that during cellular excitation, Ca^{2+} entering the cell could achieve high, local concentrations in the area adjacent to its point of entry. If the large, Ca^{2+}-dependent K^+-channels are located close to the internal orifices of Ca^{2+}-channels then the opening of the K^+-channels could well be promoted by very local increases in cytosolic free $[Ca^{2+}]$.

McCann & Welsh (1986) reported that TEA (10–25 mM), applied to the cytosolic surface of plasmalemmal patches from canine trachealis, caused voltage-dependent blockade of large Ca^{2+}-dependent K^+-channels in that unitary current amplitude was reduced at strongly positive potentials. These authors showed, too, that TEA was approximately 10 times more potent when applied to the external surface of the patch. A similar potency difference for externally- and internally-applied TEA was observed in the present study. TEA (10 mM) applied to the cytosolic surface of our patches of bovine trachealis caused minor reduction in unitary current amplitude at both positive and negative potentials (Figure 4a). This indicates that TEA caused weak voltage-independent channel blockade as reported for the large Ca^{2+}-dependent K^+-channels of intestinal and arterial smooth muscle (Benham *et al.*, 1985). When 1 mM TEA was applied to the external surface of patches from bovine trachealis it very markedly reduced unitary current amplitude at both positive and negative potentials. Clearly we have shown that the large Ca^{2+}-dependent K^+-channel of bovine trachealis can be inhibited by TEA and the greater potency of TEA when applied to the external as opposed to the cytosolic surface of the patches is consistent with the suggestion (Benham *et al.*, 1985) that the site of action of TEA is close to the outer end of the channel.

The application of Ba^{2+} (1–10 mM) to the cytosolic surface had no effect on the amplitude of unitary currents carried by large Ca^{2+}-dependent K^+-channels in membrane patches from rabbit jejunum (Benham *et al.*, 1985) but, at negative potentials, caused minor reduction in such currents in patches from canine trachealis (McCann & Welsh, 1986). The results of the present study (Figure 4a) suggest that internally-applied Ba^{2+} has relatively little effect on the amplitude of unitary currents carried by the large Ca^{2+}-dependent K^+-channel in bovine trachealis. A major effect of internally-applied Ba^{2+} was to reduce the probability of channel opening at positive potentials (Figure 5). In this respect we have confirmed similar observations made in rabbit jejunum (Benham *et al.*, 1985). At negative potentials, however, internally-applied Ba^{2+} increased the probability of channel opening. In trachealis muscle of the dog and ox, therefore, the differing effects of Ba^{2+} on channel opening at positive and negative potentials

lead to inversion of the P_{open}/voltage relationship (McCann & Welsh, 1986; present study).

The greater ability of internally- as opposed to externally-applied Ba^{2+} to inhibit channel opening at positive potentials, and the ability of raised extracellular [K^+] to antagonize Ba^{2+}, were among factors which led Benham *et al.* (1985) to propose that Ba^{2+} enters the open channel and causes blockade at a site close to its outer orifice. However, the failure of Ba^{2+} to reduce unitary current amplitude (Figures 3 and 4) may suggest that Ba^{2+} does not act to cause open channel blockade but rather to cause the channel to enter an inactivated state. The ability of Ba^{2+} to increase P_{open} at negative potentials may reflect an ability of Ba^{2+}, under these conditions, to substitute for Ca^{2+} in promoting channel opening.

In the present study, Cs^+ appeared to be a more effective inhibitor of the large Ca^{2+}-dependent K^+-channel when applied to the external as opposed to the cytosolic surface of the patches. In this respect externally-applied Cs^+ (10 mM) markedly reduced the amplitude of unitary currents at negative potentials whereas the same concentration of Cs^+ applied internally did not affect unitary current amplitude (Figures 4a and 6b) at any holding potential. A similar voltage-dependency of the channel blocking action of externally-applied Cs^+ and a similar external: internal inhibitory potency ratio has been reported for the large Ca^{2+}-dependent K^+-channel in smooth muscle cells from rabbit jejunum and guinea-pig mesenteric artery (Benham *et al.*, 1986).

In summary, the large Ca^{2+}-dependent K^+-channel that we have detected in bovine trachealis has many properties (specific conductance, voltage-dependence, Ca^{2+}-dependence, inhibitor sensitivity) in common with the large Ca^{2+}-dependent K^+-channels that have been observed in smooth muscle (airways, vascular and intestinal) from other mammalian species. Berry *et al.* (1991) showed that P_{open} of the large Ca^{2+}-dependent K^+-channel of bovine trachealis was not modified by glibenclamide, phentolamine, RP 49356 or cromakalim. This suggests that opening of the large Ca^{2+}-dependent K^+-channel does not underlie the ability of cromakalim-like drugs to relax airways smooth muscle *in vitro*.

The large Ca^{2+}-independent K^+-channel

Since we recorded large, Ca^{2+}-independent K^+-channel activity in inside-out but not outside-out patches, we considered the possibility that our recordings of such activity were artefacts resulting from the formation at the electrode tip of a plasmalemmal vesicle rather than an inside-out patch. However, two findings argue against this possibility. Firstly, the recorded unitary currents did not exhibit the features of capacity coupling to be expected from the presence of a plasmalemmal vesicle. Secondly, channel activity was readily modified by the addition of inhibitors to the bathing medium, suggesting that drug access to the channels was not impaired by the presence of a membrane barrier.

The sensitivity of the large, Ca^{2+}-independent K^+-channels in inside-out patches to inhibitors applied to the cytosolic surface in many ways resembled the sensitivity of the large, Ca^{2+}-dependent K^+-channels in outside-out patches to the same inhibitors applied to the external surface. This led us to wonder whether our recordings of large Ca^{2+}-independent K^+-channels were taken from patches that had adopted an outside-out rather than an inside-out configuration. However, the voltage-dependency of the opening of the large, Ca^{2+}-independent K^+-channels was in the direction expected for inside-out patches. Furthermore, while quinidine (100 μM) applied to the external surface of outside-out patches inhibited the large Ca^{2+}-dependent K^+-channel, the same agent applied to the cytosolic surface of inside-out patches failed to inhibit the activity of the large Ca^{2+}-independent K^+-channels. We conclude, therefore, that our recordings of the large Ca^{2+}-independent K^+-channels were not artefacts created either as a result of vesicle formation or as a result of the patch adopting an outside-out rather than an inside-out configuration.

The large, Ca^{2+}-independent K^+-channel detected in inside-out patches of bovine trachealis (present study) resembles the slow, potential-sensitive K^+-channels described by Benham & Bolton (1983) in smooth muscle cells from rabbit jejunum. Neither channel was sensitive to [Ca^{2+}] on the cytosolic side of the patch. Both channels were inhibited by relatively low (100 μM–1 mM) concentrations of TEA applied to the cytosolic surface of the patch. The voltage-dependencies of the two channels were similar. Furthermore, the conductance of the Ca^{2+}-independent K^+-channels was similar to that of the slow potential-sensitive K^+-channels observed in rabbit jejunum under comparable ionic gradients (i.e. 62 pS and 50 pS respectively measured at 0 mV with high K^+ concentration on one side of the patch and low K^+ concentration on the opposite side of the patch).

Role of the large Ca^{2+}-dependent and large Ca^{2+}-independent K^+-channels in determining the outward rectifying behaviour of the trachealis cell

We have shown (i) that large Ca^{2+}-dependent K^+-channels are present in great abundance in plasmalemmal patches of bovine trachealis; (ii) that such channels can be inhibited by the external application of TEA (1 mM); (iii) that such channels can be inhibited by the external application of charybdotoxin (100 nM); and that (iv) the larger of the two components of outward current evoked by a depolarizing ramp can be inhibited by charybdotoxin. These four findings are consistent with the proposal of McCann & Welsh (1986) that the large Ca^{2+}-dependent K^+-channel may play an important role in determining the strong outward rectifying behaviour of trachealis cells. This proposal receives further support from the finding that, in guinea-pig trachealis cells, charybdotoxin (100 nM) causes the conversion of spontaneous electrical slow waves into spike-like action potentials (authors' unpublished observations).

The above considerations run contrary to the suggestion of Kotlikoff (1989) that charybdotoxin-insensitive K^+-channels play the more important role in determining the outward rectifying behaviour of the trachealis cell. In support of Kotlikoff's (1989) suggestion, it could be argued that the large Ca^{2+}-dependent K^+-channel is unlikely to be involved in determining outward rectification because (under conditions where cytosolic [Ca^{2+}] is 1 μM) the channel activates only at potentials more inside-positive than −25 mV. However, our measurements of the relationship between P_{open} and membrane potential were made at a temperature of 22°C. It is possible that, at 37°C, the P_{open}/membrane potential relationship may lie to the left of its position at 22°C. Alternatively, local cytosolic Ca^{2+} concentrations might achieve values greater than 1 μM and hence ensure the opening of the large Ca^{2+}-dependent K^+-channel at values of membrane potential much closer to the normal resting value. These possibilities await the test of experiment.

The relationship between P_{open} and membrane potential for the large, Ca^{2+}-independent K^+-channel suggests that channel opening would occur in response to a very small displacement of membrane potential from its resting value in the direction of depolarization. Accordingly, this channel could be expected to play an important role in determining the outward rectifying behaviour of the trachealis cell. Our attempts to assess the charybdotoxin sensitivity of this channel and hence its role in carrying the charybdotoxin-insensitive component of the outward current induced by a depolarized ramp were frustrated by our failure to record the channel activity in outside-out patches.

This work was supported by the National Asthma Campaign and the Wellcome Trust. We thank Dr R.J. Lang for advice with regard to the patch-clamp recording technique. We thank Mrs J. Robinson for expert technical assistance.

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(Received November 20, 1990
Revised December 17, 1990
Accepted December 20, 1990)

Br. J. Pharmacol. (1991), **102**, 879–882

Convulsive thresholds in mice during the recovery phase from anaesthesia induced by propofol, thiopentone, methohexitone and etomidate

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1 Convulsive thresholds were measured with intravenous pentylenetetrazol in mice during the recovery phase after intravenous anaesthetic doses of propofol (10 and 20 mg kg^{-1}), thiopentone (30 mg kg^{-1}), methohexitone (10 mg kg^{-1}), and etomidate 3 mg kg^{-1}).

2 The convulsive threshold rose after each agent, indicating an anticonvulsant action for all the drugs tested; this declined to control values with initial half times of: 1.56 min (propofol 10 mg kg^{-1}); 1.03 min (propofol 20 mg kg^{-1}): 1.02 min (methohexitone); 3.35 min (etomidate); 13.7 min (thiopentone).

3 At no time during the recovery phase of any agent did the convulsive threshold fall below control values, which might indicate an epileptogenic effect of the drug.

4 The threshold was depressed below control values by intravenous administration of Ro 15-4513, a partial inverse agonist at the benzodiazepine receptor, thus indicating the ability of this pentylenetetrazol test to demonstrate a proconvulsant effect.

5 We conclude that the abnormal movements or convulsions associated with recovery from anaesthesia with short-acting intravenous anaesthetics may not be the result of an intrinsic proconvulsant action of the drugs.

Keywords: Anticonvulsant tests; pentylenetetrazol; intravenous anaesthetics; propofol; thiopentone; methohexitone; etomidate; epilepsy; recovery from anaesthesia

Introduction

Following a series of adverse clinical reports in 1987, the Committee for the Safety of Medicines (1987) issued a warning concerning the possible risk of seizures after anaesthesia induced with propofol. In the same year Hodkinson *et al.* (1987) observed epileptic activity on the electroencephalogram in three patients with temporal lobe epilepsy after the administration of propofol. Since then a number of case findings (Cameron, 1987; Hopkins, 1988; Victory & Magee, 1988; Jones *et al.*, 1988) have reported on the occurrence of epileptic seizures after propofol-induced anaesthesia. In all these cases the seizures occurred during the recovery phase. In contrast, Wood *et al.* (1988) described a case of status epilepticus which was resistant to combined treatment with diazepam, phenytoin, phenobarbitone and chlormethiazole, but which responded rapidly to a single 100 mg bolus of propofol, the seizures subsequently being controlled by a propofol infusion.

In a previous study conducted by the present authors (Lowson *et al.*, 1990) propofol was shown to display marked anticonvulsant properties in mice, as judged by protection against seizures induced by electroshock and the convulsant, pentylenetetrazol (PTZ). Protection was significant even at doses which caused no sedation and was much greater at higher doses. For comparison, the effects of thiopentone were studied; it was found that the anticonvulsant effects of propofol were similar to or greater than those of thiopentone at doses which caused the same degrees of sedation or anaesthesia. However, in that study anticonvulsant activity was examined at the time of peak behavioural effect and effects during the recovery phase of anaesthesia were not investigated.

The present study was undertaken to examine convulsive threshold in mice during the recovery phase of anaesthesia induced with propofol, and to make comparisons with other intravenous induction agents, viz. thiopentone, methohexitone and etomidate. We chose the intravenous route for administration of anaesthetic agents to reproduce more closely the rapidly changing brain concentrations of these drugs which are encountered in clinical practice when these agents are used for induction of anaesthesia. Under such conditions we looked for a proconvulsive effect during the recovery phase.

Methods

This investigation was conducted on 275 male, Tuck No. 1 mice, weight 25–38 g. They were maintained in cages of five on a 12 h light/dark cycle with free access to water and a standard diet. The mice were divided into groups to receive one of the following intravenous anaesthetics: propofol (ICI Pharmaceuticals: 10 mg ml^{-1} in 10% soyabean oil emulsion); 20 mg kg^{-1}, $n = 55$; 10 mg kg^{-1}, $n = 35$; thiopentone (May & Baker: 25 mg ml^{-1}) 30 mg kg^{-1}, $n = 50$; methohexitone (Eli Lilley: 10 mg ml^{-1}); 10 mg kg^{-1}, $n = 40$; etomidate (Janssen: 10 mg ml^{-1}); 3 mg kg^{-1}, $n = 55$.

Five further mice were given an intravenous injection of Ro 15-4513; 1 mg kg^{-1} (Hoffman-La Roche; 0.1 mg ml^{-1} in a propylene glycol based vehicle, Gent *et al.*, 1988).

In addition 25 control mice did not receive any anaesthetic. These were tested in groups for convulsive thresholds at intervals throughout the whole study. Controls with administration of vehicle alone were not performed because we have previously shown that none of the vehicles used in this study influenced the minimum convulsant dose of PTZ (Gent *et al.*, 1988; Lowson *et al.*, 1990).

Assessment of recovery

After induction of anaesthesia by i.v. injection of anaesthetic into a lateral tail vein recovery was scored as follows: (1)

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response to tail pinch elicited by a pair of artery forceps; (2) recovery of the righting reflex; (3) recovery of the ability to balance along the edge of a plastic tray; (4) recovery of full coordination and normal exploratory activity.

The time taken to achieve each of the above responses was recorded in initial groups of five mice which were allowed to make a full recovery and in all other animals until the time of testing with PTZ. The time (mean and range) taken for the mice to achieve each stage of recovery was determined for each test agent.

Measurement of convulsive threshold

A slow infusion (0.3 ml min^{-1}) of a solution of PTZ (Sigma London; 15 mg ml^{-1} in 154 mM saline) was given into the other tail vein of the unrestrained mouse until a clear clonic convulsion was elicited. This was the end-point of the test, and mice were killed immediately after this convulsion had occurred. The minimum convulsant dose of PTZ (MCD of PTZ) was thus obtained for each mouse and the mean (± s.e.mean) calculated for each group of 5 mice. The earliest time at which testing was performed was the time of recovery of righting reflex. This gave an initial high value from which the rate of decline of MCD of PTZ with time could be determined. It was not considered necessary to test at earlier times as we had demonstrated in a previous study (Lowson *et al.*, 1990) that for propofol and thiopentone the 'anaesthetized' state was associated with highly significant protection against seizures. Further measurements of MCD of PTZ were made in groups of five mice each at various times after the recovery of the righting reflex: propofol (20 mg kg^{-1}) every 2 min from 4 to 22 min and at 26 min; propofol (10 mg kg^{-1}) every 2 min from 2 to 14 min; thiopentone at 4 min, 10 min and every 10 min thereafter to 80 min; methohexitone every 2 min from 2 to 14 min; etomidate every 2 min from 4 to 14 min and every 4 min thereafter to 30 min.

As we were interested in discovering if any of the anaesthetic agents caused a proconvulsant effect during the recovery phase we felt it was important to show that the method used was capable of revealing a proconvulsant action. The MCD of PTZ was therefore measured in a group of five mice 1 min after they had been given an intravenous bolus dose of Ro 15-4513, a partial inverse agonist at the benzodiazepine receptor (Lister & Nutt, 1988).

Data analysis

Statistical comparisons for recovery data were performed with the Mann-Whitney U-test to avoid any assumptions concerning the distribution of the data. A value of $P < 0.05$ was considered statistically significant. When comparisons of MCD of PTZ were made between experimental and control mice, Student's t test was used to compare each group of 5 experimental values with the most recently obtained group of control values. The computer programme CSTRIP (Sedman & Waynes, 1976) was used to analyse the changes in convulsive threshold with time. Although this programme is more usually used to analyse changes in drug levels, to derive pharmacokinetic parameters, we felt that its use was appropriate to obtain estimates of half-times for the decrease in MCD of PTZ after administration of the different anaesthetic agents.

Results

Behavioural assessment

The times taken for animals to display response to tail pinch, righting reflex, balancing ability and full recovery after each anaesthetic agent are shown in Table 1. The recovery times for the earlier phases were comparable for all groups. However, times to full recovery were significantly longer in etomidate- and thiopentone-treated animals when compared with propofol at both doses and methohexitone ($P < 0.05$; Mann-Whitney U Test).

Convulsive thresholds

There were no significant differences in MCD of PTZ between any of the control groups, which were tested at different times throughout the study. The overall mean MCD of PTZ in control mice was 40.7 ± 0.74 mg kg^{-1} (mean ± s.e.mean).

At no time during the course of these investigations did the group mean MCD of PTZ in animals given any of the anaesthetic agents fall significantly below that of the corresponding control group. However, the MCD of PTZ for the group given Ro 15-4513 was 24.8 ± 0.81 mg kg^{-1}, a value significantly ($P < 0.001$) below that of the control group.

The MCD of PTZ declined with time after all anaesthetic agents (Figure 1). With propofol (20 mg kg^{-1}), thiopentone and etomidate, the initial MCD of PTZ obtained at 4 min post induction was the highest value recorded in the study for each agent; thereafter the MCD of PTZ fell towards control values. Values at 4 min were significantly higher after propofol (20 mg kg^{-1}) than those after thiopentone which in turn were significantly higher than the 4 min values recorded following etomidate ($P < 0.05$). The time taken for the group mean MCD of PTZ to decline to a value not significantly different from controls was 18 min for propofol (20 mg kg^{-1}) and 26 min for etomidate. With thiopentone, values had not fallen to control levels at 80 min post induction, indicating a prolonged anticonvulsant effect during and after the recovery phase of thiopentone induced anaesthesia. With propofol 10 mg kg^{-1} and methohexitone the highest MCD of PTZ values recorded in the study were at 2 min, at which time animals had recovered their righting reflex. The 2 min values after propofol (10 mg kg^{-1}) were significantly greater than those after methohexitone. The MCD of PTZ had returned to control levels by 10 min after propofol (10 mg kg^{-1}) and 12 min after methohexitone.

Computer analysis of the values for MCD of PTZ to obtain polyexponential parameter estimates showed that, for each set of data, the best fit was with two exponentials; the correlation coefficient was greater than 0.98 in all cases. For the initial, rapid phase of the decreasing anticonvulsant effect, the half times were as follows: for propofol (10 mg kg^{-1}), 1.56 min; for

Table 1 The times taken to reach four stages of recovery after intravenous anaesthesia

	Recovery time: mean (range) min			
	Tail pinch	*Righting reflex*	*Balancing ability*	*Coordination and exploratory activity*
Propofol 20 mg kg^{-1}	2.60	4.09	7.90	13.90
	(2–4)	(3–5)	(6–10)	(12–16)
Propofol 10 mg kg^{-1}	1.03	2.00	3.50	6.80
	(1–2)	(1–3)	(3–4)	(6–8)
Methohexitone	1.26	2.62	4.83	9.33
	(1–2)	(2–3)	(4–6)	(8–10)
Thiopentone	2.12	3.84	11.76	22.84
	(1–3)	(2–5)	(8–16)	(18–25)
Etomidate	1.84	3.78	9.56	19.78
	(1–3)	(2–5)	(8–12)	(18–22)

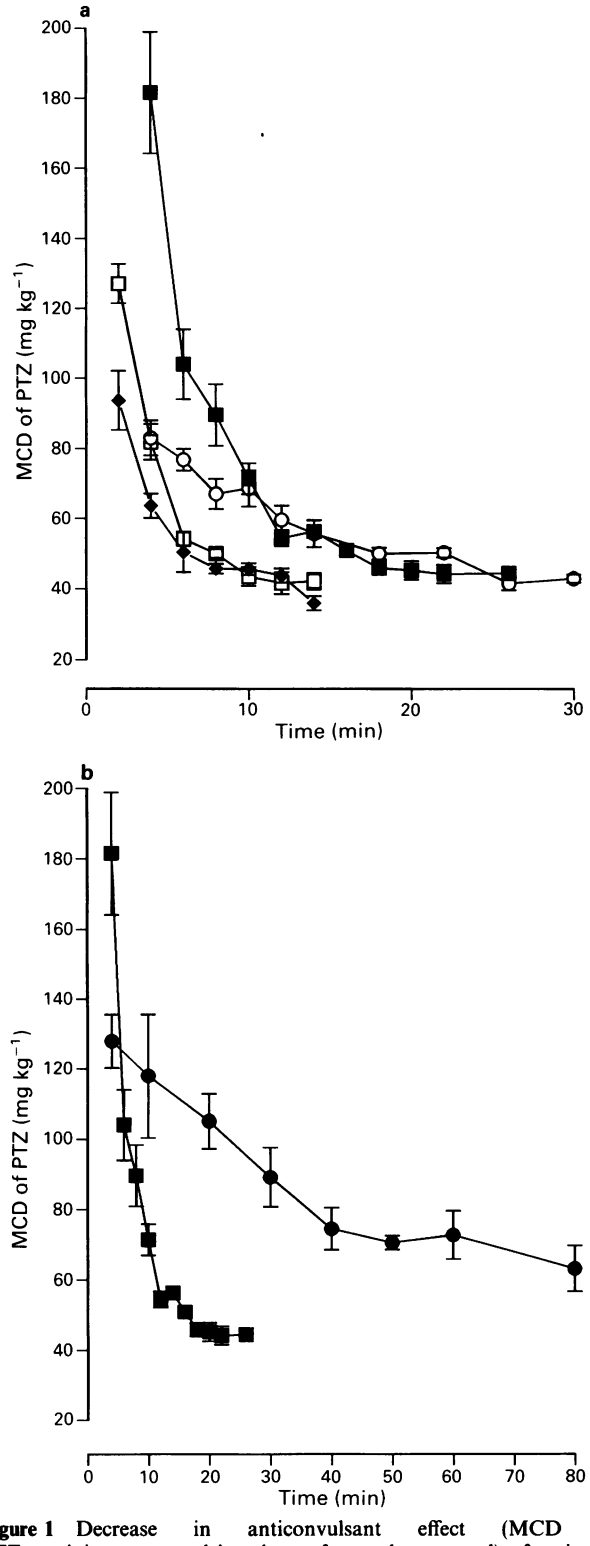

Figure 1 Decrease in anticonvulsant effect (MCD of PTZ = minimum convulsive dose of pentylenetetrazol) after intravenous injection of anaesthetic agents. Points represent means (n = 5), bars show standard error of the mean. (a) (■) propofol (20 mg kg^{-1}); (□) propofol (10 mg kg^{-1}); (○) etomidate (3 mg kg^{-1}); (◆) methohexitone (10 mg kg^{-1}); (b) (■) propofol (20 mg kg^{-1}); (●) thiopentone (30 mg kg^{-1}).

propofol (20 mg kg^{-1}), 1.03 min; for methohexitone, 1.02 min; for etomidate, 3.35 min; and for thiopentone 13.67 min. Thus the initial decline was fast after propofol and methohexitone, intermediate after etomidate and relatively slow after thiopentone. A similar pattern was seen in the half times for the second, slower phase; that for propofol (20 mg kg^{-1}) and methohexitone being similar (19 min), with etomidate longer at 40 min but thiopentone much longer at 170 min.

Involuntary movements

We observed involuntary movements in some mice in all groups. Following administration of etomidate all mice exhibited myoclonus, of varying severity during the early recovery phase (3–8 min). Differentiation between myoclonus and clonic seizures induced with PTZ at times proved difficult and may partly account for the low MCD of PTZ recorded at 4 min for etomidate. The myoclonus was made substantially worse when the animals were touched. Opisthotonus was a frequent occurrence following methohexitone; it was observed early in the recovery phase (1–2 min).

After both propofol and thiopentone a fine tremor of the mouse's head and limbs was occasionally observed in the early recovery phase. More frequently we observed rapid, repetitive movements of the limbs (more often of the hind limbs) at around the time of recovery of the righting reflex. The latter movements were more common following propofol anaesthesia. They were exacerbated if mice were laid supine, reduced if mice were laid prone (the normal position) and largely stopped by the application of a strong sensory stimulus, e.g., tail pinch. Such movements superficially resembled myoclonus. The convulsion following PTZ was easily distinguished from these and the fact that sensory stimulation stopped the abnormal movements rather than triggering or exacerbating them (as was the case after etomidate) led us to believe that these movements represented semi-purposeful actions made by animals as they were awakening from anaesthesia and regaining limited coordination.

Discussion

In these experiments we sought to study convulsive threshold during the recovery phase following anaesthesia induced with different intravenous anaesthetic agents. We were interested to discover if a proconvulsant effect could be demonstrated at this time and in the light of reports of epileptic seizures occurring during the recovery phase of anaesthesia induced with propofol, we were particularly interested in this agent. Propofol was therefore tested at two doses, a dose of 20 mg kg^{-1} as used by Glen *et al.* (1985) and a lower dose of 10 mg kg^{-1} which produced a more rapid recovery. However, at neither dose could we detect a proconvulsant effect during the recovery phase. We did not continue our studies beyond 26 min for propofol 20 mg kg^{-1} and 14 min for propofol 10 mg kg^{-1}. In both groups the group mean MCD of PTZ had been shown to be not significantly different from control values at three preceding test times and in both cases these times were well beyond the time for full recovery, the longest recovery times for propofol being 16 min (20 mg kg^{-1}) and 8 min (10 mg kg^{-1}). After 8 min of recovery the MCD of PTZ was significantly higher with thiopentone than with propofol. This is in contrast to our previous findings when the two drugs were given intraperitoneally; at equivalent levels of sedation in the previous study the MCD of PTZ was much higher in propofol-treated animals (Lowson *et al.*, 1990). This difference may be due to differences in the route of administration of the anaesthetics and the fact that the convulsant testing in the former study was performed at the time of peak behavioural effect for different doses. This study has provided no evidence that either propofol, thiopentone, methohexitone or etomidate produce preconvulsant activity during the recovery phases from anaesthesia induced with single bolus doses of these agents. However, the method used in the present investigation was well able to detect proconvulsant activity, as was demonstrated by the significant reduction in convulsive threshold induced by the partial inverse agonist at the benzodiazepine receptor, Ro 15-4513. One must be mindful of the fact, however, that it is possible for a drug to be proconvulsant yet not interact with PTZ.

These studies were conducted on a pure bred strain of

laboratory animals. The advantage of using such animals is that responses produced by a pharmacological agent will be consistent and will be minimally affected by individual pharmacokinetic or pharmacodynamic differences. However, a disadvantage arising from this consistency of response is that a pure bred strain of animals does not accurately mimic the likely range of responses obtained from a heterogeneous population such as human patients. Individuals differ in their convulsive thresholds and therefore in their susceptibility to seizures, whatever their cause. It is of interest that in three out of the four case reports describing epileptic seizures in patients recovering from a propofol-induced anaesthetic there was a personal or close family history of epilepsy.

We are therefore driven to speculate on the mechanism which might be responsible for these involuntary, often epileptiform movements which we have observed in mice during the recovery phase of short-acting anaesthetics. One possibility, as suggested above, is that they are semi-purposeful, uncoordinated grooming/righting reflexes. Another possibility is that the depressant effect of an anaesthetic may be removed from different parts of the central nervous system at different rates, perhaps as a result of perfusion differences. This could conceivably lead to an imbalance between excitatory and inhibitory influences on a potential epileptic focus, or areas involved in the control of movement. Such an effect might be most clearly seen in those drugs which are removed most rapidly; our data for behavioural recovery and loss of anticonvulsant activity (as measured by MCD of PTZ) show that this is the case for propofol (at both doses) and methohexitone. The shorter half time for the effect of the higher dose of propofol compared with the lower dose of the same agent seems incongruous; this is probably artifactual and due to the initial very high level of MCD of PTZ after 20 mg kg^{-1} propofol.

The occurrence of seizures after propofol is obviously a rare event considering the widespread use of this agent. However, in the light of the case reports described (Cameron, 1987; Hopkins, 1988; Victory & Magee, 1988; Jones *et al.*, 1988) and the results of the present study we feel that when anaesthesia is to be induced with a single bolus dose of an intravenous anaesthetic agent it would be prudent to select thiopentone in individuals with epilepsy or a strong family history of this disease. In the experiments described here it is clear that, whereas at the time of recovery from the anaesthetic effects of propofol, methohexitone and etomidate, there is still some residual protection against seizures, this is far more marked, and lasts for much longer, with thiopentone. The prolonged anticonvulsant action exhibited by thiopentone during the recovery phase will provide a greater degree of protection against seizures during this susceptible period than will propofol or any other of the intravenous anaesthetic agents commonly used.

Our results, however, would not preclude the use of propofol in epileptic patients; its powerful anticonvulsant effects may be useful in the control of drug resistant seizures in intensive care (Wood *et al.*, 1988), but prior to cessation of propofol administration a longer acting anticonvulsant must be administered and well-controlled, known epileptics are usually taking such medication.

The authors would like to thank Mrs S. Barlow and Mrs M.E. Bruce for secretarial assistance and ICI Pharmaceuticals for financial support and supplies of propofol.

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(Received August 20, 1990
Revised December 4, 1990
Accepted December 20, 1990)

Br. J. Pharmacol. (1991), **102**, 883–886

Inability of an opioid antagonist lacking negative intrinsic activity to induce opioid receptor up-regulation *in vivo*

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1 It has recently been suggested that opioid antagonists may be divided into those possessing negative intrinsic activity (e.g. naloxone) and those with neutral intrinsic activity (e.g. MR2266).

2 MR2266 was chronically administered to rats by subcutaneous infusion at a dose of $0.3\,mg\,kg^{-1}\,h^{-1}$ for 1 week.

3 This dose reduced ingestive behaviour and blocked the antinociceptive effects of a κ-agonist, indicating occupation of opioid receptors *in vivo*.

4 No supersensitivity could be detected to the antinociceptive actions of μ or κ agonists, either one or two days after cessation of treatment.

5 No up-regulation of μ, δ or κ binding sites was observed.

6 Since naloxone induces both supersensitivity and receptor up-regulation under equivalent conditions, the results suggest that negative intrinsic activity may be required for these phenomena to occur.

Keywords: μ opioid receptors; κ opioid receptors; supersensitivity; up-regulation; MR2266

Introduction

The availability of opioid receptor antagonists has facilitated the study of the actions mediated by the endogenous opioid peptides. The behavioural and neurochemical consequences of opioid antagonist administration have generally been interpreted as the result of the blockade of action of endogenous opioid peptide systems, the assumption being that antagonists do not themselves influence cellular processes. However, for the δ opioid receptors of NG108-15 neuroblastoma-glioma hybrid cells, where δ opioid agonists stimulate guanosine triphosphatase (GTPase) activity, there is now evidence that two types of competitive antagonists can be distinguished (Costa & Herz, 1989). Thus, (−)-5,9-diethyl-2-(3-furyl-methyl)-2'-hydroxy-6,7-benzomorphan (MR2266) shows no intrinsic activity at these δ receptors, neither stimulating nor inhibiting cellular GTPase. Naloxone, however, inhibits GTPase activity in the absence of any opioid agonist, apparently exhibiting negative intrinsic activity. This raises the possibility that the *in vivo* effects of opioid antagonists may not simply be due to blockade of the action of endogenous agonists, but may rather reflect an intrinsic action of the antagonist itself.

Recent evidence has demonstrated that the ability of chronic opioid treatment to affect receptor sensitivity and receptor number is critically dependent upon the intrinsic activity of the opioid (Morris, 1991). Thus, opioid agonists with high intrinsic activity will induce a subsensitivity (tolerance) and a down-regulation of opioid binding sites when administered *in vitro* (Law *et al.*, 1983) or chronically *in vivo* (Zukin & Tempel, 1986; Tao *et al.*, 1987; Morris & Herz, 1989). Opioid agonists with only low intrinsic activity appear to be unable to induce changes in receptor number, either *in vitro* or *in vivo* (Law *et al.*, 1983; Morris & Herz, 1989). The antagonists naloxone or naltrexone, chronically administered *in vivo*, will induce an up-regulation of opioid binding sites and a supersensitivity to subsequent opioid treatment (Lahti & Collins, 1978; Tang & Collins, 1978; Tempel *et al.*, 1982; 1984; Millan *et al.*, 1988; Morris *et al.*, 1988). There is no indication as to whether these effects result from the blockade of endogenous agonist action, or from 'negative intrinsic activity' of the antagonists themselves. However, the evidence available suggests that these general principles of receptor regulation apply equally to μ, δ and κ opioid receptors (Tao *et al.*, 1987; Millan *et al.*, 1988; Morris *et al.*, 1988; Morris & Herz, 1989).

An investigation into the effect of chronic MR2266 administration to rats on opioid receptor sensitivity and opioid receptor number may therefore identify the mechanisms involved in antagonist-induced supersensitivity and up-regulation. If these phenomena are caused by blockade of endogenous agonist action, then MR2266 should be able to induce these effects. Conversely, if 'negative intrinsic activity' is a prerequisite, then MR2266 treatment should not have any effect on receptor number or sensitivity. We have therefore studied the effect of chronic MR2266 infusion, under experimental conditions where chronic administration of naloxone induces both a supersensitivity to opioid agonists and an up-regulation of opioid binding sites (Millan *et al.*, 1988; Morris *et al.*, 1988). In parallel experiments to control for non-specific effects of MR2266, its inactive stereoisomer, (+)-5,9-diethyl-2-(3-furyl-methyl)-2'-hydroxy-6,7-benzomorphan (MR2267), was also administered chronically.

Methods

Adult male Sprague-Dawley rats (160–180 g) were implanted with Alzet osmotic minipumps (model 2001) subcutaneously under ether anaesthesia. The pumps contained either $50\,mg\,ml^{-1}$ MR2266 in 30% propylene glycol or $50\,mg\,ml^{-1}$ MR2267 in 30% propylene glycol. This represents a dose of approximately $0.3\,mg\,kg^{-1}\,h^{-1}$. Some rats received minipumps containing the vehicle alone. Food and water intake were monitored throughout the experiment.

After three days, basal nociceptive thresholds were assessed by the tail pressure test and tail radiant heat test, as previously described (Millan *et al.*, 1988). The ability of morphine or U50488H to raise nociceptive thresholds was then tested. The drugs were administered subcutaneously in isotonic saline, and nociceptive thresholds were assessed at a time after injection (45 min for morphine and 20 min for U50488H) previously determined to correspond to the peak effect (Millan *et al.*, 1988).

Seven days following implantation, the minipumps were removed, under ether anaesthesia. One day later, nociceptive

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thresholds were determined both before and after the administration of either morphine or U50488H. Nociceptive thresholds were also tested two days after removal of the minipumps. One group of animals were killed one day following minipump removal, and the brains processed for quantitative receptor autoradiography, as described elsewhere (Morris *et al.*, 1988; Morris & Herz, 1989). Cryostat sections were incubated in 50 mM Tris buffer containing (a) 5 nM [^{3}H]-D-Ala^2, MePhe^{4}Glyol enkephalin ([^{3}H]-DAMGO, 34 Ci mmol^{-1}, Amersham) to label μ-sites; (b) 5 nM [^{3}H]-D-Ala^2, D-Leu^5, enkephalin ([^{3}H]-DADL, 38 Ci mmol^{-1}, Amersham) + 1 μM DAMGO to label δ sites or (c) 1 nM (−)-[^{3}H]-bremazocine ([^{3}H]-Brem, 32 Ci mmol^{-1}, NEN) + 1 μM DAMGO + 1 μM DADL, to label κ-sites. Non-specific binding was assessed on adjacent sections to those used for total binding with (a) 1 μM DAMGO; (b) 1 μM DADL; (c) 5 μM U50488H. In some experiments, a range of concentrations of ligands were used, covering two orders of magnitude, in order to estimate the binding capacity of the sites. After washing, sections were dried and apposed to LKB Ultrofilm for 8–16 weeks.

The following drugs were generous gifts: morphine (Merck Chem. Inc., Darmstatdt, F.R.G.), U50488H (Dr R. Lahti, Upjohn Co. Kalamazoo, U.S.A.), MR2266 and MR2267 (Dr H. Merz, Boehringer, Ingelheim, F.R.G.).

Results

MR2266, as compared to MR2267 or vehicle alone, caused a reduction in daily food and water intake. In animals receiving infusions of MR2266, the mean daily food intake over the seven day period was 19.22 ± 0.56 g (mean ± s.e.mean, $n = 14$) significantly lower ($P < 0.01$ one-way ANOVA and Student's Newman-Keuls test) than the value of 21.13 ± 0.36 g ($n = 14$) in animals receiving MR2267. Mean daily water intake was 29.76 ± 1.59 g ($n = 14$) in animals receiving MR2266, and was 35.22 ± 1.54 g ($n = 14$) in animals receiving MR2267, ($P < 0.05$). The effects persisted throughout the period of administration, indicating the efficacy of the treatment. On the day following removal of the pumps, food and water intake returned to normal (not shown). Basal nociceptive thresholds were not significantly modified by MR2266. Thus, in animals receiving MR2267, ($n = 14$), basal nociceptive thresholds to heat were 3.43 ± 0.03 s, 3 days after commencing infusion, and were 3.58 ± 0.10 s, 1 day following discontinuation of treatment. Similarly, basal thresholds to pressure were 134.06 ± 8.12 g and 128.62 ± 9.41 g during and after infusion respectively. In animals receiving MR2266, thresholds to heat during and after treatment were 3.41 ± 0.09 s and 3.59 ± 0.08 s, respectively. Thresholds to pressure were 127.66 ± 9.01 g and 119.62 ± 8.80 g respectively. None of the differences were statistically significant (one-way ANOVA and Student's Newman-Keuls test).

Infusion of MR2266 led to a pronounced blockade of the antinociception elicited by U50488H (Figure 1). While the effect of morphine was also attenuated, this only attained statistical significance in the tail-flick test to heat. One day following removal of the pumps, there was no alteration in the antinociceptive action of U50488H either 30 min (Figure 1) or 75 min (not shown) after its administration. Similarly, the effect of morphine was not significantly modified. Two days following minipump removal, there was also no difference in the effect of morphine or U50488H (not shown).

The effect of 7 days administration of MR2266 and MR2267 on the levels of μ, δ and κ binding in various brain regions is shown in Table 1. The binding conditions used have been shown to be selective for each binding site (Morris & Herz, 1986; 1987; Morris *et al.*, 1988; Morris & Herz, 1989). Neither compound produced a significant change in binding levels in any region examined, as compared to animals receiving an infusion of vehicle. Saturation analysis revealed no change in either binding affinity or capacity (not shown).

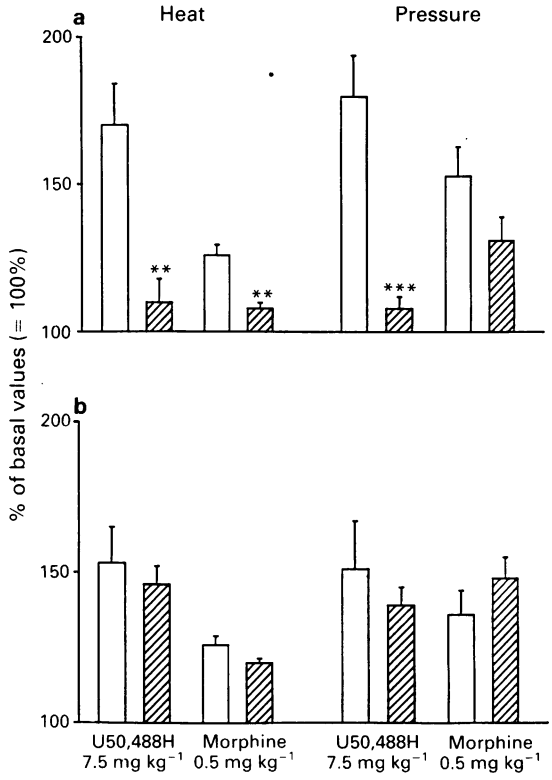

Figure 1 Antinociceptive action of U50488H or morphine in rats (a) being infused with or (b) following infusion with, MR2266 (hatched columns) or MR2267 (open columns), (0.3 mg kg^{-1} h^{-1}). In (a), experiments were performed 3 days after implantation and in (b), 1 day following removal. These were independent groups of animals. Antinociception is expressed as a percentage of basal values (= 100%). Mean of $n > 6$; s.e.mean shown by vertical bars. ** $P < 0.01$ and *** $P < 0.001$ vs MR2267 (Student's Newman-Keuls test).

Discussion

The depression of food and water intake caused by MR2266 is consistent with previous findings (Sanger *et al.*, 1981; Leander, 1984; Millan & Morris, 1988), and provides evidence that the drug was active throughout the period of treatment. Similarly the fact that ingestive behaviour returned to normal, one day after removal of the minipumps indicates that the concentration of MR2266 in the brain had fallen rapidly upon cessation of treatment.

While MR2266 is frequently regarded as an opioid antagonist which is preferentially active at κ receptors, it is, in terms of affinity, only slightly more active at κ receptors than μ or δ receptors (Magnan *et al.*, 1982; Wehyenmeyer & Mack, 1985; Miller *et al.*, 1986). This explains the ability of MR2266, as employed here, not only to abolish the effect of U50488H, but also to attenuate that of morphine. These data emphasise that, under chronic administration, as with acute administration, MR2266 cannot be used as a selective κ antagonist.

In the majority of *in vivo* experiments, MR2266 has been used to block the effect of an opiate agonist. However, it has also been used alone in order to interrupt any κ receptor-mediated activity of the endogenous opioids. In general, the behavioural effects of MR2266 administration have then been interpreted as a result of the blockade of μ or κ receptors (Sanger *et al.*, 1981; Leander, 1984).

However, there is a single report that MR2266 may have some slight ability to inhibit contractions in the guinea-pig ileum myenteric plexus-longitudinal muscle bioassay (Magnan *et al.*, 1982). This preparation appears to contain functional μ and κ opioid receptors, implying that MR2266 may possess weak partial agonist activity at either one or both of these receptor types. Other workers have failed to detect any agonist activity of MR2266 in this preparation (Miller *et al.*,

Table 1 Specific binding of [^{3}H]-D-Ala^2, MePhe^4 glyol enkephalin ([^{3}H]-DAMGO), [^{3}H]-D-Ala^2, D-Leu^5 enkephalin ([^{3}H]-DADL) and [^{3}H]-bremazocine to μ-, δ- and κ-binding sites respectively, in individual brain areas from rats treated chronically with MR2267, MR2266 or vehicle (Veh)

	[^{3}H]-*DAMGO*[1]			[^{3}H]-*DADL*[2]			[^{3}H]-*bremazocine*[3]		
Brain area	*Veh*	*MR2267*	*MR2266*	*Veh*	*MR2267*	*MR2266*	*Veh*	*MR2267*	*MR2266*
Frontal cortex	70.0 ± 6.0[a]	72.0 ± 6.0[a]	73.2 ± 5.1[a]	25.7 ± 1.2[b]	28.1 ± 0.8[b]	24.3 ± 1.8[b]	13.0 ± 1.2[c]	13.0 ± 0.8[c]	10.6 ± 0.4[c]
Claustrum	92.0 ± 4.9	73.0 ± 8.2	92.1 ± 4.4	41.4 ± 2.9	37.9 ± 1.5	39.3 ± 1.7	31.0 ± 3.7	34·6 ± 1.4	29.0 ± 3.6
Amygdala-bl	104.6 ± 5.8	90.9 ± 4.0	99.1 ± 2.0	39.1 ± 2.0	38.0 ± 2.1	38.3 ± 4.2	26.0 ± 2.7	23.3 ± 1.9	26.5 ± 1.1
Hippocampus									
-ml	110.8 ± 5.4	124.0 ± 8.2	123.9 ± 6.9	14.5 ± 1.1	14.5 ± 0.9	12.8 ± 2.0	15.2 ± 0.9	16.7 ± 3.2	15.4 ± 2.2
-CA_3	65.3 ± 10.0	70.3 ± 5.4	61.4 ± 6.7	15.3 ± 1.9	14.6 ± 2.0	14.0 ± 1.3	11.6 ± 0.9	12.8 ± 0.7	11.8 ± 0.7
Striatum									
-patches	230.6 ± 13.1	220.5 ± 10.4	212.1 ± 14.2	—	—	—	—	—	—
-matrix	55.0 ± 3.5	41.8 ± 7.4	42.8 ± 5.7	27.7 ± 1.3	24.2 ± 1.1	23.8 ± 1.0	18.3 ± 1.0	18.3 ± 0.8	12.9 ± 2.1
Thalamus-vpl	25.2 ± 4.6	24.1 ± 2.8	18.1 ± 4.0	ND	ND	ND	8.6 ± 1.0	9.1 ± 0.9	7.2 ± 2.0
Hypothalamus-vm	59.1 ± 4.5	56.8 ± 3.8	66.4 ± 4.2	9.1 ± 1.5	9.7 ± 1.4	10.2 ± 0.9	ND	ND	ND
Zona Incerta	44.0 ± 5.1	41.9 ± 1.7	48.1 ± 2.9	ND	ND	ND	16.5 ± 3.2	13.1 ± 1.2	14.0 ± 3.1
SGSC	124.0 ± 5.4	114.0 ± 3.3	99.4 ± 9.1	ND	ND	ND	19.6 ± 2.8	29.0 ± 2.6	23.6 ± 0.9
PAG-dorsolateral	74.3 ± 4.1	83.0 ± 5.3	72.3 ± 9.5	ND	ND	ND	15.2 ± 1.5	18.7 ± 1.6	18.4 ± 1.7
S. nigra-pars retic.	31.4 ± 2.0	35.6 ± 0.7	25.5 ± 4.7	ND	ND	ND	17.0 ± 1.1	21.1 ± 2.6	18.0 ± 1.6

Values are expressed as mean ± s.e.mean from groups of 3–4 animals, and are in fmol mg^{-1} tissue.
[1] 5 nM [^{3}H]-DAMGO; [2] 5 nM [^{3}H]-DADL + 1 μM DAMGO; [3] 1 nM [^{3}H]-bremazocine + 1 μM DAMGO + 1 μM DADL.
[a] Layer III, [b] Layer V, [c] Layer VI.
Abbreviations: bl – basolateral nucleus, amygdala; ml – molecular layer, hippocampus; vpl – ventral posterolateral nucleus, thalamus; vm – ventromedial nucleus, hypothalamus; SGSC – superior grey layer, superior colliculus; PAG – periaqueductal grey area; s. nigra, pars retic – substantia nigra, pars reticulata. ND – not detectable. No significant differences were found in any region by one-way ANOVA.

1986). In pharmacological bioassays where only high intrinsic activity opioid ligands are active, MR2266 shows only antagonist activity (Hayes & Kelly, 1985; McKnight *et al.*, 1985).

We have demonstrated previously (Millan & Morris, 1988; Millan *et al.*, 1988; Morris *et al.*, 1988) that when naloxone is administered under equivalent conditions to those used here (7 days of continuous subcutaneous infusion) it will abolish the antinociceptive effects of acutely administered opioid agonists. However, naloxone is able to induce a behavioural supersensitivity to opioids administered up to four days following removal of the pumps and to cause a profound increase in the levels of opioid binding sites in many regions of rat brain. In this study, despite the attenuation of opioid actions in the presence of MR2266, no supersensitivity to μ or κ agonists could be detected following pump removal. Consistent with this, no changes in μ, δ or κ binding could be detected in any brain area.

The possibility that sufficient MR2266 remained after the chronic treatment to interfere with the binding assay and the behavioural assessment of opioid actions appears to be highly unlikely. Ingestive behaviour had returned to normal values, no supersensitivity could be detected even three days after pump removal, and, when saturation analysis of opioid binding was performed, no reduction in binding affinity was observed. Furthermore, any suggestion that the dose of MR2266 was insufficient to induce an up-regulation of receptors and supersensitivity appears untenable, since the dose was sufficient to attenuate the actions of morphine and U50488H. Therefore, it is difficult to explain the lack of supersensitivity and up-regulation on the basis of the concentrations of MR2266 attained *in vivo* being either too high or too low.

Partial agonists at opioid receptors appear to be unable to induce long-term changes in receptor number (Law *et al.*, 1983). Thus, chronic administration of diprenorphine, a weak partial agonist at δ and κ receptors (Miller *et al.*, 1986; Costa & Herz, 1989), does not affect the number of δ and κ binding sites (Morris & Herz, 1989). Similarly, chronic administration of nalorphine, which is a partial agonist at κ receptors, also leaves the number of κ binding sites unchanged (Morris & Herz, 1989). Partial opioid agonists can also be distinguished from opioids with strong positive or negative intrinsic activity according to the effect of sodium ions on binding parameters. Sodium ions will increase the binding of naloxone, and decrease the binding of agonists with positive intrinsic activity. The effects of sodium on diprenorphine binding are complex (Kosterlitz *et al.*, 1987), and the effects on MR2266 binding have unfortunately not yet been reported. Nevertheless, the presence of the sodium effect in brain tissue suggests that antagonists with negative intrinsic activity may be able to produce unique effects, on G-protein coupling in neurones *in vivo*, which are unrelated to the blockade of endogenous agonist action.

Two possibilities should therefore be considered as to why MR2266 and naloxone show a different profile of activity after chronic administration. Firstly, that antagonists possessing either 'neutral' or 'negative' intrinsic activity are able to induce receptor up-regulation and supersensitivity, but MR2266 administration has no effect because MR2266 is acting as a partial agonist at μ, δ and κ receptors. This would be consistent with the report suggesting that MR2266 can produce some slight inhibition of contractions via the μ or κ receptors of the guinea-pig ileum-myenteric plexus preparation (Magnan *et al.*, 1982). However, the lack of any GTPase activity in NG108-15 neuroblastoma-glioma cells (Costa & Herz, 1989), an unequivocal demonstration of efficacy, implies that MR2266 is not a partial agonist at δ receptors under physiological conditions. Therefore, the lack of δ binding site up-regulation observed here favours an alternative explanation.

The other possibility is that binding site up-regulation may only be seen after the chronic administration of antagonists with 'negative intrinsic activity'. MR2266, as a 'neutral' antagonist or weak partial agonist, does not induce this effect on either μ, δ or κ sites, while naloxone can up-regulate each receptor class. This would imply that the phenomena of receptor up-regulation and supersensitivity are not a direct consequence of the blockade of tonic activity mediated by the endogenous opioids, but are rather a reflection of an action intrinsic to the ligand. This can be easily accommodated within the current models of receptor-G protein interaction. The 'negative' antagonist can be viewed as inhibiting the tonic activation of G proteins resulting from spontaneous receptor coupling. Since agonist-induced receptor and G protein activation is clearly linked to a suppression of receptor sensitivity and number (desensitization and down-regulation), this would

remove any tonic suppression that existed. Interestingly, there are reports of pharmacological differences between naloxone and MR2266 that are apparently unrelated to potency or receptor selectivity (France & Morse, 1989).

Thus, the evidence presented here appears more consistent with the second explanation, and therefore has some general implications. *In vivo* experiments on the effects of MR2266 administration should certainly be interpreted with some caution, in that the actions of MR2266 are clearly different from those of more classical antagonists. In addition, the results suggest a reinterpretation of the mechanisms involved in receptor up-regulation and supersensitivity.

We are grateful to Professor A. Herz for his support and encouragement throughout this study, and to Alison Neill for typing services.

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(Received July 26, 1990
Revised December 13, 1990
Accepted December 20, 1990)

Br. J. Pharmacol. (1991), **102**, 887–895

The pharmacology of fluparoxan: a selective α_2-adrenoceptor antagonist

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1 This paper describes the pharmacology of the novel α_2-adrenoceptor antagonist fluparoxan (GR 50360) which is currently being studied clinically as a potential anti-depressant. Idazoxan and yohimbine were included in many studies for comparison.

2 In the rat isolated, field-stimulated vas deferens and the guinea-pig isolated, field-stimulated ileum preparations, fluparoxan was a reversible competitive antagonist of the inhibitory responses to the α_2-adrenoceptor agonist UK-14304 with pK_B values of 7.87 and 7.89 respectively. In the rat isolated anococcygeus muscle, fluparoxan was a much weaker competitive antagonist of the contractile response to the α_1-adrenoceptor agonist phenylephrine with a pK_B of 4.45 giving an $\alpha_2:\alpha_1$-adrenoceptor selectivity ratio of greater than 2500.

3 In the conscious mouse, fluparoxan (0.2–3.0 mg kg^{-1}) was effective by the oral route and of similar potency to idazoxan in preventing clonidine-induced hypothermia and antinociception. In the rat, UK-14304-induced hypothermia (ED_{50} = 1.4 mg kg^{-1}, p.o. or 0.5 mg kg^{-1}, i.v.) and rotarod impairment (ED_{50} = 1.1 mg kg^{-1} p.o. or 1.3 mg kg^{-1}, i.v.) were antagonized by fluparoxan. Fluparoxan, 0.67–6 mg kg^{-1}, p.o., also prevented UK-14304-induced sedation and bradycardia in the dog.

4 In specificity studies fluparoxan had low or no affinity for a wide range of neurotransmitter receptor sites at concentrations up to at least 1×10^{-5} M. It displayed weak affinity for 5-HT_{1A} (pIC_{50} = 5.9) and 5-HT_{1B} (pK_i = 5.5) binding sites in rat brain.

5 We conclude that fluparoxan is a highly selective and potent α_2-adrenoceptor antagonist. The density of rat brain [^{3}H]-dihydroalprenolol binding sites was reduced by 26% when fluparoxan was administered chronically for 6 days at a dose of 12 mg kg^{-1} orally twice daily. The down-regulation of β-adrenoceptors by fluparoxan is consistent with its antidepressant potential.

Keywords: α_2-Adrenoceptor antagonist; fluparoxan; GR 50360; idazoxan

Introduction

The classification of α-adrenoceptors into α_1 and α_2 subtypes is well recognised (see McGrath *et al.*, 1989). Both agonists and antagonists for these subtypes are known and have proved to be useful therapeutically. However, highly selective antagonists at α_2-adrenoceptors are relatively novel, e.g. Merck 912 (Pettibone *et al.*, 1987), CH38083 (Vizi *et al.*, 1988) and RS-15385-197 (Clark *et al.*, 1989), and await therapeutic evaluation. The autoreceptor on noradrenergic neurones mediating the feedback inhibitory effects of noradrenaline is widely recognised as belonging to the α_2-subtype (Langer, 1980). Antagonists at this receptor facilitate noradrenergic neurotransmission as evidenced by a rapid increase in noradrenaline turnover (Anden *et al.*, 1982; Walter *et al.*, 1984). An α_2-adrenoceptor antagonist could therefore be beneficial in conditions such as depression which have been associated with a deficiency of noradrenaline in the CNS (Schildkraut, 1965).

Several novel α_2-adrenoceptor antagonists with varying degrees of α_2- to α_1-adrenoceptor selectivity have been described since the appearance of imiloxan in 1981 (Michel & Whiting, 1981) and idazoxan in 1983 (Doxey *et al.*, 1983). We describe here the *in vitro* and *in vivo* pharmacology of the novel benzodioxinopyrrole, fluparoxan (formerly known as GR50360), (Figure 1), a potent and selective α_2-adrenoceptor antagonist that is currently undergoing clinical evaluation. Preliminary accounts of some of the data reported here were presented to the British Pharmacological Society (Halliday *et al.*, 1988a,b).

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Methods

Rat vas deferens

The prostatic halves of vasa deferentia were carefully removed from adult Sprague-Dawley rats (300–400 g; Charles River) killed by stunning and cervical dislocation.

The tissues were mounted under 0.5 g tension at 31°C in oxygenated Krebs-Henseleit solution. Electrical stimulation was provided through two stainless steel ring electrodes, 20 mm apart, placed around the tissue and consisted of supramaximal rectangular monophasic pulses of 2 ms duration and approximately 20 V amplitude delivered every 10 s. Twitch responses were recorded isometrically by use of Ormed Dynamometer UF1 strain-gauge transducers. UK-14304 or clonidine was added to the bath in incremental doses to produce cumulative inhibitory concentration curves. For antagonist studies the antagonist drugs were added 25–30 min before addition of the agonist.

Guinea-pig ileum

Guinea-pig terminal ileum (3 cm lengths) was obtained from male Dunkin Hartley guinea-pigs (450–550 g; Porcellus) killed by cervical dislocation. The tissues were set up under 0.5 g

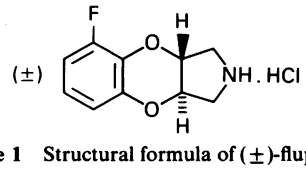

Figure 1 Structural formula of (±)-fluparoxan.

tension in 12.5 ml jacketed organ baths in oxygenated Krebs-Henseleit solution at 36°C and subjected to electrical field stimulation as described by Drew (1978).

Twitch responses were recorded isometrically as for rat vas deferens. After equilibration, the tissues were exposed to either fresh Krebs-Henseleit solution or Krebs-Henseleit solution containing antagonist for 15–30 min. Successive doses of UK-14304 were then added to the bath to produce a cumulative inhibition of the twitch response, each dose being added when the previous dose had produced its maximal inhibitory effect.

Rat anococcygeus muscle

Anococcygeus muscles were dissected from male Sprague-Dawley rats (300–400 g; Charles River) killed by stunning and cervical dislocation. Tissues were soaked overnight for 17 h at 4°C in Krebs-Henseleit solution containing 6-hydroxydopamine, 1×10^{-3} M, to destroy presynaptic sites of uptake and noradrenaline release as described by Doggrell (1980). The anococcygeus muscles were then mounted under 1 g tension in 12.5 ml organ baths and washed repeatedly with oxygenated Krebs-Henseleit solution at 36°C for 1 h. Thereafter the tissues were attached to isometric strain gauge transducers and the tension reduced to 0.5 g. The tissues were primed with high doses of phenylephrine (final bath concentration of 6×10^{-6} M) and a 30 s contact time until reproducible contractions were obtained. The maximum contractile response of each tissue to either phenylephrine or methoxamine was measured and tissues were then washed with fresh Krebs-Henseleit solution and incubated with either this solution or Krebs-Henseleit solution containing antagonist for a period of 15–30 min. Concentration-response curves were obtained by cumulative addition of either phenylephrine or methoxamine, each dose being added after the previous dose had produced its maximum effect.

Rabbit aorta

Lengths of aorta (8 cm approx.) were removed from adult rabbits (Froxfield NZW) killed by cervical dislocation. Excess fatty tissue was removed from the aorta which was then threaded on to a 20 mm diameter glass rod and cut helically into strips approximately 4 mm wide and 4 cm long. The strips were mounted under 0.5 g tension in oxygenated Krebs-Henseleit solution at 37°C in 15 ml organ baths. Agonist-induced contractures were recorded isometrically. Four cumulative dose-response curves to phenylephrine were obtained for each tissue strip. The antagonist was introduced at least 15 min before the fourth curve. The shift in the agonist potency between the third and fourth curves was then calculated after correcting for any sensitivity change calculated from tissues not exposed to antagonist. The largest sensitivity change was usually between the first and second curves, hence the design employed here.

Estimation of potency in vitro

Lateral displacements of agonist concentration-response curves were measured at the control half-maximal response level. The negative logarithm of the dissociation constant of each antagonist (pK_B) was calculated using the Schild/Gaddum equation (Gaddum, 1957) from the relationship: $pK_B = \log (\text{dose-ratio} - 1) - \log [\text{antagonist}]$ where dose-ratio (DR) is the ratio of the agonist concentration producing half maximal responses (IC_{50}) in the presence and absence of the antagonist at the molar concentration [antagonist]. Individual pK_B determinations were made using 3 or 4 concentrations for each antagonist such that a mean ($\pm$s.e.) pK_B value could be calculated. The results were also plotted according to the method of Arunlakshana & Schild (1959) to yield Schild plot gradients. The effect of each concentration of antagonist was measured on 1–6 preparations of a given tissue, each from a separate animal.

Antagonism of clonidine-induced hypothermia in the mouse

Male mice (Glaxo CRH 20–26 g), housed in groups of 5 at room temperature (22°C), were given gum acacia 5% w/v (vehicle), fluparoxan or idazoxan orally in a dose volume of 10 ml kg^{-1}. After 60 min the oesophageal temperature of each mouse was recorded with an electronic thermistor probe (Comark) and clonidine, 0.15 mg kg^{-1}, was given intraperitoneally. Temperatures were remeasured 30 min later and the changes in temperature were calculated.

Antagonism of clonidine-induced antinociception in the mouse

Male mice (Glaxo CRH 20–26 g) were given vehicle (5% w/v gum acacia 10 ml kg^{-1}), fluparoxan, idazoxan or yohimbine; 60 min later either saline or clonidine, 0.05 mg kg^{-1}, was given subcutaneously. After another 30 min each mouse received an intraperitoneal injection of 0.25 ml phenylbenzoquinone, 0.02% w/v, and was observed for 10 min for the appearance of abdominal constriction accompanied by stretching.

Antagonism of UK-14304-induced hypothermia and rotarod impairment in the rat

Male hooded rats (70–120 g; Glaxo) were given vehicle (5% w/v gum acacia-oral route; 0.9% w/v sodium chloride solution-intravenous route), fluparoxan or idazoxan by either the oral or intravenous (tail vein) routes (10 ml kg^{-1} and 5 ml kg^{-1} dose volume respectively). After 30 min the oesophageal temperature of each rat was recorded with an electronic thermistor probe (Comark) and either 0.9% w/v sodium chloride solution or UK-14304 (2 mg kg^{-1}) was given subcutaneously (5 ml kg^{-1} dose volume). Oesophageal temperature was recorded again after an additional 30 min; 10 min later each rat was placed on an accelerating rotarod (Jones & Roberts, 1968) and the time it remained on the revolving drum was measured.

Antagonism of UK-14304-induced sedation and bradycardia in the dog

Studies were performed in beagles (6 males, 6 females; Glaxo; 6.8–10 kg weight range). Fluparoxan was given orally (0.22–6 mg kg^{-1} in gelatin capsules) 30 min before a subcutaneous injection of UK-14304 (0.05 mg kg^{-1}). Sedation and heart rate were measured before giving fluparoxan and at various times after injection of UK-14304. For sedation, both spontaneous activity and reactivity to an audible stimulus (hand clap) were assessed on pairs of dogs housed in their home kennel and again when placed alone in an unfamiliar environment. Behaviour each time was scored from 0 (inactive) to 6 (maximal activity) such that by combination of the 4 behavioural scores a maximum activity score of 24 was possible. Heart rate was measured by counting the number of pulses in the femoral artery in 30 s.

Estimation of potency in vivo

The potencies of test compounds in antagonizing α_2-adrenoceptor agonist-induced behavioural effects were calculated in the form of ED_{50} values. For antagonism of antinociceptive effects the data were quantal and ED_{50} values were used to represent the dose of antagonist reducing the incidence of the response to half that of the control animals. This was obtained from the best line fit to a log dose-logit plot of the quantal data. Confidence limits (95%) were obtained by use of Fieller's theorem. For antagonism of the hypothermia and sedation, the ED_{50} value represents the dose of antagonist reducing the magnitude of the response to 50% of that in animals given agonist alone. Half maximal response values

and standard errors were obtained by fitting dose-response curves to the data by the computer programme ALLFIT (De Lean *et al.*, 1978).

Evaluation of activity or affinity at non α-adrenoceptor sites

β-Adrenoceptors Affinity for β_1-adrenoceptors was assessed in the rat (male 200 g; Glaxo AHA strain) isolated, electrically stimulated left atrium by a method based on that of Blinks (1965). The isolated atrium was impaled on a two pin electrode and placed in a 2 ml organ bath in oxygenated Krebs-Henseleit solution and stimulated at 1 Hz and 2 ms using just suprathreshold voltage.

Activity at β_2-adrenoceptors was measured in guinea-pig (Dunkin Hartley male 300–400 g; Porcellus) isolated trachea as described by Coleman & Nials (1989).

Dopamine receptors The effect of fluparoxan at functional peripheral dopamine receptors was assessed by use of dopamine-induced increases in mesenteric blood flow in the anaesthetized dog for D_1-receptor-mediated effects as described by Hilditch & Drew (1984) and dopamine-induced inhibition of electrically stimulated increases in heart rate of the anaesthetized cat for D_2-receptor-mediated effects (Dallas *et al.*, 1986).

Radioligand binding assays for the D_2 site were performed on rat striatal membranes. Corpora striata were obtained from male hooded rats (200–250 g; Glaxo) killed by stunning and decapitation. The tissues were homogenized in 30 volumes of 50 mM Tris-HCl buffer at 4°C and pH 7.4 in an Ultra-Turrax homogeniser (lowest setting for two periods of 10 s with 30 s on ice in between). The homogenate was centrifuged (40000 *g* for 20 min at 4°C) and the resulting pellet resuspended in 30 vol of fresh Tris-HCl buffer. The centrifugation was repeated and the final pellet resuspended in 30 vol of Tris-Krebs buffer containing (mM): Tris-HCl 50, NaCl 120, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2 and EDTA 1. Assays were performed on 1 ml of homogenate (approx. 225 μg protein) in a final volume of 3 ml of Tris-Krebs buffer containing 150 pM [^{3}H]-spiperone (35 Ci mmol^{-1}, Dupont NEN) and 50 nM ketanserin to prevent binding to 5-HT_2 sites. (+)-Butaclamol (1 μM) was used to define specific binding. Incubation was performed at 25°C for 75 min and terminated by filtration through Whatman GF/B filters in a Brandel Cell Harvester followed by washing of the filters (3 × 5 ml buffer at 4°C).

Muscarinic acetylcholine receptors Terminal ileum was obtained from male Lister Hooded rats (200–250 g; Glaxo). Responses were recorded isometrically (Ormed Dynamometer UF1 transducer) from 2 cm lengths of tissue under 0.5 *g* tension in oxygenated Krebs-Henseleit solution in 10 ml organ baths at 37°C. Fluparoxan was tested at concentrations up to 1×10^{-5} M against the contractile response to muscarine with a 5 min dosing cycle.

For radioligand binding assays rat heart and cerebral cortex membranes were prepared in a HEPES buffer (20 mM HEPES + 100 mM sodium chloride + 0.5 mM EDTA at pH 7.4; buffer for heart membranes also contained 3 mM magnesium chloride). Radioligands were [^{3}H]-N-methylscopolamine (72 Ci mmol^{-1}, Amersham International, 0.2 nM) for the heart assays and [^{3}H]-pirenzepine (87 Ci mmol^{-1}, Dupont NEN, 0.8 nM) for cortex. Atropine (1 μM) was used to define specific binding and assays were terminated after 90 min at 22°C by centrifugation (Lazareno & Roberts, 1989).

Histamine receptors Lengths of terminal ileum obtained from male Dunkin Hartley guinea-pigs (450–500 g; Porcellus) were set up as described above. The effect of fluparoxan on histamine H_1-receptors was assessed from concentration-response curves obtained to histamine in tissues preincubated for 30 min in fresh Krebs-Henseleit solution or Krebs-Henseleit solution containing fluparoxan. Assays for histamine H_2-receptor activity were performed on histamine-induced tachycardia in guinea-pig atrium as described by Humphray *et al.* (1982).

5-Hydroxytryptamine (5-HT)-receptors Possible activity of fluparoxan at functional 5-HT_1-like receptors in dog saphenous vein and 5-HT_2 receptors in rabbit isolated aorta was assessed as described by Feniuk *et al.* (1985). Affinity for 5-HT_{1A} and 5-HT_{1B} binding sites was measured with rat (male hooded; Glaxo; 150–200 g) hippocampal and striatal membranes respectively. Hippocampi from 8 rats were pooled and homogenized in an Ultra-Turrax at its lowest setting for 10 s at 5°C in 10 vol of 50 mM Tris-HCl buffer, pH 7.4. The membrane preparation was centrifuged at 40000 *g* for 15 min and the pellet was resuspended in 10 volumes of Tris-HCl buffer. A further centrifugation and re-suspension of the membranes was followed by a 10 min incubation at 37°C to remove endogenous 5-HT. Following the final centrifugation, the resulting pellet was resuspended in 10 vol of 50 mM Tris-HCl buffer, pH 7.4 at 37°C. Binding assays were performed using 100 μl aliquots (equivalent to 0.16–0.2 mg protein). The membranes were incubated for 15 min at 37°C with [^{3}H]-8-hydroxy-2-(di-n-propylamino) tetralin (85 Ci mmol^{-1}, Amersham International 0.5–1.0 mM) and the test compound in a final volume of 250 μl. 5-HT, 1×10^{-5} M, was used to define specific binding. Assays were terminated by filtration through Whatman GF/B filters using a Brandel Cell Harvester. Affinity for 5-HT_{1B} binding sites in rat striatum was determined with [^{125}I]-cyanopindolol (2088 Ci mmol^{-1}, Amersham International) according to Hoyer *et al.* (1985).

Noradrenaline uptake

Male Hooded rats (250–300 g; Glaxo) were killed by stunning and decapitation. Brains were quickly removed and the occipital cortex dissected out. Occipital cortices from three rats were placed in 5.1 ml of ice-cold 0.32 M sucrose solution in a 10 ml Potter-Elvehjem glass mortar fitted with a Teflon pestle having a clearance of 0.25 mm. The tissue was homogenized twice at 840 r.p.m. each time using six complete up and down strokes of the pestle over a 30 s period with a 60 s pause with the mortar on ice in between. The homogenate was then centrifuged at 1000 *g* and 4°C for 10 min. The supernatant (crude synaptosomal preparation) was used immediately for uptake studies. Assays were performed in triplicate using 0.1 ml of the synaptosomal preparation added to 1.8 ml of an oxygenated Krebs-Henseleit buffer at 37°C. The buffer had the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.1, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 1.3, *D*-glucose 10, ascorbic acid 0.5 and pargyline HCl 0.1; pH 7.5 and contained either no drug, fluparoxan or desmethylimipramine with or without ouabain (final concentration 10 mM). After 10 min, 0.1 ml of [^{3}H]-noradrenaline (41 Ci mmol^{-1}, Dupont NEN) in Krebs-Henseleit buffer was added (final concentration 10 nM) and the tubes incubated in a shaking water-bath for 7 min. The samples were then filtered through Whatman GF/B filters using a Brandel Cell Harvester. The filters were washed with cold (4°C) buffer (2 × 5 ml) and the amount of radioactivity retained measured by liquid scintillation spectroscopy.

Specific and energy-dependent noradrenaline uptake was defined as the difference between that in the presence and absence of ouabain. The concentration of fluparoxan and desmethylimipramine inhibiting by 50% the specific noradrenaline uptake was determined from concentration inhibition-dose-response curves analysed using the logistic curve-fitting programme ALLFIT (De Lean *et al.*, 1978).

Effect on [^{3}H]-dihydroalprenolol binding sites

Male Sprague-Dawley rats (300 g; Charles River) were given water (5 ml kg^{-1}), fluparoxan (6 or 12 mg kg^{-1}), amitriptyline (30 mg kg^{-1}) or fluparoxan (6 mg kg^{-1}) + amitriptyline (30 mg kg^{-1}) by the oral route twice daily (at 08 h 00 min and

17 h 00 min) for 6 days. Fifteen hours after the final dose the rats were killed by stunning and decapitation, their brains rapidly removed and the cerebral cortices obtained and cleared of adhering white matter. Each cerebral cortex (350–450 mg wet wt.) was rapidly frozen in liquid nitrogen and stored at −70°C for up to three weeks. Membranes for radioligand binding assays were prepared from the thawed cerebral cortices as described by Wise & Halliday (1985). The binding assays were performed on 0.2 ml aliquots of cerebral membranes incubated initially in triplicate in 50 mM Tris-HCl buffer (final volume of 1 ml, pH 7.8) at 25°C for 10 min. Thereafter, 0.1 ml [^{3}H]-dihydroalprenolol (1-[propyl-^{3}H(N)]dihydroalprenolol HCl, 40 Ci mmol^{-1}, Dupont NEN) was added and the mixture incubated at 25°C for 25 min followed by filtration through Whatman GF/B filters in a Brandel Cell Harvester. The filters were washed (3 × 5 ml Tris-HCl buffer at 25°C) and the retained tritium measured by addition of the filters to 10 ml Pico-Fluor 30 scintillation fluid (United Technologies Packard) for approximately 15 h followed by conventional liquid scintillation spectrometry. (−)-Isoprenaline (200 μM) was included in some tubes and specific binding was calculated as the difference between binding in its absence and presence.

The density of binding sites (B_{max}) and their affinity (K_D) were determined by Scatchard analysis (Scatchard, 1949) of the binding of six concentrations (0.1–2.0 nM) of [^{3}H]-dihydroalprenolol.

In some studies fluparoxan, amitriptyline and (−)-isoprenaline were tested over a range of concentrations for their ability to displace specific binding of [^{3}H]-dihydroalprenolol (0.3 nM) from cerebrocortical membranes from untreated rats. The respective concentrations producing 50% displacement of specific binding (IC_{50}) and hence K_i values and Hill slopes were determined by log probit analysis.

Drugs and solutions

Tissues were bathed in a modified Krebs-Henseleit solution (Apperley *et al.*, 1976) unless otherwise stated. The solution was gassed with 95% O_2 and 5% CO_2. For rat vas deferens and ileum preparations the buffer solution contained 2.5 mM $CaCl_2$ and for guinea-pig ileum experiments 2 μM (+)-propranolol was included. Reagents used were A.R. grade and purchased from commercial sources. The following drugs were used: fluparoxan (GR50360, *trans*-(±)-5-fluoro-2,3,3a,9a-tetrahydro-1H[1,4]-benzodioxino-[2,3-c]pyrrole hydrochloride, Glaxo Group Research Ltd); amitriptyline hydrochloride (Sigma); atropine sulphate (Sigma); (+)-butaclamol hydrochloride (Research Biochemicals Inc.-RBI); clonidine hydrochloride (Sigma); desmethylimipramine hydrochloride (Ciba-Geigy); dopamine hydrochloride (Sigma); histamine hydrochloride (Sigma); 6-hydroxydopamine hydrochloride (Sigma); 5-hydroxytryptamine creatinine sulphate (Sigma); idazoxan hydrochloride (Glaxo Group Research Ltd); (−)-isoprenaline sulphate (Sigma); ketanserin (RBI); methoxamine hydrochloride (Burroughs Wellcome); muscarine chloride (Sigma); ouabain octahydrate (Sigma); pargyline hydrochloride (Sigma); phenylbenzoquinone (Kodak); α-phenylephrine hydrochloride (Koch Light); (+)-propranolol hydrochloride (ICI); spiperone hydrochloride (RBI); UK-14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline, Pfizer); yohimbine hydrochloride (Sigma).

Drug solutions were prepared immediately before use and for *in vivo* experiments the doses refer to the free base.

Results

Antagonism of α_2-adrenoceptor selective agonists on the rat vas deferens and guinea-pig ileum

UK-14304 and clonidine produced a concentration-dependent inhibition of the twitch response of the rat isolated field-stimulated vas deferens. Inhibition in excess of 90% was produced by UK-14304, 2 × 10^{-8} M, and clonidine, 1 × 10^{-7} M, respectively. Fluparoxan (0.25–50 × 10^{-7} M), idazoxan (0.25–10 × 10^{-7} M) and yohimbine (1–100 × 10^{-7} M) produced concentration-dependent, parallel rightward displacements of agonist concentration-inhibitory response curves with no suppression of the maximum attainable inhibitions. The results for fluparoxan are shown in Figure 2. The Schild plots (Arunlakshana & Schild, 1959) yielded straight lines with gradients not significantly different from unity except for the antagonism of clonidine by fluparoxan where the gradient was 1.29. The pK_B values and Schild plot gradients are shown in Table 1.

Fluparoxan (0.1–10 × 10^{-6} M), idazoxan (0.25–10 × 10^{-7} M) and yohimbine (0.1–10 × 10^{-6} M) were also potent and competitive antagonists of the inhibitory action of UK-14304 on the guinea-pig isolated and field-stimulated ileum.

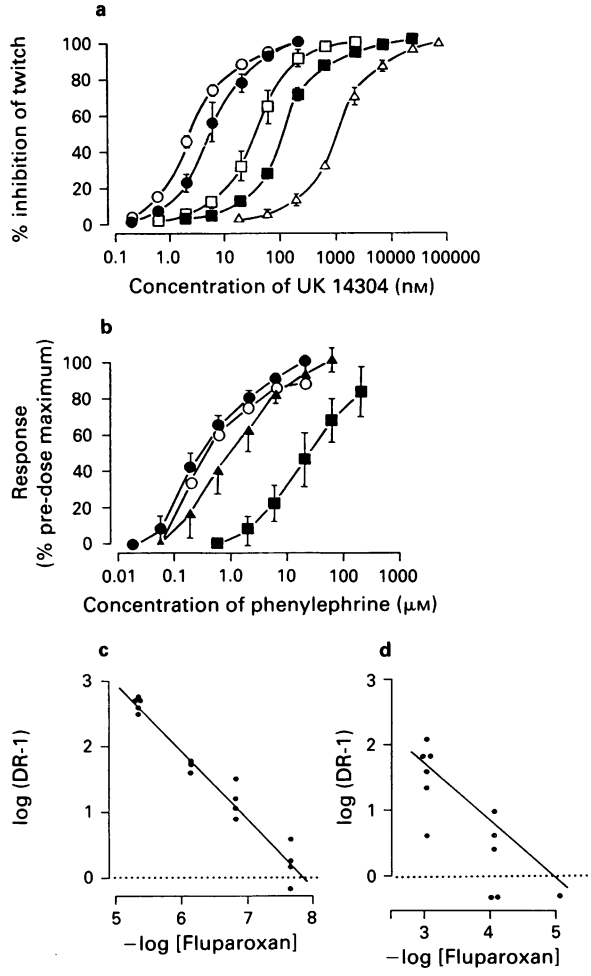

Figure 2 Antagonism by fluparoxan of UK-14304-induced inhibition of the twitch response of the rat isolated and field-stimulated vas deferens (a) and of phenylephrine-induced contractions of the rat isolated anococcygeus muscle (b). In (a) results are the mean of 4–6 experiments for UK-14304 alone (○) and UK-14304 in the presence of 25 nM (●), 160 nM (□), 800 nM (■) and 5000 nM (△) fluparoxan; vertical bars show s.e.mean. In (b) results are the mean of 1–6 experiments for phenylephrine alone (●) and phenylephrine in the presence of 10 μM (○), 100 μM (▲) and 1000 μM (■) fluparoxan; vertical bars show s.e.mean. Concentration-response curves were obtained by cumulative addition of UK-14304 or phenylephrine and a separate tissue was used for each curve. Experimental data illustrated in (a) and (b) are shown plotted according to Arunlakshana & Schild (1959) in (c) and (d) respectively. In (c) and (d) each point represents the result from a separate tissue compared with the mean control response in tissues treated with either UK-14304 or phenylephrine in the absence of fluparoxan and straight lines were fitted by linear regression analysis.

Table 1 Antagonist potencies at α_2- and α_1-adrenoceptors in rat vas deferens and anococcygeus muscle respectively

		Mean pK_B value ± s.e. (Schild plot slope)		
Agonist	*Tissue*	*Fluparoxan*	*Idazoxan*	*Yohimbine*
UK-14304	Vas deferens	7.87 ± 0.05 (1.02)	7.73 ± 0.06 (1.02)	7.59 ± 0.04 (1.08)
Clonidine	Vas deferens	7.91 ± 0.07 (1.23)*	8.12 ± 0.06 (0.98)	7.48 ± 0.07 (1.10)
Phenylephrine	Anococcygeus muscle	4.45 ± 0.15 (1.08)	6.27 ± 0.13 (0.90)	6.25 ± 0.08 (0.91)
Methoxamine	Anococcygeus muscle	4.88 ± 0.04 (1.16)	NT	NT

* Slope significantly different from unity (linear regression analysis).
pK_B = (dose-ratio − 1) − log [antagonist] where dose-ratio is the ratio of the agonist concentration producing half maximal responses in the presence and absence of the antagonists at the molar concentration [antagonist].

The effect of increasing concentrations of fluparoxan on the inhibitory potency of UK-14304 and the resulting Schild plots are shown in Figure 3. For fluparoxan, idazoxan and yohimbine the mean pK_B values ± standard errors obtained were 7.89 ± 0.07 (Schild slope 1.04), 8.13 ± 0.04 (Schild slope 0.92) and 7.64 ± 0.06 (Schild slope 0.98) respectively. In a separate study of the antagonism of UK-14304 in the rat vas deferens, fluparoxan was compared with its two enantiomers. There were no differences in potencies. Thus for the racemate (+)-fluparoxan and (−)-fluparoxan the respective mean pK_B values (±standard errors and Schild plot gradients) were 7.86 ± 0.07 (slope = 1.09), 7.88 ± 0.08 (slope = 0.99) and 7.68 ± 0.06 (slope = 1.04).

Antagonism of contractions produced by α_1-adrenoceptor selective agonists on the rat anococcygeus muscle

Fluparoxan ($0.1–10 \times 10^{-5}$ M), idazoxan ($0.01–10 \times 10^{-5}$ M) and yohimbine ($0.1–10 \times 10^{-5}$ M) were also competitive antagonists of phenylephrine-induced contractions of the rat isolated anococcygeus muscle. The effects of increasing concentrations of fluparoxan are shown in Figure 2. All three compounds were weaker antagonists of α_1-adrenoceptors than of α_2-adrenoceptors. Fluparoxan had at least 50 times lower affinity than that of the other two antagonists. The affinity constants and Schild slopes obtained are shown in Table 1

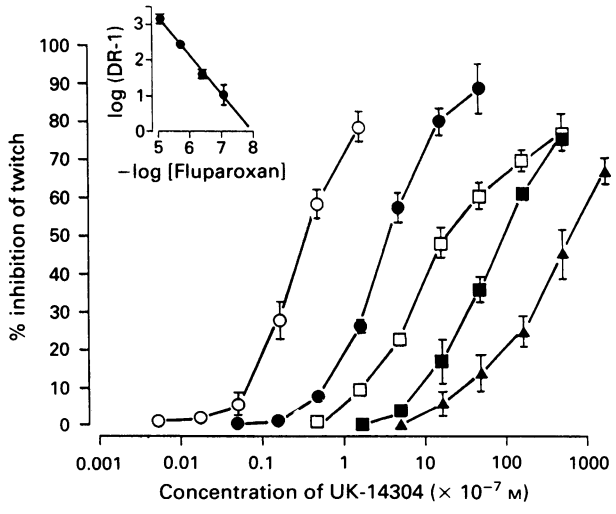

Figure 3 Antagonism by fluparoxan of UK-14304-induced inhibition of the twitch response of the guinea-pig isolated and field stimulated ileum. The results are means of 4–5 experiments for UK-14304 alone (○), and UK-14304 in the presence of 0.1 μM (●), 0.5 μM (□), 2.5 μM (■) and 10 μM (▲) fluparoxan; vertical bars show s.e.mean. The inset shows the data plotted according to Arunlakshana & Schild (1959). Values are mean responses obtained from doses of UK-14304 producing 50% inhibition of the twitch (IC_{50}) in individual tissues compared with the mean IC_{50} value in control tissues; vertical bars show s.e.mean.

and the Schild plot for the antagonism of phenylephrine by fluparoxan in Figure 2. On this tissue preparation there were also no differences between the affinities of the enantiomers of fluparoxan and the racemate against phenylephrine concentration-response curves (mean pK_B values ± s.e. = (±)-fluparoxan: 5.00 ± 0.26, (+)-fluparoxan: 4.57 ± 0.14, (−)-fluparoxan: 4.95 ± 0.16).

Antagonism of phenylephrine-induced contraction of the rabbit aortic strip

Fluparoxan behaved as a competitive antagonist of phenylephrine-induced contractions of the aortic strip producing a mean (±s.e.) pK_B value of 4.32 ± 0.06 (slope = 1.01). In separate studies with idazoxan, concentrations in excess of 1×10^{-5} M occasionally produced a weak contraction of the tissue (up to 10% of the phenylephrine maximum). Idazoxan also behaved as a competitive antagonist of phenylephrine in this tissue (mean pK_B = 5.79 ± 0.08; slope = 0.83).

Antagonism of clonidine-induced hypothermia and antinociception in the mouse

In the conscious mouse, fluparoxan and idazoxan were of similar potency in preventing the hypothermia produced by clonidine 0.15 mg kg^{-1} i.p. Clonidine caused a fall in oesophageal temperature of 1.77 ± 0.33°C (mean ± s.e.; n = 10). This was prevented dose-dependently by oral fluparoxan, 0.2–3.0 mg kg^{-1} (15%–93%).

In a second study, clonidine-induced hypothermia was 2.5 ± 0.2°C (mean ± s.e.; n = 16) which was prevented dose-dependently by oral idazoxan 0.13–3.0 mg kg^{-1} (16%–76%). The oral doses of fluparoxan and idazoxan required to reduce by half the clonidine induced hypothermia were 0.68 ± 0.10 and 0.84 ± 0.09 mg kg^{-1} respectively.

In another study, fluparoxan and its two enantiomers were of similar potency in producing dose-related inhibitions of the hypothermia caused by clonidine. The oral ED_{50} values for the racemate, (+)-fluparoxan and (−)-fluparoxan for inhibition of hypothermia were 0.66 ± 0.10, 0.73 ± 0.13 and 0.46 ± 0.06 mg kg^{-1} respectively.

In the antinociception study, an intraperitoneal injection of phenylbenzoquinone caused a characteristic abdominal constriction response that was abolished by pretreatment with clonidine 0.05 mg kg^{-1} s.c. Oral doses of fluparoxan, 0.38–3 mg kg^{-1}, idazoxan, 0.25–2.0 mg kg^{-1} and yohimbine, 0.63–5 mg kg^{-1}, prevented this antinociceptive action of clonidine dose-dependently and with similar potencies (Figure 4). The ED_{50} values were 0.75 (0.54–1.07), 0.65 (0.4–1.03) and 1.10 (0.28–1.94) mg kg^{-1} orally respectively.

Antagonism of UK-14304 induced hypothermia and impaired rotarod performance in the rat

In the rat UK-14304, 2 mg kg^{-1} s.c., produced hypothermia of 4–5°C and a marked impairment of rotarod performance.

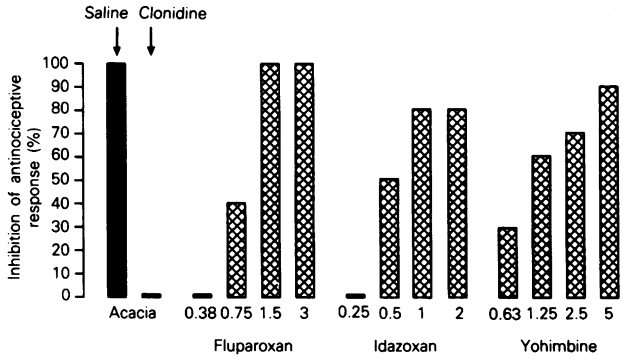

Figure 4 Antagonism of clonidine-induced antinociception (phenylbenzoquinone-induced abdominal constriction) in the mouse. All animals, except the saline controls, received clonidine, 0.05 mg kg^{-1} s.c., and either vehicle, fluparoxan, idazoxan or yohimbine orally at one of the stated doses. The proportion of animals exhibiting at least one abdominal constriction response over the following 10 min is indicated by the shaded histograms.

Both effects of the α_2-adrenoceptor agonist were prevented dose-dependently by fluparoxan and idazoxan (Figure 5). Fluparoxan was of similar potency by either route of administration unlike idazoxan which, although similar in potency to fluparoxan following intravenous dosing, was much weaker when given orally.

Antagonism of UK-14304-induced sedation and bradycardia in the dog

UK-14304 (0.05 mg kg^{-1} s.c.) caused marked sedation and bradycardia in beagles that was apparent after 30 min, peaked at 2–3 h and persisted for more than 5 h.

Fluparoxan, 0.22–6 mg kg^{-1} orally, prevented both effects of UK-14304 in a dose-related fashion and the antagonism

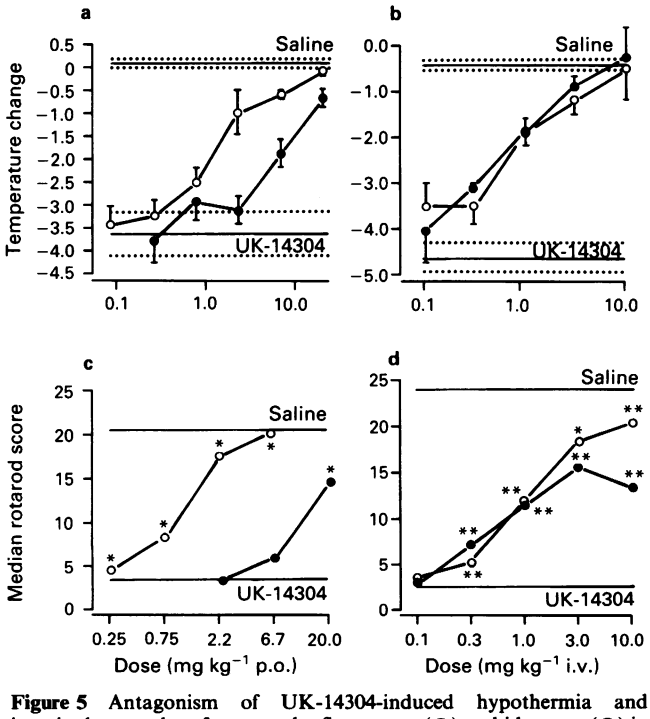

Figure 5 Antagonism of UK-14304-induced hypothermia and impaired rotarod performance by fluparoxan (○) and idazoxan (●) in the rat. Values show mean temperature changes (a, b) or median rotarod scores (c, d) of treated rats compared with vehicle/saline or vehicle/UK-14304 treated (horizontal lines) controls using the oral (a, c) or intravenous (b, d) routes of administration; vertical bars show s.e.mean. Antagonism of rotarod impairment was significant where indicated (*$P < 0.05$, **$P < 0.01$ by Mann-Whitney U-test).

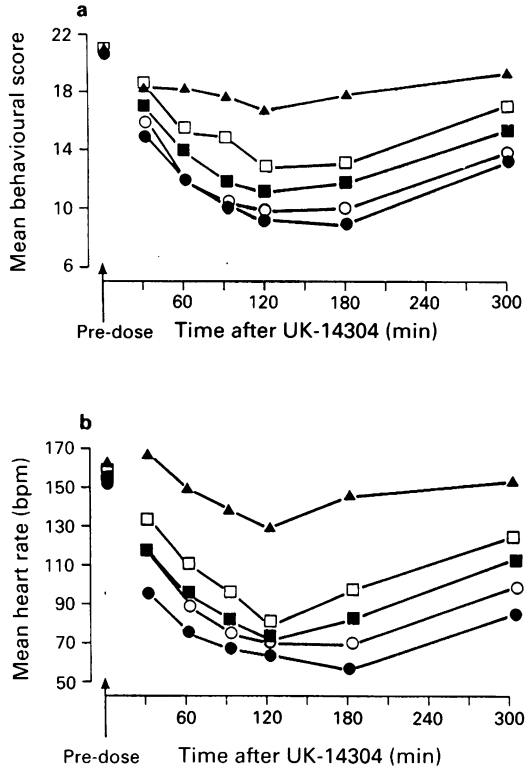

Figure 6 Antagonism of UK-14304-induced sedation (a) and bradycardia (b) in the dog. Values are means ($n = 10$) at the indicated times for dogs given UK-14304 (0.05 mg kg^{-1} s.c.) 30 mins after placebo (●), or fluparoxan 0.22 (○), 0.67 (■), 2 (□), or 6 (▲) mg kg^{-1} orally. Behaviour was scored subjectively, a reduced score representing sedation. Heart rate was obtained by palpating the femoral artery. Predose values were obtained before giving either treatment.

was maintained over the 5 h testing period (Figure 6). Analysis of the relationship between peak effect and dose of fluparoxan showed no differences in the ED_{50} values against UK-14304-induced sedation and bradycardia (ED_{50} values of 2.5 ± 0.1 mg kg^{-1} and 1.9 ± 0.6 mg kg^{-1} orally, respectively).

Actions of fluparoxan at non-α-adrenoceptor sites

Fluparoxan, 1–10 × 10^{-5} M, was either inactive or weakly active at most non-α-adrenoceptor sites investigated (Table 2). At functional muscarinic sites in rat ileum there was no activity at 1 × 10^{-5} M and only weak affinity for muscarinic M_1 binding sites in rat cerebral cortex and M_2 sites in rat heart. No antihistamine activity was detected at either H_1- or H_2-histamine receptors at concentrations up to 3 × 10^{-5} M in rat atria. There was a five fold displacement of the isoprenaline dose-response curve preincubated with 3 × 10^{-5} M fluparoxan indicating weak β_1-adrenoceptor antagonism but no activity at β_2-adrenoceptors in guinea-pig ileum at 3 × 10^{-5} M. Fluparoxan was found to have some affinity for some 5-HT sites. There was weak affinity for 5-HT_{1A} ($pIC_{50} = 5.9$) and 5-HT_{1B} ($pK_i = 5.5$) binding sites in rat brain but no agonist or antagonist activity at functional 5-HT_1-like and 5-HT_2 receptors in dog saphenous vein and rabbit aorta respectively.

At dopamine receptors fluparoxan was more than 200 times weaker than haloperidol in preventing [^{3}H]-spiperone binding to rat striatal membranes. At D_2-receptors in the anaesthetized cat, fluparoxan, 0.1, 1.0 and 10.0 mg kg^{-1} i.v. caused 1.2, 1.3 and 1.4 fold displacements respectively of dopamine-induced falls of electrically-stimulated increases in heart rate. Fluparoxan and desmethylimipramine inhibited the accumulation of [^{3}H]-noradrenaline by rat occipital cortex synaptosomes in a concentration-dependent manner

Table 2 Activity of fluparoxan at receptors other than α-adrenoceptors

Receptor type	*Tissue* (response)	*Competing drug*	*Fluparoxan* (max. conc. tested, μM)	*Comments*
Muscarinic M_3	Rat ileum (contraction)	Muscarine	10	Inactive
Muscarinic M_2	Rat heart (binding)	[^{3}H]-N-methylscopolamine	100	$pIC_{50} = 4.4$
Muscarinic M_1	Rat cerebral cortex (binding)	[^{3}H]-pirenzepine	100	$pK_i = 4.0$
5-HT_1-like	Dog saphenous vein (contraction)	5-HT	25	Inactive
5-HT_{1A}	Rat hippocampus (binding)	[^{3}H]-8-OH-DPAT	100	$pIC_{50} = 5.9$
5-HT_{1B}	Rat striatum (binding)	[^{3}H]-cyanopindolol	100	$pK_i = 5.5$
5-HT_2	Rabbit aorta (contraction)	5-HT	50	Inactive
Histamine H_1	Guinea-pig ileum (contraction)	Histamine	30	Inactive
Histamine H_2	Guinea-pig atria (rate)	Histamine	30	Inactive
β_1-Adrenoceptor	Rat atria (force of contraction)	Isoprenaline	30	$pK_B \simeq 5$
β_2-Adrenoceptor	Guinea-pig trachea (relaxation)	Isoprenaline	30	Inactive
Dopamine D_2	Rat striatum (binding)	[^{3}H]-Spiperone	1	13% displacement of binding

In functional models fluparoxan was tested for both agonist and antagonist activity. In binding assays pIC_{50} represents the negative logarithm of the molar concentration of fluparoxan preventing by 50% the specific binding of the competing drug. In some studies a pK_i value was obtained by the method of Cheng & Prusoff (1973). pK_B is as defined in Table 1.

giving IC_{50} values of 8.8×10^{-5} M and 5×10^{-9} M respectively.

Effects of rat cerebrocortical [^{3}H]-dihydroalprenolol binding sites

Both fluparoxan and amitriptyline were very weak displacers of [^{3}H]-dihydroalprenolol binding to cerebrocortical membranes from untreated rats with pK_i values (Hill slopes) of 4.05 (0.71) and 3.64 (1.05) respectively. For isoprenaline, the pK_i was 6.38 and the Hill slope was 0.69. The effect of twice daily, oral dosing with fluparoxan 6 and 12 mg kg^{-1}, amitriptyline 30 mg kg^{-1} or a combination of both drugs for 6 days on the density of binding sites is shown in Table 3. The number of binding sites (B_{max}) was reduced by fluparoxan (9% and 21%) and amitriptyline (31%) without affecting their affinity (K_D) for the radioligand. The greatest reduction (40%) was produced in rats treated with the combination of fluparoxan, 6 mg kg^{-1}, and amitriptyline 30 mg kg^{-1}.

Discussion

Fluparoxan behaves as a novel, competitive antagonist having very high selectivity for α_2-adrenoceptors. The pK_B values against the α_2-adrenoceptor responses induced by the selective agonists UK-14304 and clonidine in rat vas deferens and guinea-pig ileum (Brown *et al.*, 1979; Drew, 1978) ranged from 7.86–7.91. Agonist concentration-response curves were displaced in a parallel fashion and the data gave straight line Schild plots which, with one exception, had slopes which were not significantly different from unity indicating competitive antagonism. The Schild-plot slope for the antagonism of clonidine by fluparoxan in the rat vas deferens was 1.23. The reason for the high value is not clear and requires further investigation. However, the pK_B values obtained for idazoxan and yohimbine were in agreement with generally quoted literature values. Fluparoxan was a much weaker competitive antagonist of the α_1-adrenoceptor selective agonists phenylephrine and methoxamine in rat anococcygeus muscle and rabbit aorta preparations. The pK_B values obtained in rat vas deferens and anococcygeus muscle showed fluparoxan to have at least 1000 fold α_2-: α_1-adrenoceptor selectivity making it one of the most selective α_2-adrenoceptor antagonists so far described.

Many α-adrenoceptor antagonists with an imidazoline moiety display partial agonist activity (Ruffolo, 1983). For example, idazoxan displays partial α_1-adrenoceptor agonism in some tissues including rabbit isolated aorta (Dalrymple *et al.*, 1983). In the present study, idazoxan, at high concentrations, sometimes caused a weak contraction of the rabbit aorta preparation. In contrast, fluparoxan, which does not have an imidazoline group, did not show any α-adrenoceptor agonist activity in this or any other preparation examined.

In studies to measure the α_2-adrenoceptor antagonism produced *in vivo*, fluparoxan was effective by the oral route and of similar potency to idazoxan in preventing clonidine-induced hypothermia and antinociception in the mouse. Similarly, in the rat, fluparoxan prevented the hypothermia and impaired rotarod performance caused by UK-14304. Fluparoxan showed similar potency by both the oral and intravenous routes of administration indicating good oral bioavailability.

To interpret these antagonist effects it is important to consider the nature of the agonist-induced response. The hypothermia produced by clonidine, although in part a consequence of stimulation of α_2-adrenoceptors in the brain, could have a histamine H_2-receptor mediated component (Bugajski *et al.*, 1980). While amphetamine-like drugs and β-adrenoceptor agonists also prevent clonidine-induced hypothermia (unpublished data) fluparoxan does not have histamine H_2-receptor antagonist, β-adrenoceptor agonist or amphetamine-like activity.

The prevention of clonidine- and UK-14304-induced hypothermia is therefore most probably attributable to blockade of

Table 3 Effect of twice daily dosing of fluparoxan and amitriptyline for 6 days on rat cerebrocortical [^{3}H]-dihydroalprenolol binding sites

Compound	*Dose* (mg kg^{-1} twice daily)	B_{max} (fmol mg^{-1} protein)	% *change*	K_D (nM)	n
Water		149 ± 8		0.49 ± 0.02	7
Fluparoxan	6	136 ± 6	−9	0.53 ± 0.07	7
Fluparoxan	12	111 ± 10*	−26	0.48 ± 0.05	7
Amitriptyline	30	103 ± 7*	−31	0.56 ± 0.12	8
Amitriptyline + fluparoxan	30 6	89 ± 7*	−40	0.42 ± 0.04	8

* Significant decrease in B_{max} compared to placebo-treated group ($P < 0.05$, unpaired t test).

central α_2-adrenoceptors. The antinociceptive activity of clonidine also appears to be α_2-adrenoceptor-mediated (Paalzow & Paalzow, 1976; Skingle *et al.*, 1982), but could have been produced both at peripheral and at central sites (Bentley *et al.*, 1977; Spaulding *et al.*, 1979; Reddy *et al.*, 1980). Impairment of rotarod performance appears to reflect the sedative effects of α_2-adrenoceptor agonists and is believed to be a centrally-mediated action (Drew *et al.*, 1979). In the dog, fluparoxan prevented both the sedative and cardiovascular responses to UK14304 with a sustained duration of action. The *in vivo* findings with fluparoxan are therefore indicative of potent and effective antagonism of α_2-adrenoceptors at sites in the CNS. Further studies would be needed to show whether the compound displays differential activity at pre- and postsynaptic α_2-adrenoceptors in the CNS, as could be shown by comparing potencies against clonidine induced mydriasis and hypoactivity (Heal *et al.*, 1989), or for α_{2A} and α_{2B} subtype binding sites (Bylund, 1988).

Fluparoxan is a racemate. Dabire *et al.* (1983) reported significant differences in the selectivity and potency of the enantiomers of idazoxan. In contrast, there were no significant differences between the enantiomers of fluparoxan in terms of α_2- and α_1-adrenoceptor antagonism in rat tissues *in vitro* and for their oral potency in preventing clonidine-induced hypothermia in the mouse. This was the anticipated result because fluparoxan is essentially a planar molecule and the two enantiomers are virtually superimposable. This contrasts with the enantiomers of idazoxan where the imidazoline side chain lies outside of the plane of the benzodioxan moiety.

In specificity studies, fluparoxan showed no appreciable affinity or activity at histamine H_1- or H_2-receptors, muscarinic acetylcholine receptors, β_2-adrenoceptors, dopamine D_1 or D_2 receptors or functional 5-HT_1-like receptors in the dog saphenous vein. The antagonist action of fluparoxan at β_1-adrenoceptors was about 1000 times less than that at α_2-adrenoceptors. Fluparoxan displaced [^{3}H]-8-OH-DPAT and [^{3}H]-cyanopindolol binding to 5-HT_{1A} and 5-HT_{1B} sites respectively, but only at concentrations between 100 and 1000 times its affinity for α_2-adrenoceptors.

Twice daily administration of fluparoxan to rats for six days resulted in a significant reduction in the number of cerebrocortical [^{3}H]-dihydroalprenolol binding sites with no reduction in affinity. Other α_2-adrenoceptor antagonists have been shown to produce this change (Swann *et al.*, 1981; Brown *et al.*, 1990) which could reflect an adaptation to a chronically increased synaptic concentration of noradrenaline since α_2-adrenoceptor antagonists are known to increase noradrenaline turnover (Anden *et al.*, 1982; Walter *et al.*, 1984; Scheinin & Virtanen, 1986). Uptake inhibitors and monoamine oxidase inhibitors also bring about this adaptive change (Sellinger-Barnette *et al.*, 1980).

Inhibition of uptake is unlikely to explain why fluparoxan decreases [^{3}H]-dihydroalprenolol binding site density because it has only very weak affinity for the uptake site. Furthermore, in separate unreported studies we have found that, unlike known monoamine oxidase inhibitors, fluparoxan does not prevent the depressant effects of tetrabenazine or the pressor and mydriatic effects of orally administered tyramine in rodents. The greatest reduction in the density of [^{3}H]-dihydroalprenolol binding sites occurred in rats given both fluparoxan and amitriptyline. This has been found with other α_2-adrenoceptor antagonists when coadministered with uptake inhibitors (Johnson *et al.*, 1980) and is consistent with a synergistic effect on noradrenaline turnover (Halliday & Ford, 1983).

In conclusion, fluparoxan is a novel, competitive α_2-adrenoceptor antagonist in isolated tissues with a very high α_2- to α_1-adrenoceptor selectivity ratio. The results of *in vivo* studies indicate good bioavailability and a potent action mediated at α_2-adrenoceptors in the CNS. The most likely therapeutic use for fluparoxan is in the treatment of depression, and in common with known antidepressant drugs fluparoxan reduces the density of rat cerebrocortical [^{3}H]-dihydroalprenolol binding sites (Banerjee *et al.*, 1977).

We should like to acknowledge colleagues in Medicinal Chemistry Department, Glaxo Group Research Ltd., Greenford, especially Dr A. Borthwick, Dr P.C. Cherry and Dr A.J. Crame, for the synthesis of fluparoxan and its enantiomers and the synthesis of idazoxan. (+)-Propranolol hydrochloride and UK14304 were generous gifts from ICI and Pfizer respectively. We are also grateful to Sharon McBain and Glenn Mason for excellent technical assistance.

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(Received September 7, 1990
Revised November 26, 1990
Accepted December 4, 1990)

Br. J. Pharmacol. (1991), **102**, 896–903

The release of a non-prostanoid inhibitory factor from rabbit bronchus detected by co-axial bioassay

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1 Methacholine relaxed phenylephrine-contracted aorta of the rat with the endothelium intact. This effect was inhibited by haemoglobin, methylene blue, gossypol, phenidone and L-N^G-nitroarginine methyl ester (L-NAME). Rat aorta denuded of endothelium failed to relax in response to methacholine, histamine and the peptidoleukotrienes C_4, D_4 and E_4.

2 Methacholine and histamine but not leukotrienes C_4, D_4 and E_4 relaxed phenylephrine-contracted rat aorta without endothelium when surrounded by rabbit epithelium-intact bronchus. The muscarinic antagonist atropine antagonized the methacholine-induced relaxation.

3 Removal of the epithelium either mechanically or chemically, abolished methacholine-induced relaxation of rat aorta in the co-axial bioassay. These data indicate that the epithelium is responsible for the observed relaxant effect to methacholine and histamine.

4 The cyclo-oxygenase inhibitor, indomethacin, the phospholipase A_2 inhibitor, mepacrine and the lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), failed to inhibit methacholine-induced relaxation of rat aorta in the co-axial bioassay. This indicates that the epithelium-derived inhibitory factor (EpDIF) is not a product of the cyclo-oxygenase or lipoxygenase pathway or a product derived from activation of phospholipase A_2.

5 Haemoglobin, methylene blue, phenidone, gossypol and L-NAME failed to inhibit the relaxation of rat aorta in the co-axial bioassay. These results demonstrate that EpDIF detected in the co-axial bioassay is not endothelium-derived relaxing factor (EDRF) or nitric oxide. Similarly, catalase was without effect.

6 EpDIF is unlikely to be a peptide since papain and α-chymotrypsin failed to alter the methacholine-induced relaxation of rat aorta in the co-axial bioassay. Furthermore, thiorphan, captopril and aprotinin were also without effect, suggesting that EpDIF is not a substrate for airway peptidases.

7 The results presented in this paper demonstrate the release of a vasoactive epithelium-derived inhibitory factor (EpDIF) from rabbit intrapulmonary bronchi by use of a co-axial bioassay preparation.

Keywords: EpDIF; rabbit bronchus; epithelium; co-axial bioassay

Introduction

The epithelium not only acts as a physical barrier to many large macromolecules (Hogg, 1981) but is also important for mucociliary clearance (Sleigh *et al.*, 1988) and ion transport (Hogg & Eggleston, 1984). The epithelium may also act as a metabolic sink for various agents including adenosine (Holyrode, 1986; Advenier *et al.*, 1988) and neuropeptides (Djokic *et al.*, 1989; Fine *et al.*, 1989; Frossard *et al.*, 1989; Naline *et al.*, 1989). Furthermore, the epithelium has the capability of producing a variety of arachidonic acid-derived metabolites including prostaglandin E_2, leukotriene B_4, 5-HETE's and 15-diHETE's (Orehek *et al.*, 1975; Holtzman *et al.*, 1983; Eling *et al.*, 1985; Hunter *et al.*, 1985; Brunelleschi *et al.*, 1987; Butler *et al.*, 1987; Barnett *et al.*, 1988; Jacoby *et al.*, 1988) in response to a variety of stimuli including exogenous arachidonic acid, the calcium ionophore A23187, bradykinin, platelet activating factor (PAF) and eosinophil-derived major basic protein (MBP). More recently, the production of a non-prostanoid inhibitory factor by the epithelium has also been demonstrated (Ilhan & Sahin, 1986; Hay *et al.*, 1987a; Guc *et al.*, 1988a,b; Fernandes *et al.*, 1989).

It has been proposed that the non-specific bronchial hyperresponsiveness found in asthma may be a consequence of damage to the airway epithelium (Hogg & Eggleston, 1984). Indeed, viral infection and exposure to a variety of chemical agents which may trigger asthma are thought to induce epithelial cell damage. Furthermore, epithelial cell damage has been observed in bronchial biopsies taken from asthmatics (Laitinen *et al.*, 1985; Jeffery *et al.*, 1989) and both the number of epithelial cells collected from bronchoalveolar lavage and the extent of epithelial cell damage found in bronchial biopsies histologically, is inversely correlated with airways responsiveness to spasmogens (Wardlaw *et al.*, 1988; Beasley *et al.*, 1989; Jeffery *et al.*, 1989). In contrast, no damage to the airway epithelium has been reported in mild asthmatics with underlying bronchial hyperresponsiveness (Lozewicz *et al.*, 1990). It is conceivable that damage to the airway epithelium may compromise many of the epithelial cell functions listed above. One aspect of epithelial cell function that has been of particular interest is the capability of the epithelium to modulate bronchoconstrictor responses by the release of an epithelium derived relaxing/inhibitory factor (EpDIF; Vanhoutte, 1988; Goldie *et al.*, 1990). Many studies have shown that mechanical removal of airway epithelium may increase the sensitivity of airway smooth muscle preparations to various spasmogens *in vitro* (for review, see Fedan *et al.*, 1988), providing indirect evidence for an epithelium-derived relaxing factor.

Recently, a novel assay preparation has been used to demonstrate the release of an EpDIF and in the present study we have used a similar bioassay to investigate the release and characteristic of a vasoactive EpDIF from the epithelium of rabbit intrapulmonary bronchi.

Methods

Tissue preparation

Male Wistar rats (200–300 g) were stunned by a blow to the head and killed by cervical dislocation. The abdominal aorta was removed, cleared of connective tissue and placed in oxygenated ice-cold Krebs-Henseleit solution. The endothelium was removed by gentle rubbing of the mucosal surface with a

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cotton wool probe and the aorta was cut into zig-zag strips. In some experiments, care was taken to avoid damage to the endothelium. Endothelium-intact or denuded preparations were suspended under a load of 500 mg in an organ bath containing Krebs-Henseleit solution aerated with 95% O_2 and 5% CO_2 at 37°C and equilibrated for 45 min with changes in the bath fluid every 15 min. Any loss in tone during this period was compensated for by reapplying tension to 500 mg. Changes in the isometric tension were measured with a Grass force-displacement transducer (FTO3C) and recorded on a 4 channel Lectromed.

Pharmacological testing of aortic preparations

Rat aortic preparations, with or without endothelium, were contracted with (−)-phenylephrine (0.05 μM). Once the contractile response had reached a plateau, either methacholine (100 μM), histamine (100 μM), leukotriene C_4 (100 nM), leukotriene D_4 (100 nM) or leukotriene E_4 (100 nM) was added to the bath to confirm the absence or presence of a functionally intact endothelium. Prior to the addition of leukotriene C_4 and leukotriene D_4 to the organ bath, the aortic preparations were incubated for 30 min with L-serine borate (45 mM; Charette & Jones, 1987) and L-cysteine (3 mM; Hay *et al.*, 1987b) respectively. To demonstrate that endothelium-denuded preparations retained the ability to relax, (±)-isoprenaline (1 μM) was added to phenylephrine-contracted aorta. All preparations were then washed 3 times in a 10 min period by gravity drainage of the bathing medium. This procedure was repeated in indomethacin pretreated preparations (5 μM; 30 min) in the absence or presence of the guanylate cyclase inhibitor methylene blue (10 μM, 30 min), the EDRF blocking agent haemoglobin (10 μM, 10 min) or phenidone (10 μM, 30 min), and the EDRF synthesis inhibitors, gossypol (30 μM, 20 min) or L-NAME (100 μM, 15 min).

Co-axial bioassay

White New Zealand rabbits (1.8–3 kg) of either sex were anaesthetized with diazepam (5 mg ml^{-1}; 2.5 mg kg^{-1}) and fentanyl citrate (0.315 mg ml^{-1}; 0.4 ml kg^{-1}) and killed by exsanguination while under anaesthesia. The lungs were quickly removed and placed in oxygenated ice-cold Krebs-Henseleit solution. Intrapulmonary bronchi were removed from the lung and cleared of alveolar parenchymal tissue and visible blood vessels and cut into tubes (approximately 7 mm in length and 0.5–3 mm, i.d.).

After it had been established that the endothelium had been removed from rat aorta, the bronchial preparations were placed above the aortic preparations which had been resuspended under 500 mg tension. At least 20 min was allowed to elapse before the aorta was positioned within the bronchus and a further 10 min was allowed to elapse before addition of drugs.

(−)-Phenylephrine (10 μM) was used to contract the rat aorta which was surrounded by airway tissue. Cumulative concentrations of either methacholine (0.1–100 μM), histamine (0.1–300 μM), or leukotriene C_4, D_4 or E_4 (0.1–100 nM) were superimposed on the phenylephrine-contracted rat aorta.

In some experiments, the co-axial assembly was incubated for 45 min with the muscarinic receptor antagonist, atropine (0.03–0.1 μM), in the presence of indomethacin (5 μM). Dose-ratios were calculated and Schild regression was used to calculate the pA_2 and slope value for atropine according to the method of Arunlakshana & Schild (1950).

In other experiments, the effects of various inhibitors on methacholine-induced relaxation were performed. Cumulative concentrations of methacholine were added to indomethacin (5 μM) pretreated co-axial bioassay preparations in the absence or presence of the phospholipase A_2 inhibitor, mepacrine (10 μM) or the lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA, 10 μM). Furthermore, the effects of methylene blue, L-NAME (concentrations given above) and catalase (100 u ml^{-1}, 20 min) on methacholine-induced relaxations were also assessed. The effects of haemoglobin, gossypol and phenidone (concentrations given above) were investigated by the addition of these inhibitors to the organ bath following completion of the methacholine concentration-effect curve.

In another series of experiments, cumulative concentration-effect curves to methacholine were constructed in indomethacin (5 μM) pretreated co-axial bioassay preparations in the absence or presence of various peptidases including α-chymotrypsin (2 units ml^{-1}; 10 min), papain (2 units ml^{-1}; 10 min), the peptidase inhibitor aprotinin (1.75 units ml^{-1}; 30 min), the neutral endopeptidase inhibitor, thiorphan (10 μM; 30 min) or the angiotensin converting enzyme inhibitor, captopril (10 μM; 30 min).

In other experiments, after the addition of cumulative concentrations of methacholine to phenylephrine-contracted aorta, the co-axial bioassay assemblies were washed for 15 min and then the bronchial tubes were removed and the bronchial epithelium removed by either insertion of a cotton wool probe or by infusion with 3-[(3-cholamidopropyl)dimethylammonio] 1-propanesulphonate (CHAPS) for 4 min (20 mg ml^{-1}; 2.25 ml min^{-1}). The bronchial tubes were then placed above the aortic preparations which had been resuspended under 500 mg tension and allowed to equilibrate as described previously. Cumulative concentration-effect curves to methacholine were then superimposed on phenylephrine-contracted rat aorta in the co-axial bioassay. To confirm that either procedure did not damage bronchial smooth muscle, rings from control and epithelium-denuded bronchial tubes were suspended under 1 g tension for 45 min, followed by addition of cumulative concentrations of methacholine. Contractile potency pD_2, and maximum tension generated for methacholine (E_{max}) in epithelium-intact and epithelium-denuded preparations were compared. Confirmation of the removal of the epithelium was obtained from histological examination of 8 μm paraffin sections of rabbit bronchus stained with haemotoxylin-eosin.

Uptake studies

It was noted that greater concentrations of phenylephrine were required to contract endothelium-denuded rat aorta in a co-axial bioassay. Therefore, we investigated the possibility that this was a consequence of tissue uptake of phenylephrine.

Following the construction of a concentration-effect curve to phenylephrine in endothelium-denuded rat aortic preparations, rabbit bronchial tubes were cut longitudinally and epithelium-intact or denuded preparations were suspended alongside the rat aorta (approx. 2 mm separation). Concentration-effect curves to phenylephrine were then repeated in rat aortic preparations. This was again repeated but in the presence of the uptake$_1$ inhibitor, cocaine (5 μM; 30 min) and the uptake$_2$ inhibitor, corticosterone (100 μM; 30 min) or with oxytetracycline (100 μM; 30 min). Indomethacin (5 μM) was present throughout these experiments.

Preparation of haemoglobin

The reduced form of haemoglobin was prepared according to the method of Martin *et al.* (1985). Briefly, oxidized haemoglobin (approximately 1 mM) was incubated for 15 min with 1 M sodium dithiosulphite ($Na_2S_2O_4$) then dialysed for 2 h at 4°C. The presence of reduced haemoglobin was confirmed spectrophotometrically (extinction coefficient 576 nm = 15990 M^{-1} cm^{-1}).

Statistical analysis

All results expressed as mean ± s.e.mean. The relaxant or contractile potency of a drug is expressed as $pD_2 = -\log_{10} EC_{50}$. Some of the data was expressed as the derived EC_{50} (antilog pD_2) together with 95% confidence limits. Differences between means in the uptake studies and in the co-axial bio-

assay studies with the different inhibitors used, were analysed by Student's t test for paired data, while data represented in Table 1 were analysed by Student's t test for non-paired data. In both cases, n was taken as the number of preparations tested.

Differences in relaxation responses expressed as % values were analysed by the Mann-Whitney U test, since the variances of the two groups were significantly different as assessed by an F test. All tests were considered significant at the 0.05 level.

In some situations, mean and s.e.mean were calculated using the number of animals (N), which was accompanied by the number of preparations used.

Drugs

Drugs used in this study were obtained from the following sources: aprotinin, atropine sulphate, catalase, CHAPS, α-chymotrypsin (Type 1S), cocaine hydrochloride, corticosterone hydrochloride, gossypol, haemoglobin (rabbit), histamine hydrochloride, indomethacin, isoprenaline hydrochloride, mepacrine, methacholine hydrochloride, methylene blue, L-nitroarginine methyl ester (L-NAME), nordihydroguaiaretic acid (NDGA), oxytetracycline hydrochloride, papain, phenidone, phenylephrine hydrochloride, sodium nitroprusside (Sigma Chem. Co.); leukotriene C_4, D_4, E_4 (a kind gift by Dr D.W.P. Hay; Smith, Kline and Beecham Inc, King of Prussia, PA, U.S.A.); captopril (Squibb & Sons); thiorphan (Penninsula Lab. Europe). Krebs-Henseleit solution consisted of (mM): NaCl 117.6, KCl 5.4, $NaHCO_3$ 25, KH_2PO_4 1.03, $MgSO_4$ 0.57, D-glucose 11.1 and $CaCl_2$ 2.5. The majority of drugs were dissolved in Krebs-Henseleit solution. NDGA and corticosterone were dissolved in 100% ethanol. Isoprenaline was dissolved in 0.9% NaCl containing ascorbic acid. (20 $\mu g\,ml^{-1}$). Indomethacin was dissolved in 0.5% Na_2CO_3. Phenidone was dissolved in 0.9% saline containing 0.1 M HCl. Stock solutions (1 mM) leukotriene C_4 (LTC_4), and LTD_4 were dissolved in methanol and LTE_4 (1 mM) dissolved in ethanol and stored at −20°C. Absolute concentrations of the leukotrienes were measured spectrophotometrically (LTC_4, LTD_4: extinction coefficient 280 nm = 40,000 $M^{-1}\,cm^{-1}$; LTE_4 extinction coefficient 280 nm = 35,200 $M^{-1}\,cm^{-1}$). L-Serine borate was prepared by dissolving equimolar concentrations (2 M) of L-serine and boric acid to distilled water and buffered at pH 7.4 with NaOH (10 M).

Results

Endothelium-intact aorta

Phenylephrine (0.05 μM) increased vascular tone by 224 ± 15 mg (N = 6 rabbits, providing 16 (n) preparations). Both methacholine and histamine reversed phenylephrine (0.05 μM)-induced tone in endothelium-intact rat aorta. The mean relaxant potency (pD_2) for methacholine was 6.49 ± 0.09 (N = 3, 8). Both methacholine and histamine reversed phenylephrine-induced tone by 54 ± 7% (N = 6, 16) and 42 ± 9% (N = 3, 8) respectively. Methylene blue (10 μM), haemoglobin (10 μM), L-NAME (100 μM), gossypol (30 μM) and phenidone (10 μM) abolished the methacholine-induced relaxation (data not shown).

Endothelium-denuded aorta

Phenylephrine contracted rat endothelium-denuded aorta in a concentration-dependent manner. The contractile potency (pD_2) and the maximal tension generated (E_{max}) to phenylephrine was 8.36 ± 0.11 and 286 ± 27 mg respectively (N = 6, 36). Mechanical removal of the endothelium with a cotton wool probe completely abolished methacholine- and histamine-induced relaxation of phenylephrine-contracted rat aorta (Figure 1a,b). The leukotrienes also failed to relax endothelium-denuded rat aorta (Figure 2a,c,e). This confirmed that the endothelium had been effectively removed. The phenylephrine-induced contractile response (264 ± 7 mg

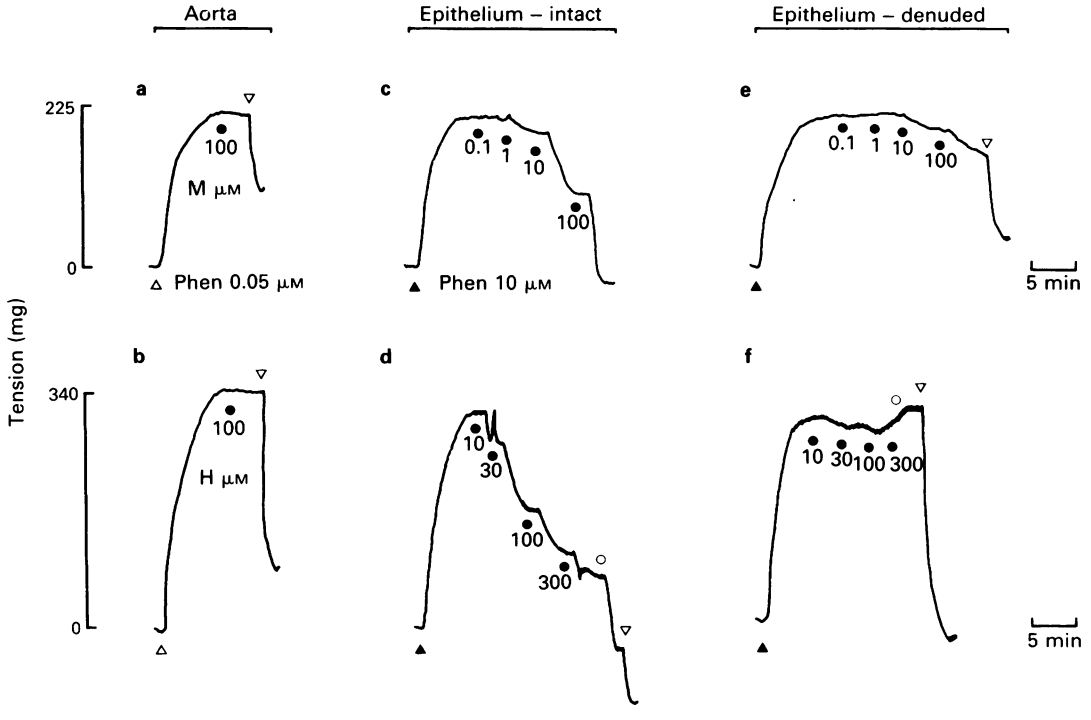

Figure 1 Lack of effect of (a) methacholine (M, 100 μM, ●) and (b) histamine (H 100 μM, ●) in reversing phenylephrine (Phen, 0.05 μM, △)-induced tone in rat endothelium-denuded aorta. Response to phenylephrine (10 μM, ▲)-contracted rat aorta surrounded by rabbit epithelium-intact bronchus in a co-axial bioassay to (c) methacholine (0.1–100 μM) and (d) histamine (10–300 μM). No relaxation response to (e) methacholine and (f) histamine was observed when the epithelium was removed. Methacholine (○, 100 μM) and isoprenaline (▽, 1 μM) was used to reverse tone. Indomethacin (5 μM) present throughout the co-axial bioassay experiments.

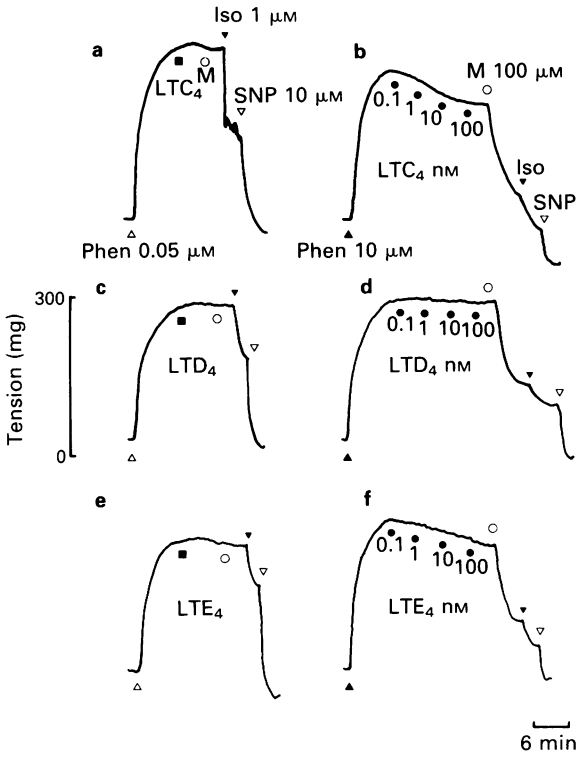

Figure 2 Effect of (a,b) leukotriene C_4 (LTC_4 100 nM, ■; 0.1–100 nM, ●), (c,d) leukotriene D_4, (LTD_4 100 nM, ■; 0.1–100 nM, ●), and (e,f) leukotriene E_4 (LTE_4 100 nM, ■; 0.1–100 nM, ●) in phenylephrine (Phen 0.05 μM, △; Phen 10 μM, ▲)-contracted rat aorta alone (a,c,e) or in co-axial bioassay (b,d,f). In all preparations methacholine (M 100 μM, ○), isoprenaline (Iso 1 μM, ▼) and sodium nitroprusside (SNP 10 μM, ▽) were used to reverse tone. Indomethacin (5 μM) was present throughout.

N = 45, 217) was reversed in response to isoprenaline (1 μM) by 83 ± 3% (N = 45, 217; Figure 1a,b).

Co-axial bioassay

Methacholine and histamine caused concentration-dependent relaxation of phenylephrine (10 μM)-precontracted rat aorta without endothelium which was suspended inside a tube of rabbit intrapulmonary bronchi (Figure 1c,d). The relaxant potency (EC_{50}; 95% CL) of methacholine and histamine was 1.94 μM (1.55–2.23; N = 30, 125) and 14.1 μM (7.25–27.5; N = 11, 26) respectively. Both methacholine and histamine reversed phenylephrine-induced tone by 138 ± 9% (N = 30) and 107 ± 18% (N = 11) respectively. Histamine was less effective compared with methacholine at releasing EpDIF as it produced a relaxation response which was 72 ± 6% (N = 11) of that to mechacholine (Figure 1d). Neither LTC_4, LTD_4 or LTE_4 (0.1–100 nM) reversed phenylephrine-induced tone in rat aortic preparations in the co-axial bioassay (Figure 2b,d,f). Histological examination of the intrapulmonary bronchi revealed an intact epithelium (Figure 3a).

Following mechanical or chemical removal of the epithelium, both methacholine and histamine failed to relax the rat aorta in the co-axial bioassay in some preparations (Figure 1e,f; mechanical removal shown only). Histological examination of the intrapulmonary bronchi confirmed the absence of an intact epithelium (Figure 3b,c). In epithelium-intact bronchi, methacholine reversed phenylephrine-induced tone by 131 ± 21% (n = 24 preparations), which was significantly attenuated in preparations denuded of epithelium mechanically (36 ± 5%, n = 32, $P < 0.01$ Mann-Whitney U test). Similarly, the methacholine-induced reversal of phenylephrine-induced tone in epithelium-intact preparations (104 ± 8%, n = 8) was significantly attenuated in preparations

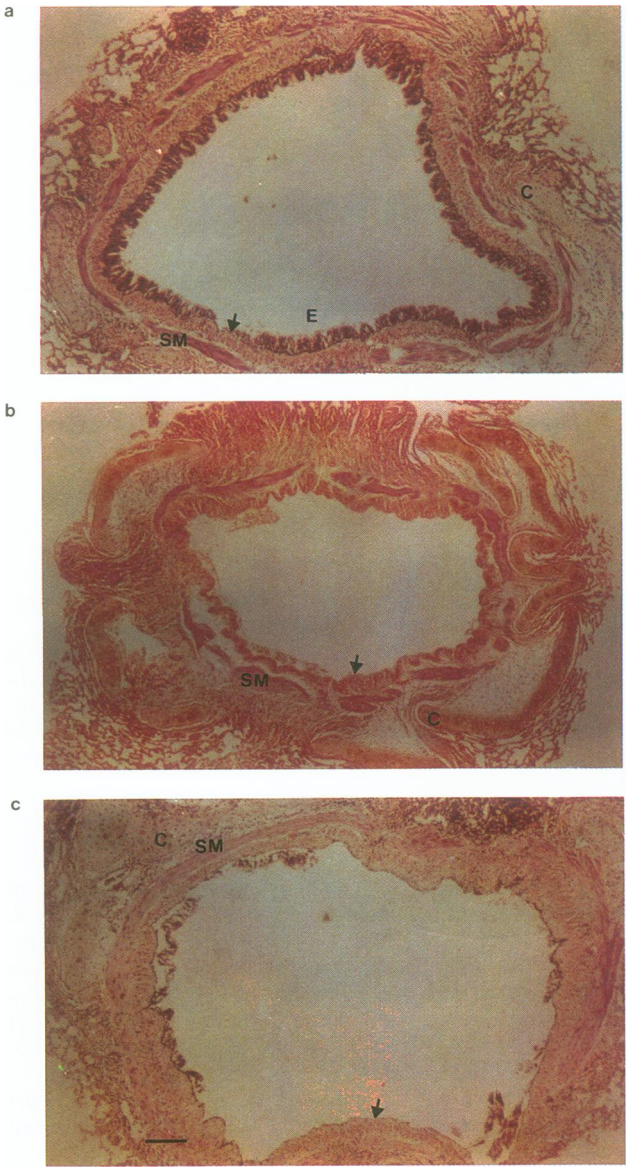

Figure 3 Photomicrographs of 8 μm paraffin sections from rabbit intrapulmonary bronchi with intact epithelium (a) and in bronchi in which the epithelium was removed either mechanically (b) or chemically with CHAPS (c). E = epithelium, SM = smooth muscle, C = cartilage and arrow indicates submucosa. Bar = 100 μm.

denuded of epithelium with CHAPS (38 ± 12%, n = 9, $P < 0.01$ Mann-Whitney U test). Rings taken from bronchial tubes in which the epithelium was removed mechanically or by CHAPS demonstrated increased sensitivity to methacholine (Table 1, $P < 0.05$ unpaired t test). No change in E_{max} was observed as a result of epithelium removal with either method (Table 1, $P > 0.05$, unpaired t test).

The muscarinic receptor antagonist, atropine (0.3–3 μM), concentration-dependently antagonized the methacholine-induced relaxation response. Analysis of the rightward shifts in the concentration-effect curves to methacholine in the presence of various concentrations of atropine by Schild analysis revealed a pA_2 value of 8.86 ± 0.16 and a slope of 1.13 ± 0.08 (n = 12).

The relaxant potency of methacholine was 2.5 μM (1.8–3.4; n = 12) which was not significantly altered in the presence of indomethacin (2.6 μM, 1.8–3.8; n = 12, $P > 0.05$, paired t test). Furthermore, the relaxant potency to methacholine was not altered by mepacrine (10 μM), NDGA (10 μM), methylene blue (10 μM), L-NAME (100 μM) or catalase (100 u ml^{-1}; Table 2). Haemoglobin (10 μM, n = 9), phenidone (10 μM, n = 10) and

Table 1 Contractile potency (pD_2) and maximum contraction (E_{max}) in epithelium-intact bronchi and in bronchi denuded of epithelium by mechanical means or by CHAPS

	pD_2	E_{max}	n
Mechanically removed			
Control	5.65 ± 0.06	1054 ± 133	12
Denuded	5.96 ± 0.07*	807 ± 140#	18
CHAPS-treated			
Control	5.58 ± 0.05	1440 ± 246	9
Denuded	5.89 ± 0.08*	973 ± 117#	7

Results expresed as mean ± s.e.mean. $pD_2 = -\log_{10} EC_{50}$, E_{max} expressed in mg tension. n represents the number of preparations tested.
* Contractile potency to methacholine significantly increased in epithelium-denuded preparations compared with epithelium-intact preparations ($P < 0.05$, unpaired t test).
# No difference in E_{max} in control and denuded preparations.

gossypol (30 μM, $n = 9$) also failed to reverse methacholine-induced relaxations (data not shown).

The peptidases α-chymotrypsin (2 units ml^{-1}) and papain (2 units ml^{-1}) failed to alter the relaxant potency of methacholine (Table 3). Similarly, the relaxant potency of methacholine was not significantly altered in the presence of aprotinin (1.75 units ml^{-1}), thiorphan (10 μM) or captopril (10 μM; Table 3).

Sandwich preparations

A loss in tissue sensitivity to phenylephrine was observed in consecutive concentration-effect curves by 2.0 (1.0–3.9; $n = 6$, 95% confidence limits) and 3.1 (1.4–7.2; $n = 6$) fold respectively, in rat endothelium-denuded aorta. The contractile potency to phenylephrine was significantly reduced in preparations which were placed alongside rabbit epithelium-intact or denuded bronchus in a 'sandwich' by 15.8 fold (10.2–24.6; $n = 12$) and 13.5 fold (8.5–21.4) respectively (Table 4; $P < 0.05$). The uptake$_1$ inhibitor, cocaine (5 μM) and the uptake$_2$ inhibitor, corticosterone (100 μM), failed to prevent the loss of tissue sensitivity to phenylephrine in the presence of epithelium-intact (16 fold; 8.8–29.2, $n = 6$) or epithelium-denuded (15.3 fold, 9.6–24.2; $n = 6$) bronchi (Table 4; $P > 0.05$). In contrast, oxytetracycline (100 μM) significantly attenuated the loss in vascular smooth muscle sensitivity to phenylephrine in the presence of epithelium-intact (4 fold, 2.3–7.0; $n = 6$) and epithelium-denuded (3.1 fold, 1.7–5.5; $n = 6$) bronchi (Table 4; $P < 0.05$).

Table 2 Mean relaxant potency (EC_{50}) for methacholine in phenylephrine-contracted rat aorta surrounded by an epithelium-intact rabbit bronchus in the absence or presence of various inhibitors

Control	*Treatment*	n
	NDGA (10 μM)	
2.2	3.2*	7
(1.2–4.3)	(1.4–7.6)	
	Methylene blue (10 μM)	
3.0	2.3*	8
(1.6–5.4)	(1.0–4.9)	
	Mepacrine (10 μM)	
3.6	4.4*	10
(1.8–7.2)	(2.3–8.3)	
	L-NAME (100 μM)	
3.2	2.4*	10
(2.1–4.8)	(1.2–4.8)	
	Catalase (100 u ml^{-1})	
3.4	2.4*	12
(2.6–4.5)	(1.4–4.2)	

Results expresed in μM. Values in parentheses indicate 95% confidence limits. n = the number of preparations tested in 3–5 rabbits.
* No significant difference cf control ($P > 0.05$, paired t test). Indomethacin (5 μM) present throughout. NDGA = nordihydroguaiaretic acid; L-NAME = L-N^G-nitroarginine methyl ester.

Table 3 Lack of an effect of various peptidases and peptidase inhibitors on the relaxant potency (EC_{50}) of methacholine in reversing phenylephrine-contracted rat aorta in a co-axial bioassay

Control	*Treatment*	n
	α-Chymotrypsin (2 units ml^{-1})	
1.2	1.2*	6
(0.52–2.75)	(0.59–2.44)	
	Papain (2 units ml^{-1})	
1.12	1.20*	7
(0.48–2.61)	(0.55–2.64)	
	Captopril (10 μM)	
3.09	3.55*	7
(1.76–5.43)	(2.53–4.98)	
	Thiorphan (10 μM)	
2.09	1.26*	7
(1.06–4.11)	(0.90–1.76)	
	Aprotinin (1.75 units ml^{-1})	
1.78	2.09*	8
(1.09–2.90)	(1.21–3.60)	

Results expressed in μM. Values in parentheses indicate 95% confidence limits. n = the number of preparations tested in 3–4 rabbits.
* No significant difference cf. control ($P > 0.05$, paired t test). Indomethacin 5 μM present throughout.

Discussion

The results from this study demonstrate that the epithelium of rabbit intrapulmonary bronchus is capable of releasing an inhibitory factor which relaxes vascular smooth muscle. These results confirm earlier reports that an inhibitory factor is released from guinea-pig tracheal epithelium (Ilhan & Sahin, 1986; Guc *et al.*, 1988a,b; Hay *et al.*, 1987a; Fernandes *et al.*, 1989) and human (Fernandes *et al.*, 1989) and canine (Gao &

Table 4 Contractile potency (pD_2) of (−)-phenylephrine in endothelium-denuded rat aorta in the absence or presence of a strip of rabbit bronchus and the effect of various uptake inhibitors

	Sandwich preparations[a]		
Aorta alone	*Control*[b]	*Cocaine/ Corticosterone*	*Oxytetracycline*
	Epithelium intact		
8.38 ± 0.09 (12)	6.87 ± 0.11* (12)	6.65 ± 0.13# (6)	7.36 ± 0.18** (6)
	Epithelium denuded		
8.38 ± 0.07 (12)	6.89 ± 0.11* (12)	6.72 ± 0.10# (6)	7.44 ± 0.14** (6)

Results expressed as mean ± s.e.mean. Numbers in parentheses indicate the number of preparations (n) tested.
[a] Rabbit bronchial preparations with intact or denuded epithelium placed in close proximity to endothelium-denuded rat aorta.
[b] No uptake inhibitors present.
* pD_2 value for phenylephrine is significantly lower in the presence cf. absence of airway tissue ($P < 0.05$, paired t test).
# pD_2 value for phenylephrine in sandwich preparations in the presence of cocaine and corticosterone is not significantly different cf. the value in control ($P < 0.05$, paired t test).
** pD_2 value for phenylephrine in sandwich preparations in the presence of oxytetracycline is significantly greater cf. the value in control ($P < 0.05$, paired t test).

Vanhoutte, 1989) bronchial epithelium. We have demonstrated that the release of this factor by methacholine was mediated by muscarinic receptors since atropine inhibited methacholine-induced relaxation in a concentration-dependent manner, consistent with an earlier report (Fernandes *et al.*, 1989). Similarly, histamine but not LTC_4, LTD_4 and LTE_4 is capable of stimulating the release of this inhibitory factor, as in the guinea-pig (Fernandes *et al.*, 1989; Fernandes & Goldie, 1990). In bronchial preparations in which the epithelium was removed by mechanical rubbing of the intimal surface, significant attenuation of the relaxation response induced by methacholine was observed, confirming the epithelial-dependence of this response. Similarly, perfusion of bronchial tubes with the detergent CHAPS, shown to remove endothelial cells effectively (Bhardwaj & Moore, 1988), also attenuated the relaxation response to methacholine. Histological analysis of tissues from bronchi in which the epithelium was removed either by mechanical rubbing or by chemical treatment with CHAPS revealed extreme epithelial cell denudation, with no apparent damage to the underlying tissue structures.

It is possible that the relaxation response to methacholine may have been due to the physical effect of a constricting bronchial tube about the vascular preparation and it is also plausible that mechanical and chemical removal of the epithelium may have damaged the ability of the airway smooth muscle to contract. Hence, the absence of a relaxant response may have been erroneously attributed to the absence of an epithelium. To investigate this possibility, we determined the functional integrity of the airway smooth muscle. The contractile potency to methacholine was significantly increased in bronchial rings taken from bronchi used in the co-axial bioassay where the epithelium had been removed by mechanical or chemical means, consistent with *in vitro* studies in a variety of species (see Fedan *et al.*, 1988). Furthermore, the maximum tension generated in rings from bronchial tubes with or without epithelium were similar. Together these results demonstrate that the relaxation response is therefore epithelium-dependent and not an artefact of the co-axial bioassay preparation.

Recently, it was shown that acetylcholine-induced contraction of guinea-pig trachea resulted in a reduction in the oxygen tension within the lumen of the trachea. This agonist-induced fall in oxygen tension was suggested to be responsible for the relaxation response which is observed in the co-axial bioassay (Gunn & Piper, 1990). In contrast to these findings, Fernandes & Goldie (1990) demonstrated that LTC_4 and LTD_4 failed to relax rat aorta within the co-axial bioassay, even though these are potent contractile agonists in guinea-pig trachea (Hay *et al.*, 1987b). Similarly, various spasmogens including the leukotrienes and acetylcholine failed to relax rat aorta when surrounded by rat trachea in a co-axial bioassay, even though these agonists are also potent spasmogens in rat trachea (R.G. Goldie personal communication). These findings rule out the likelihood that the observed relaxations are primarily due to luminal hypoxia in co-axial preparations.

We also found that a greater concentration of phenylephrine was required to contract the rat aortic preparation when rabbit bronchus was present in the organ bath. This observation may be attributed to the non-specific binding of phenylephrine to rabbit bronchus since oxytetracycline, which has been shown to inhibit binding of catecholamines to elastin and collagen in blood vessels (Powis, 1974), inhibited the loss in tissue sensitivity to phenylephrine whereas the conventional $uptake_1$ and $uptake_2$ inhibitors were without affect.

We have also attempted to determine the chemical nature of this inhibitory factor. EpDIF was neither a product of the cyclo-oxygenase nor lipoxygenase pathway as indomethacin, mepacrine and NDGA failed to inhibit the relaxant response induced by methacholine. Although mepacrine and NDGA are relatively non-specific in effect (Griffith *et al.*, 1984), no inhibition was observed. Furthermore, prostaglandin E_2 contracts rat endothelium-denuded aorta (Lai *et al.*, 1988) and LTC_4, LTD_4 and LTE_4 also failed to relax this preparation (this study). Haemoglobin and phenidone, inhibitors of EDRF (Griffith *et al.*, 1984; Martin *et al.*, 1985) and gossypol, an inhibitor of EDRF synthesis and/or release (Forstermann *et al.*, 1986) at concentrations known to inhibit EDRF (also confirmed in this study), failed to inhibit relaxation induced by methacholine. Furthermore, EDRF has been shown to be synthesized from L-arginine in endothelial cells which can be inhibited by L-N^G-monomethylarginine (Moncada *et al.*, 1989). Recently, L-N^G-nitroarginine (L-NOARG) has been shown to be a more potent inhibitor of EDRF production (Moore *et al.*, 1990). Similarly, the methyl ester derivative L-NAME is equipotent with L-NOARG (Moore *et al.*, 1990). However, L-NAME failed to inhibit the epithelium-dependent methacholine-induced relaxation. Taken together these results indicate that EpDIF is not EDRF or NO. These studies are also in accord with the results obtained with guinea-pig trachea (Fernandes *et al.*, 1989; Fernandes & Goldie, 1990) and canine bronchus (Gao & Vanhoutte, 1989). Although no attempt was made to measure cyclic nucleotide levels, it has recently been reported that guinea-pig EpDIF is capable of elevating intracellular levels of guanosine 3':5'-cyclic monophosphate (cycle GMP) but not adenosine 3':5'-cyclic monophosphate (cyclic AMP) in rat aorta (Hay *et al.*, 1989). These results suggest that EpDIF does not stimulate the guanylate cyclase which is activated by EDRF/NO. It is most likely that EpDIF stimulates the particulate rather than the soluble form of guanylate cyclase (Hay *et al.*, 1989).

We have also used several peptidases and peptidase inhibitors to determine whether this inhibitory factor was a peptide. The endopeptidase α-chymotrypsin and papain, at concentrations which inhibited the relaxant effect of exogenously applied vasointestinal peptide (VIP) in cat and guinea-pig trachea (Altiere & Diamond, 1984; Ellis & Farmer, 1989), failed to inhibit the effects of the EpDIF. Similarly, the protease inhibitor aprotinin, the neutral endopeptidase inhibitor thiorphan and the dipeptidyl carboxypeptidase inhibitor captopril, were all without effect. However, it remains possible that this factor is a peptide which is resistant to the peptidases used or is not degraded by enzymes sensitive to the inhibitors tested (Ellis & Farmer, 1989). Furthermore, catalase at concentrations which inhibit hydrogen peroxide-induced relaxation of airway tissue (Rhoden & Barnes, 1989) was also ineffective at inhibiting methacholine-induced relaxation, ruling out the possibility that EpDIF is an oxygen-derived free radical.

Whilst the original studies have shown that epithelial removal can increase the sensitivity of airway smooth muscle to various spasmogens, thus providing indirect evidence for an epithelium-derived relaxing factor (EpDRF), very few studies have been able to transfer and detect successfully such a factor when assayed on airway smooth muscle in superfusion cascade (Holyrode, 1986; Undem *et al.*, 1988). In contrast, it has been shown that an inhibitory factor is capable of relaxing both vascular and airway smooth muscle in superfusion cascade (Vanhoutte, 1988). Similarly, by using the co-axial bioassay it has been shown that the presence of airway epithelium modulates carbachol-induced airway smooth muscle tone (Guc *et al.*, 1988b). It is therefore possible that the vasoactive EpDIF is distinct from the EpDRF which has been suggested as an endogenous regulator of airway smooth muscle tone (Vanhoutte, 1988; Fernandes & Goldie, 1990; Goldie *et al.*, 1990). However, the lack of success in the transfer of an inhibitory factor using superfusion cascade while such a factor can be detected in co-axial bioassay may be a consequence of a greater local concentration of this substance at the site of the recipient preparation tissue within the co-axial bioassay. Factors such as dilution of the inhibitory factor and/or metabolism by oxygen radicals may hamper the detection of such a factor in superfusion cascade.

D.S. is a C.J. Martin Overseas Research Fellow of the National Health and Medical Research Council of Australia.

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(Received July 2, 1990
Revised October 11, 1990
Accepted December 7, 1990)

Br. J. Pharmacol. (1991), **102**, 904–908

A comparison of excitotoxic lesions of the basal forebrain by kainate, quinolinate, ibotenate, N-methyl-D-aspartate or quisqualate, and the effects on toxicity of 2-amino-5-phosphonovaleric acid and kynurenic acid in the rat

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1 It has been suggested that an $NMDA_1$ receptor subtype might be activated by N-methyl-D-aspartate (NMDA) and ibotenate and an $NMDA_2$ subtype by NMDA or quinolinate, and that the $NMDA_2$ site might be more susceptible to blockade by kynurenic acid.

2 Experiments were carried out to examine the ability of 2-amino-5-phosphonovaleric acid (AP5) and kynurenic acid to antagonize the neurotoxic properties of kainate, ibotenate, NMDA, quinolinate and quisqualate injected into the rat basal forebrain.

3 Following histological analysis of the injection sites, lesion volume was assessed parametrically. Each of the toxins except quisqualate was found to make lesions of parvocellular neurones within the basal forebrain with a relative order of potency: kainate $\gg$ quinolinate > ibotenate = NMDA.

4 Equimolar doses of AP5 abolished the toxicity produced by quinolinate and NMDA; toxicity to kainate and ibotenate was attenuated to ~40% of the toxin-alone condition.

5 The antagonistic properties of kynurenate were dose-dependent: equimolar kynurenate had no effect on quinolinate but attenuated the actions of ibotenate, kainate and NMDA; 2 × equimolar kynurenate had no effect on quinolinate or ibotenate but attenuated the toxicity of kainate and NMDA; and 3 × equimolar kynurenate had no effect on the toxicity of kainate or ibotenate, attenuated the actions of NMDA and abolished the toxic action of quinolinate.

6 The results are discussed in terms of the actions of the various toxins at different receptors, differentially sensitive to AP5 and kynurenate.

Keywords: Basal forebrain; neurotoxic amino acids; lesions; kynurenic acid; 2-amino-5-phosphonovaleric acid (AP5); excitatory amino acid receptors

Introduction

A number of compounds related to the putative excitatory amino acid transmitters glutamate and aspartate can cause neuronal death when injected into certain regions of the brain. These 'excitotoxins' include kainic acid, N-methyl-D-aspartate (NMDA), ibotenic acid and the more recently discovered quinolinic acid (Schwarcz *et al.*, 1983; Stone & Connick 1985; Stone *et al.*, 1987). The special importance of the latter compound lies in the fact that it is the only one of these excitotoxins endogenous to the mammalian CNS, including human (Wolfensberger *et al.*, 1983; Moroni *et al.*, 1984). The existence of such an endogenous toxin has therefore generated speculation that the gradual accumulation of quinolinate, or an increased neuronal sensitivity to it, might be a causative factor in the neuronal degeneration seen in Huntington's disease and related syndromes (Stone *et al.*, 1987).

In view of this potential clinical relevance it is clearly important to understand the pharmacology of excitotoxin receptors in the CNS, but it has so far proved difficult to accommodate excitotoxin pharmacology within the now accepted view that there are separate species of excitatory amino acid receptor, sensitive predominantly to NMDA, kainate and quisqualate (Watkins & Evans, 1981). Quinolinic acid, for example appears to activate NMDA receptors when studied in electrophysiological experiments (Stone & Perkins, 1981; Perkins & Stone, 1983a; Herrling *et al.*, 1983; Stone & Connick, 1985; Stone & Burton, 1988). However, differences in the relative sensitivity of neurones to NMDA and quinolinic acid have been described in some regions of the CNS (Perkins & Stone, 1983a,b; Stone & Burton, 1988) and the resulting suggestion, that there may be subtypes of the NMDA receptor, has been supported by evidence of a differential sensitivity of NMDA and quinolinate to blockade by antagonists (Ffrench-Mullen *et al.*, 1983; Perkins & Stone, 1985).

In studies of their neurotoxic properties it has been found that quinolinic acid differs from NMDA in showing preferential toxicity towards hippocampal CA3 neurones (Schwarcz *et al.*, 1984) and in being dependent on afferent innervation (Foster *et al.*, 1983). These and various other factors led to the proposal that an $NMDA_1$ receptor subtype might be activated by NMDA and ibotenate, while an $NMDA_2$ subtype would be activated by NMDA or quinolinate, the $NMDA_2$ site being more susceptible to blockade by kynurenic acid (Stone & Connick 1985; Stone & Burton 1988). To date however, this hypothesis has been tested only in electrophysiological studies on the neocortex (Burton *et al.*, 1988). Following the demonstration that neurones in the basal forebrain are susceptible to damage by a variety of excitotoxins (Dunnett *et al.*, 1987) the present study was undertaken to assess the ability of DL-2-amino-5-phosphonovaleric acid (AP5) and kynurenic acid to attenuate toxicity to a variety of toxins at this site.

Methods

Male Lister hooded rats (232–518 g) were used in both experiments. Following anaesthesia with Avertin (10 ml kg^{-1}), rats

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were placed in a Kopf stereotaxic frame; the skull surface was then exposed and a unilateral infusion of excitotoxin made via a 30 ga cannula connected by polyethylene tubing to a 10 μl SGE syringe mounted in a Harvard infusion pump. All injections were of 0.5 μl over 1 min, the cannula remaining in place for a further 2 min following infusion to allow for diffusion away from the tip. The stereotaxic co-ordinates used for the infusions were (in mm from bregma with the nose bar set 5 mm above the interaural line): AP + 0.8 mm, lateral +/ − 3.0 mm and vertical −7.2 mm.

The first series of experiments was to determine the relative lesioning potency of various excitotoxic amino acids in the basal forebrain. The following doses of toxin were administered (in nmol; injection volume always 0.5 μl): kainate (Sigma) 1.5 and 3; quinolinate (Sigma) 15, 30, 60 and 80; NMDA (Sigma) 15, 30, 60 and 80; quisqualate (Cambridge Research Biochemicals) 15, 30, 60 and 80; ibotenate (Cambridge Research Biochemicals) 15, 30 and 60; 80 nmol ibotenate could not be prepared in 0.5 μl so this dose was omitted. The vehicle for all excitotoxins was phosphate buffer (pH 7.4) and the pH of the solutions was adjusted to be within the range 6.32–7.4. At least 2 rats received each dose of toxin. Initial studies showed that even 3 nmol kainate produced extraordinarily intense convulsive activity, so in all cases where kainate was used Diazepam (Roche) 10 mg kg^{-1} was given s.c. in the neck 30 min prior to anaesthesia, and the dose of Avertin halved.

In the second series of experiments, the ability of AP5 (Cambridge Research Biochemicals) and kynurenate (Sigma) to block lesions induced by kainate, quinolinate, NMDA and ibotenate was examined. The highest effective dose of each toxin was used for this (kainate 3, quinolinate 80, NMDA 80 and ibotenate 60 nmol). The ability of equimolar AP5 and kynurenate, and 2 × and 3 × nmolar concentrations of kynurenate, to block toxicity were examined. In each case the antagonist was infused into the basal forebrain 15 min before the excitotoxin. AP5 was administered in a volume of 0.5 μl. Kynurenate was administered in the lowest possible volume (3, 6 and 9 nmol 0.5 μl; 60, 1.0 μl; 80, 1.34 μl; 120, 2.0 μl; 160, 2.68 μl; 180, 3.0 μl; 240, 4.02 μl). All doses of AP5 and kynurenate used were also tested for independent effects.

Following recovery from anaesthesia rats were observed at 30 min intervals and the incidence of the following noted over a 2 min period: forepaw treading, barrel rolling around the long axis, wet dog shaking, postural deviation (ipsi- or contralateral to the lesion) and rotation. Recording was discontinued after two consecutive 'normal' observations.

Rats were allowed to survive for 14–21 days and then killed by barbiturate overdose. Following transcardial perfusion with buffered saline followed by 10% formalin the brains were removed. Sections (40 μm) were cut on a bench microtome and stained with cresyl violet for light microscopic analysis with a Leitz Diaplan microscope. Lesions were identified and silhouettes drawn with a Leitz Laborlux 12 microscope fitted with a drawing tube. When lesions were present the borders were always clearly detectable. Within the borders extensive gliosis was present and parvocellular neurones were absent. The surface area of the lesion within each serial section was computed by use of a two dimensional image analyzer ('Videoplan', Kontron) and the total lesion volume expressed in mm^3 (uncorrected for tissue shrinkage during fixation) was calculated as an integral of the section surface areas. Assessment of lesion volume was undertaken without knowledge of the treatment the rat had received. The parametric data generated by this assessment were analysed by ANOVA followed when necessary by Dunnett's t test.

Results

Experiment 1: Neurotoxicity

Of the acids tested, all except quisqualate produced lesions. Lesion volume was assessed parametrically and the mean

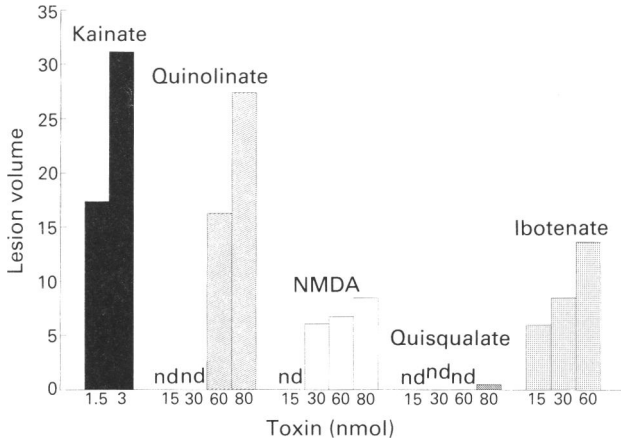

Figure 1 Comparative neurotoxicity of kainate, quinolinate, N-methyl-D-aspartate (NMDA), quisqualate and ibotenate following injection into the basal forebrain. Lesion volume was assessed parametrically in mm^3; the values shown are means. Values were computed from 2–4 rats in each case. (Kai 3, Quin 80, NMDA 60 and 80, Ibo 60 nmol: $n = 4$; all others, $n = 2$; variance (s.e.mean) was 15.0% of the mean on average.) Statistically significant differences between toxins and between different doses of the same toxin were found; see text for details.

(± s.e.) for each dose of each toxin is presented in Figure 1. The lesions were all centred on the basal forebrain, and typical damage is illustrated in Figure 2. The lesions almost invariably extended into adjacent structures. Damage was seen throughout the ventral pallidum, and larger lesions extended into the thalamus, amygdala, hypothalamus (particularly the lateral hypothalamic area), bed nucleus of the stria terminalis, horizontal limb of the diagonal band and the caudate-putamen. All of this damage appears to have been caused by diffusion of toxin away from the site of the injection. Damage remote from the site of injection was not detected.

As can be seen from Figure 1, the most potent toxin was clearly kainate, which produced very large lesions at doses 10–40 × lower than the others. There was however no significant difference between the lesion volume produced by 1.5 and 3 nmol kainate ($t = 2.269$ d.f. $= 1$ $P < 0.4$). Quinolinate was also very potent and showed dose-dependent effects ($F = 24.97$ d.f. $= 3,6$ $P < 0.001$); the two highest concentrations used (60 and 80 nmol) were effective, the two lower doses (15 and 30 nmol) not so. NMDA also showed dose-dependent effects ($F = 8.44$ d.f. $= 3,8$ $P < 0.01$) and ibotenate was neurotoxic at all doses examined, although there were no significant differences between doses ($F = 1.34$ d.f. $= 2,5$). Quisqualate was not a potent toxin. Only one rat presented with a lesion (which was very small) when administered the highest dose (80 nmol) and so quisqualate was not included in the subsequent blockade study.

Statistical comparison between 60 nmol of each toxin (and 3 nmol kainate) revealed significant differences ($F = 24.24$ d.f.3,10 $P < 0.001$). Dunnett's t test revealed differences between kainate and all other toxins (each $P < 0.01$) and between quinolinate and NMDA ($P < 0.05$). Quinolinate 60 nmol was not different from ibotenate, nor was ibotenate different from NMDA. Kainate was clearly the most potent toxin, producing lesions at doses 10–40 × less than the others. Quinolinate was judged to be the next most potent: at 60 nmol it made significantly larger lesions than NMDA, though not ibotenate. The effect of quinolinate at 80 nmol however was similar to kainate rather than NMDA or ibotenate. Overall therefore it might be concluded that kainate was the most potent toxin, followed by quinolinate, then, indistinguishable from each other, ibotenate and NMDA.

Neurotoxicity was always associated with convulsive activity, the highest doses of the effective toxins producing qualitat-

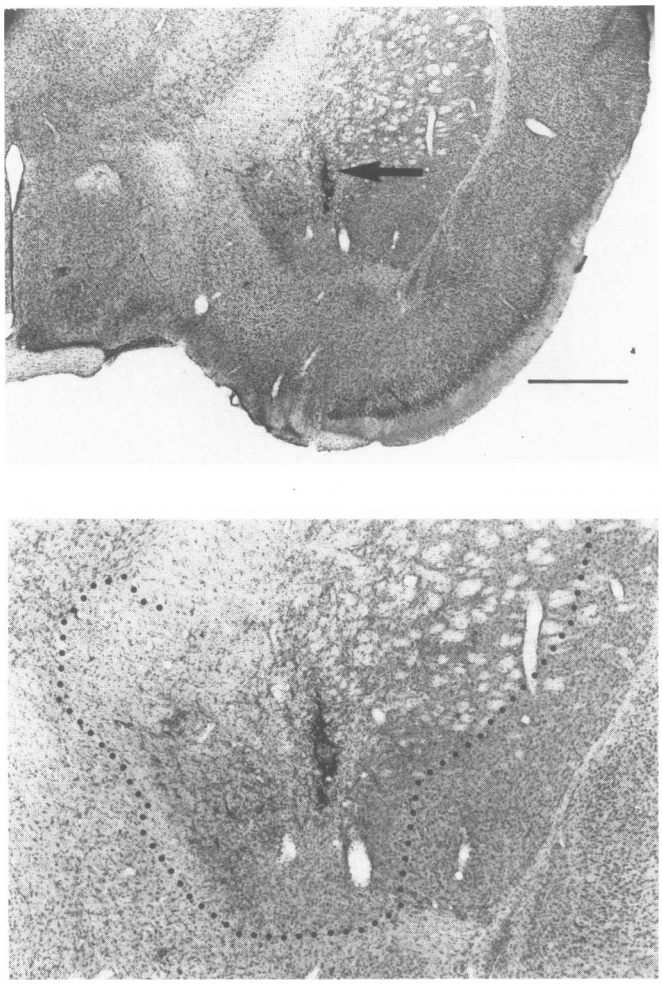

Figure 2 Representative photomicrograph of an injection site in the area of the basal forebrain (magnification × 20; bar = 1 mm). The injection site, marked with an arrow, is clearly visible. The lower picture is a magnification (× 40) of the same section. The dotted line indicates the extent of the lesion.

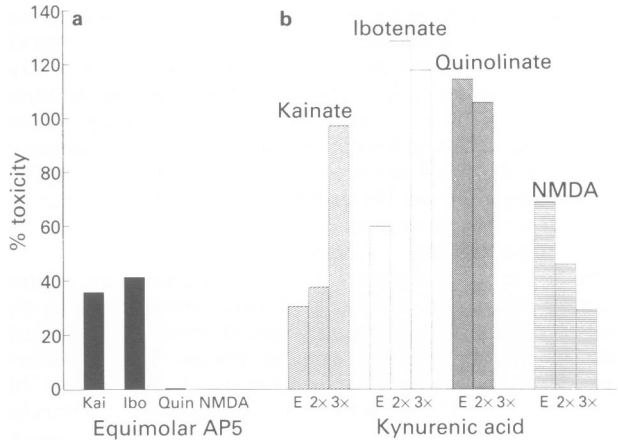

Figure 3 Antagonism by equimolar 2-amino-5-phosphonovaleric acid (AP5) (a) and by various doses of kynurenate (Kyn) (b) of neurotoxicity to kainate (Kai), quinolinate (Quin), N-methyl-D-aspartate (NMDA) and ibotenate (Ibo) following injection into the basal forebrain. (E: equimolar; 2 × : 2 × equimolar; 3 × : 3 × equimolar kynurenate.) Values are expressed as a percentage of the toxicity to the toxin alone (100% = no antagonism). Lesion volume was assessed parametrically in mm^3. The values shown are means computed from: AP5: Kai, Quin and Ibo $n = 2$, NMDA $n = 4$: equimolar Kyn: Kai $n = 3$, Quin $n = 4$, NMDA and Ibo $n = 2$; 2 × Kyn: Kai and Quin $n = 2$, NMDA and Ibo $n = 4$; 3 × Kyn: Kai and Quin $n = 3$, NMDA and Ibo $n = 4$; variance (s.e.mean) was 19.5% of the mean on average. Statistically significant differences between toxins and between the antagonistic effects of different doses of kynurenate were found; see text for details.

ively similar effects. Rats showed uncontrollable forepaw treading in air followed by rapid rolling around their long axis ('barrel rolling'); either postural deviation or active rotation was often present also. After the higher doses of kainate or quinolinate these persisted for 4–9 h, while after high doses of NMDA or ibotenate they persisted for 5–6 h. However, in comparing toxins it must be remembered that the kainate rats had been pretreated with diazepam to reduce convulsive activity, which otherwise would have lasted far longer.

Experiment 2: Blockade of neurotoxicity

AP5 and kynurenate were both tested for their ability to antagonize the effects of excitotoxins in the basal forebrain. Prior to this each was examined in all of the doses used (AP5 3–80 nmol, kynurenic acid 3–240 nmol) for neurotoxic effects of their own. No lesion was ever observed after administration of either AP5 or kynurenic acid alone; nor were convulsions observed on recovery from anaesthesia.

The ability of AP5 and kynurenate to antagonise the effects of the highest doses of kainate (3 nmol), quinolinate (80 nmol), NMDA (80 nmol) and ibotenate (60 nmol) were examined, and the results of these tests are summarised in Figure 3. Analysis of the effects of AP5 on each toxin (Figure 3a) revealed significant differences ($F = 15.912$ d.f. = 3,4 $P < 0.025$) which are clearly accounted for by AP5 abolishing the neurotoxic effects of both NMDA and quinolinate, and reducing to ~40% of normal the toxicity of kainate and ibotenate. Analysis of the effects of the three doses of kynurenate (Figure 3b) revealed no differences overall between doses ($F = 0.759$ d.f. = 2,12 NS) but significant differences between the toxins ($F = 3.847$ d.f. = 3,12 $P < 0.05$) and a statistically significant toxin × kynurenate interaction ($F = 5.006$ d.f. = 6,12 $P < 0.01$) suggesting that kynurenic acid affects the toxic properties of the four agents differently. Lower doses of kynurenic acid attenuated toxicity to kainate and to a lesser extent ibotenate and NMDA, but had no effect on quinolinate. The highest dose used however had no effect on either kainate or ibotenate toxicity, but attenuated NMDA toxicity and abolished completely the effects of quinolinate.

The effects the antagonists had on the convulsive activity of the toxins was complex. The most straightforward effect concerned quinolinate: convulsions were blocked only when toxicity was also. Kainate-induced convulsions were unaffected by either AP5 or kynurenate. NMDA-induced convulsions were almost completely abolished by both AP5 and kynurenate regardless of the effect on toxicity. Ibotenate-induced convulsions were abolished by AP5 and reduced by kynurenate regardless of toxicity.

Discussion and conclusions

The relative order of potency of the excitotoxins tested was: kainate ≫ quinolinate > NMDA = ibotenate > quisqualate. At the highest doses all except quisqualate produced clearly delineated lesions which extended beyond the basal forebrain and extensive gliosis was present within the damaged area. If one were interested in a selective lesion for functional studies both concentration and volume would have to be controlled carefully. All of the effective toxins also produced convulsions. These were so severe following kainate as to make the use of diazepam essential. Convulsions were also marked after quinolinate, but not so severe after either NMDA or ibotenate. Although quisqualate was ineffective compared to the other toxins, it should be noted that in other studies it has been shown to have neurotoxic properties in this area (Dunnett *et*

al., 1987; Robbins *et al.*, 1989). This toxicity however appears to be limited to destruction of the cholinergic neurones scattered through this region, with the parvocellular neurones of the basal forebrain being spared. That sparing was replicated here; whether there was damage to cholinergic neurones cannot be determined in the absence of appropriate indices. In terms of the ability to lesion the parvocellular portion of the basal forebrain, and surrounding tissue, these data confirm previous reports (Dunnett *et al.*, 1987) and extend them by adding quinolinate to the range of toxins.

One possible source of variance in the present data was the age range of the rats used. For instance, previous reports have indicated age-related changes in the sensitivity of neurones to kainate (Gaddy *et al.*, 1979) and increased glutamate receptor binding with age (Baudry *et al.*, 1981). However, changes in susceptibility to kainate toxicity are present only in young rats (<50 days), and the increase in the density of glutamate receptor binding sites is only observed in calcium-free media. In the presence of calcium the density of glutamate binding sites is no different in hippocampal membranes taken from rats between 9 days and 2 years old. In the present study it is important to note that the group with the largest age range (3 nmol kainate) showed particularly low variability (mean lesion volume: $31.14 \pm 1.89\,mm^3$, $n = 4$). It is unlikely that age was an important factor in the present study.

The principal purpose of these experiments was to examine the ability of AP5 and equimolar kynurenate to antagonize neurotoxicity. The effects of AP5 and equimolar kynurenate on kainate were curious, in that both reduced lesion volume to $\sim 40\%$ but had no effect on the convulsive action, suggesting that the toxic and convulsive effects of kainate are dissociable. The ability of AP5 and equimolar kynurenate to attenuate kainate-induced lesions could suggest either that AP5 and kynurenate can act directly at kainate receptors; or that kainate can act to release agents which act directly or indirectly through AP5- and kynurenate-sensitive sites to produce neurotoxic effects. The first of these explanations is unlikely to be correct since in electrophysiological studies, kainate-induced excitation is resistant to AP5 (Perkins *et al.*, 1981; Watkins & Evans 1981) although kynurenic acid can block the actions of kainate (Perkins & Stone, 1982; for review see Stone & Burton, 1988). The second is consistent with the suggestion that kainate can act to release glutamate (which could act at AP5-sensitive sites) from presynaptic sites. The antagonism produced by kynurenate may be related to its ability to antagonize the effects of extracellular glycine, which can modify intra-synaptic events (Thomson *et al.*, 1989).

Since AP5 was able to block the toxic effects of both NMDA and quinolinate, both are probably acting via NMDA receptors. Such a conclusion is consistent with a substantial amount of electrophysiological and neurochemical data (Stone & Perkins 1981; Perkins & Stone 1983a; Herrling *et al.*, 1983; Lehmann *et al.*, 1983; Peet *et al.*, 1986). However, equimolar kynurenic acid was able to discriminate between NMDA and quinolinate since it attenuated NMDA-induced lesions and virtually abolished convulsions, whereas it had no antagonistic effect whatever against quinolinate. This finding supports the view that at least two subpopulations of NMDA receptor exist in the CNS, one of which is sensitive to NMDA alone and one of which responds to both NMDA and quinolinate. This scheme has been proposed primarily as a result of electrophysiological studies in which regional difference of neuronal sensitivity (Perkins & Stone, 1983a,b), and different susceptibilities to antagonism (Perkins & Stone 1985; Ffrench-Mullen *et al.*, 1988) were described. However, it is clear that the discriminative profile of kynurenate in the present study is dose-dependent. At higher doses, it continues to attenuate the effects of NMDA but abolishes the effects of quinolinate. This is consistent with earlier work in which kynurenate has been found to block quinolinate excitations and toxicity more readily than the corresponding effects of NMDA (Foster *et al.*, 1984; Perkins & Stone 1985).

The attenuation rather than abolition of ibotenate toxicity in the basal forebrain by an equimolar dose of AP5 is similar to that previously reported in the hippocampus (Schwarcz *et al.*, 1982). The insensitivity of ibotenate-induced lesions to higher doses of kynurenate is consistent with previous work in the striatum (Foster *et al.*, 1984) and is also consistent with the receptor models proposed by Stone *et al.* (1987) and Stone & Burton (1988). In these models the NMDA receptor subtype activated by ibotenate was considered to be less susceptible to blockade by kynurenate. The present work is therefore also consistent with the recent quantitative analysis of amino acid receptors in the mouse neocortex in which the pA_2 values for AP5 and kynurenate were significantly greater against ibotenate than either NMDA or quinolinate (Burton *et al.*, 1988).

Overall the significance of the present work lies in the fact that it emphasises the apparent heterogeneity of amino acid receptor pharmacology. It is becoming clear that whereas AP5 is a pure competitive antagonist at NMDA receptors, the pharmacology of kynurenate is more complex. Kynurenate antagonism of NMDA and related agonists yields Schild slopes significantly greater than unity (Burton *et al.*, 1988). This is probably explained by kynurenate having at least two sites and mechanisms of action, one competitive at the receptor site and a second, non-competitive, at the recently discovered glycine modulatory site (Birch *et al.*, 1988; Danysz *et al.*, 1989). It is thus possible that the relative activity of kynurenate towards a series of amino acids may vary between brain regions depending on the importance of the glycine site, the endogenous levels of glycine and the activity of local amino acid releasing pathways. Monaghan *et al.* (1988) for example have reported that glycine affects glutamate binding to a different extent in different brain regions.

A.J.M.C. was supported by the Science and Engineering Research Council of Great Britain. M.H.H. was supported by the Medical Research Council of Great Britain.

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(Received April 24, 1990
Revised October 18, 1990
Accepted November 29, 1990)

Br. J. Pharmacol. (1991), **102**, 909–915

Effects of tacrine, velnacrine (HP029), suronacrine (HP128), and 3,4-diaminopyridine on skeletal neuromuscular transmission *in vitro*

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1 The effects of tacrine (9-amino-1,2,3,4-tetrahydroacridine), velnacrine (HP029, 9-amino-1,2,3,4-tetrahydroacridin-1-ol maleate), suronacrine (HP128, 9-benzylamino-1,2,3,4-tetrahydroacridin-1-ol maleate), and 3,4-diaminopyridine on neuromuscular transmission were compared on isolated nerve-muscle preparations.

2 Tacrine, HP029, and 3,4-diaminopyridine augmented responses of chick biventer cervicis preparations to nerve stimulation, with tacrine and HP029 increasing responses to exogenously applied acetylcholine. HP128 blocked responses to nerve stimulation and to carbachol, but increased responses to acetylcholine.

3 In mouse diaphragm preparations that were partially paralysed by tubocurarine or low calcium solutions, tacrine, HP029, and 3,4-diaminopyridine reversed the twitch block. HP128 deepened the block.

4 In mouse triangularis sterni preparations, tacrine and HP029 prolonged the decay phase of endplate potentials and miniature endplate potentials, but had no effect on quantal content at 36°C; above 10 μM, they reduced endplate potential amplitude. 3,4-Diaminopyridine increased quantal content without affecting the time course of the endplate potentials. HP128 (1–10 μM) had no effect on amplitude or time course of endplate potentials, but reduced their amplitude at higher concentrations.

5 Extracellular recording of nerve terminal currents from triangularis sterni preparations revealed that 3,4-diaminopyridine and HP128 had a selective blocking action on the waveform associated with K^+ currents, tacrine reduced and prolonged the K^+-related waveform, and HP029 had nonselective blocking actions only seen at high concentrations.

6 Tacrine and HP029 behave predominantly as anticholinesterase agents, while HP128 has weaker anticholinesterase actions that are masked by cholinoceptor blockade. Tacrine and HP128, but not HP029, have some blocking actions on K^+ currents of mouse motor nerve terminals.

Keywords: Tacrine; aminopyridines; acetylcholine release; inhibition of acetylcholinesterase; potassium currents; nerve action potentials; neuromuscular transmission

Introduction

Since the clinical report of effectiveness of 9-amino-1,2,3,4-tetrahydroacridine (tacrine) in alleviating some of the symptoms of Alzheimer's disease (AD) (Summers *et al.*, 1986), there has been increased interest in the pharmacology of this cholinesterase inhibitor. However, clinical use of tacrine has been associated with liver toxicity (Marx, 1987; Ames *et al.*, 1988), which may limit its clinical usefulness. Therefore, several new analogues, incuding (±)-9-amino-1,2,3,4-tetrahydroacridin-1-ol maleate (HP029, velnacrine) and (±)-9-(benzylamino)-1,2,3,4-tetrahydroacridin-1-ol-maleate (HP128, suronacrine), have been synthesized (Shutske *et al.*, 1988; 1989). These compounds are structurally similar to tacrine, they inhibit acetylcholinesterase *in vitro* and are active in a model that may be predictive of AD but have less acute toxicity in rats and mice (Shutske *et al.*, 1989). HP029 was well-tolerated in single dose studies with healthy volunteers (Puri *et al.*, 1989).

The ability of tacrine to alleviate some of the symptoms of AD is not simply correlated with its anticholinesterase activity as there are other anticholinesterases, physostigmine for example (Becker & Giacobini, 1988), that are more active as cholinesterase inhibitors but apparently less effective in AD. Therefore, the precise mechanism of action of tacrine in AD is unknown. However, its structural similarity to the diaminopyridines suggests that the clinical efficacy of tacrine could be, in part, due its ability to interfere with the activity of potassium channels (Summers *et al.*, 1986). The ability of tacrine to block potassium channels has been demonstrated in several studies (e.g. Drukarch *et al.*, 1987; Schauf & Sattin, 1987; Stevens & Cotman, 1987; Rogawski, 1987; Sim & Griffith, 1988; Halliwell & Grove, 1989; Elinder *et al.*, 1989; Harvey & Rowan, 1988; 1990), although it is not known whether the concentrations of tacrine that are effective at blocking potassium channels are clinically relevant.

The purpose of the present study was to investigate the cholinergic actions of the two new tacrine analogues, in comparison to tacrine and 3,4-diaminopyridine (3,4-DAP) at the neuromuscular junction. By using extracellular and intracellular recording techniques, we could estimate the effects of the compounds on nerve terminal action potentials, acetylcholine release, and acetylcholinesterase activity.

Methods

Chick biventer cervicis preparations

Biventer cervicis muscles and associated nerves were removed from 3–14 day old chicks that had been killed by exposure to anaesthetic ether (Ginsborg & Warriner, 1960) and mounted, with a resting tension of approximately 1 g, in 10 ml tissue baths containing a physiological salt solution of the following composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.1. The solution was maintained at 33°C and pH 7.3, and bubbled with oxygen containing 5% CO_2. Twitches were evoked by stimulating the motor nerve every 10 s with pulses of 0.2 ms duration and a voltage greater than that which produced a maximal twitch. Contractures to exogenously applied acetylcholine (1–3 mM), carbachol (20–40 μM), and KCl (30–50 mM) were obtained

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prior to the addition of the compounds and at the end of the experiment.

Mouse diaphragm nerve-muscle preparations

Twitch tension experiments were performed on the nerve-muscle hemidiaphragm preparations isolated from 15–25 g male mice (Balb C strain) that had been killed by exsanguination after anaesthetizing with ether. The preparations were mounted in 40 ml tissue baths containing the physiological salt solution described above. Experiments were carried out at room temperature and at 36°C. The phrenic nerve was stimulated at 0.1 Hz with rectangular pulses of 0.2 ms duration and voltage greater than that required to produce maximal twitches.

In order to reveal any facilitation of neuromuscular transmission in twitch tension experiments, a 70% reduction in the twitch height was induced by (+)-tubocurarine (1–5 μM) or by physiological salt solution containing low Ca^{2+} (0.5 mM) and high Mg^{2+} (2.5–5 mM). In these experiments, the concentrations of the compounds tested were increased when the twitch height had stabilized after the previous addition of the compounds.

Mouse triangularis sterni preparations

Experiments were performed on the left triangularis sterni nerve-muscle preparation (McArdle *et al.*, 1981) isolated from 15–25 g male mice (Balb C strain). The complete dissection of the muscle with its three nerves was performed under continuous perfusion with physiological salt solution (aerated with oxygen) of the following composition (mM): NaCl 154, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 11.1 and HEPES 5; the solution was adjusted to pH 7.3 with HCl.

The preparation was pinned thoracic side downwards to the base of a 2–3 ml tissue bath and perfused at a rate of 5–10 ml min^{-1} with the physiological solution described above to which 15 μM (+)-tubocurarine (for extracellular recording) or 2–5 mM Mg^{2+} (for intracellular recording) was added to prevent muscle twitching. In all Mg^{2+} experiments, the Ca^{2+} concentration was reduced to 0.5 mM, and a 20 min equilibration period allowed. When Ca^{2+} ions were replaced by Mg^{2+} ions, a solution of the following composition (mM) was used: NaCl 154, KCl 5, $MgCl_2$, 3.7, glucose 11 and HEPES 5 (pH 7.3). Experiments were performed at room temperature (20–26°C), and at 36 ± 1°C. The intercostal nerves were stimulated via a suction electrode every 2 s with pulses of 50 μs duration and supramaximal voltage. In experiments where 3,4-DAP and tetraethylammonium (TEA) were used, procaine (10–60 μM) was used to suppress repetitive activity.

Intracellular recording Neuromuscular junctions were identified by the presence of endplate potentials (e.p.ps) and miniature endplate potentials (m.e.p.ps) with rise times of 1 ms or less. E.p.ps and m.e.p.ps were monitored using conventional intracellular recording techniques. Recordings were made continuously from one endplate area before and throughout application of compounds, each endplate area (and hence each preparation) being exposed once to compounds.

Extracellular recording Presynaptic waveforms were recorded by a glass microelectrode (filled with 2M NaCl, resistance 5–15 MΩ) placed inside the perineural sheath (near endplate areas) of one of the branches of an intercostal nerve (see Mallart, 1985; Penner & Dreyer, 1986; Anderson *et al.*, 1988). The potentials were recorded, amplified and stored as for the intracellular recordings. Usually 20–25 waveforms were recorded at each time period. As the shape of the waveform recorded was very dependent on the electrode position, waveforms were monitored continuously from the same site before and throughout application of drugs. Recording sites were rejected if the signal amplitude fell by more than 10% during the first 20 min prior to the addition of the compounds. With this protocol, stable recordings could be obtained over a 180 min period in control solutions or in the presence of drugs.

Statistics

Values in the text are mean ± s.e. of at least four experiments, unless otherwise stated. Differences between means were tested by the Mann-Whitney U-test, $P < 0.05$ and $P < 0.01$ being taken as significant.

Materials

Tacrine was obtained from Aldrich Chemical Co., Gillingham, Dorset. HP029 and HP128 were gifts from Hoechst-Roussel Pharmaceuticals Inc., Somerville, New Jersey, U.S.A. Other drugs were obtained from Sigma Chemical Co., Poole, Dorset.

Results

The effects of tacrine, HP029, HP128, and 3,4-diaminopyridine on chick biventer cervicis preparations

Tacrine, HP029 and 3,4-DAP induced a concentration-dependent increase in the twitch height of indirectly stimulated preparations (Figure 1). The maximal twitch augmentations were 93, 151 and 210% for tacrine (10 μM), HP029 (30 μM) and 3,4-DAP (1 mM), respectively. Higher concentrations of tacrine and HP029 caused less augmentation. In contrast, HP128 (1–100 μM) reduced twitch height in a concentration-dependent way: 10 μM caused a 40% ± 5% reduction in twitch height (Figure 2), and 100 μM abolished the twitch response. HP128 (100 μM) also induced a large and prolonged contracture of indirectly stimulated preparations.

Tacrine and HP029 (10 μM) induced a large increase in the response to exogenously applied acetylcholine (1 mM), whereas the responses to carbachol (20–40 μM) and KCl (30–50 mM) were not significantly different from control (Figure 2a and b). In contrast, 3,4-DAP, at concentrations capable of greatly augmenting indirectly elicited twitches, had no effect on responses to exogenously applied acetylcholine, carbachol or KCl (Figure 2c). HP128 (10 μM) increased responses to added acetylcholine, but reduced the response to carbachol (Figure 2d). The response to KCl was, however, not significantly different from control (Figure 2d).

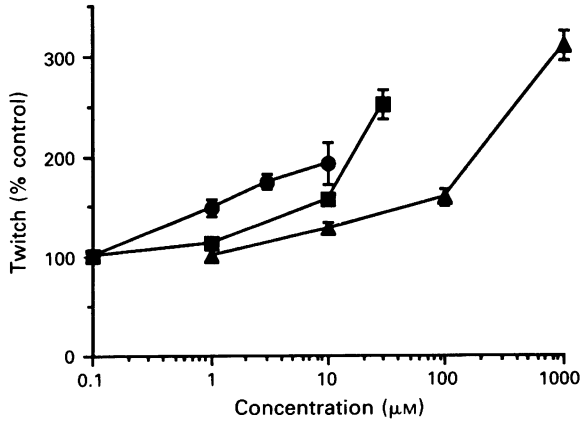

Figure 1 Effects of tacrine (●), HP029 (■), and 3,4-diaminopyridine (▲) on the responses of chick biventer cervicis nerve-muscle preparations to indirect muscle stimulation. Compounds were added cumulatively after the twitch height had stabilized in the presence of each concentration. Each point represents the maximum response for that concentration (means, with s.e. shown by vertical bars, $n = 4$).

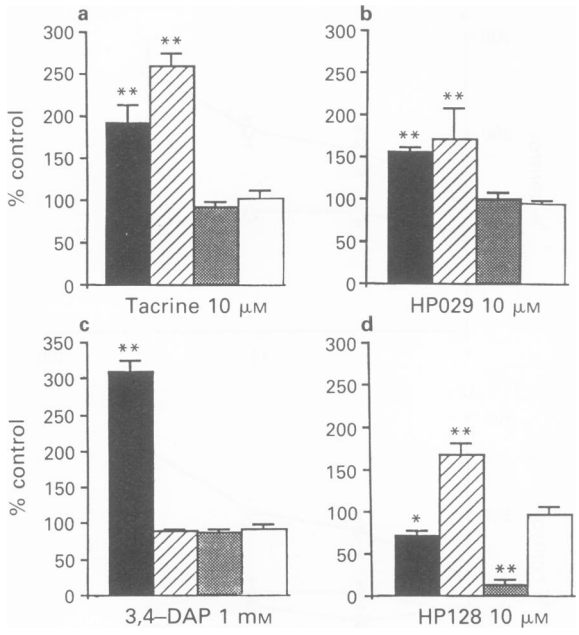

Figure 2 Effects of tacrine, HP029, HP128, and 3,4-diaminopyridine (3,4-DAP) on the responses of chick biventer cervicis nerve-muscle preparations to indirect muscle stimulation (solid columns), acetylcholine (hatched columns), carbachol (stippled columns), and KCl (open columns). Columns represent the means from 4 preparations; s.e. are shown by the vertical bars. Significantly different from control: $*P < 0.05$; $**P < 0.01$.

The effects of tacrine, HP029, HP128, and 3,4-diaminopyridine on mouse diaphragm muscle preparations

In order to investigate if the effects of tacrine and analogues were influenced by the amount of acetylcholine released, we carried out experiments where quantal content was high and where quantal content was reduced, using tubocurarine and low Ca^{2+}/high Mg^{2+}, respectively, to prevent muscle twitching. Experiments were also carried out at 36°C and at room temperature to investigate whether the slowing of action potentials at the lower temperature (see Figure 8) could cause any changes in the effects of the compounds.

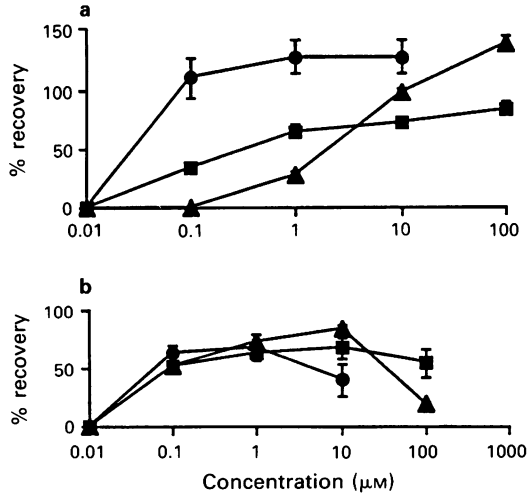

Figure 3 Reversal of (+)-tubocurarine blockade by tacrine (●), HP029 (■), and 3,4-diaminopyridine (▲) on responses of mouse hemidiaphragm nerve-muscle preparations to indirect muscle stimulation. (a) Experiments carried out at room temperature; (b) experiments carried out at 36°C. Points are means of 4 experiments, and s.e. are indicated by the bars unless smaller than symbols. Preparations were stimulated once every 10 s.

Reversal of tubocurarine Tacrine, HP029 and 3,4-DAP reversed a 70% reduction in twitch height induced by tubocurarine (1–5 μM) at both room temperature and at 36°C. At room temperature, the threshold concentration for reversal was 0.01 μM for tacrine and HP029, and 0.1 μM for 3,4-DAP. Complete reversal of tubocurarine paralysis was achieved with 0.1 μM of tacrine and about 10 μM of 3,4-DAP (Figure 3a). In the presence of higher concentrations of tacrine (1–10 μM) and 3,4-DAP (100 μM), augmentation of the maximal twitches could be induced (Figure 3a). The slope of the concentration-effect curve for HP029 was shallower than those for tacrine and 3,4-DAP, and HP029 at 100 μM did not completely reverse the effects of tubocurarine. HP128 (1–10 μM) failed to reverse the blockade produced by tubocurarine at room temperature and higher concentrations (30–100 μM) deepened the block.

At 36°C, the threshold concentration was 0.1 μM for tacrine, HP029 and 3,4-DAP (Figure 3b). However, the compounds only partially reversed the effect of tubocurarine, the degree of reversal being less than that observed at room temperature (Figure 3a). HP128 (1–10 μM) did not reverse the effect of tubocurarine and at 100 μM, HP128 deepened the block.

Reversal of low Ca^{2+}/high Mg^{2+} blockade At room temperature, tacrine and HP029 only partially reversed the 70% reduction in twitch height induced by Krebs solution containing low Ca^{2+} (0.5 mM) and high Mg^{2+} (2.5–5 mM) (Figure 4a). The threshold concentration was around 1 μM for HP029 and 0.1 μM for tacrine, and the maximum recovery was about 60% with both compounds. In contrast, 3,4-DAP (10 μM) produced complete reversal followed by twitch augmentation. At the highest concentration used (1 mM), 3,4-DAP increased twitch height to 215 ± 5% of control. HP128 (0.1–10 μM) was unable to reverse the blockade produced by low Ca^{2+} and high Mg^{2+}, and it deepened the block at concentrations above 10 μM.

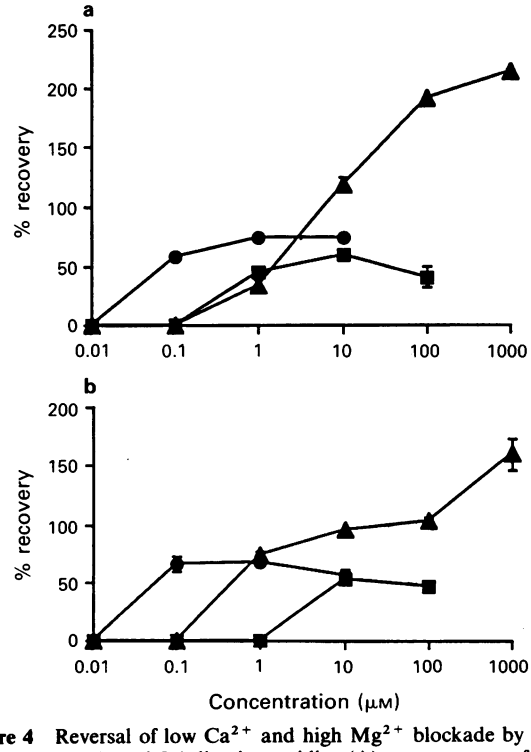

Figure 4 Reversal of low Ca^{2+} and high Mg^{2+} blockade by tacrine (●), HP029 (■), and 3,4-diaminopyridine (▲) on responses of mouse hemidiaphragm nerve-muscle preparations to indirect muscle stimulation. (a) Experiments carried out at room temperature; (b) experiments carried out at 36°C. Points are means of 3–4 experiments, and s.e. are indicated by the bars unless smaller than symbols. Preparations were stimulated once every 10 s.

At 36°C, tacrine and HP029 again only partially reversed the effect of low Ca^{2+} (0.5 mM) and high Mg^{2+} (2.5–5 mM) on indirectly stimulated twitches (Figure 4b). The threshold concentrations for tacrine and 3,4-DAP remained unchanged from those at room temperature, although HP029 was 10 times less active at 36°C (Figure 4). However, the response to 3,4-DAP was quicker in onset but the final twitch height was about half that at room temperature. HP128, once again, was unable to reverse the blockade produced by low Ca^{2+} and high Mg^{2+} and, at concentrations higher than 10 μM, HP128 deepened the blockade.

Intracellular recording

M.e.p.ps in control preparations were about 0.3 to 0.6 mV in amplitude and had a time constant of decay of around 0.6–0.8 ms at a resting membrane potential of −60 to −80 mV. Tacrine (1 μM) and HP029 (10 μM) produced 40% and 15% increases in both m.e.p.p. and e.p.p. amplitude, respectively (Figures 5 and 6). However, higher concentrations of tacrine and HP029 reduced the amplitude of m.e.p.ps to control levels and of the e.p.ps to below control levels. In addition, both tacrine (> 0.1 μM) and HP029 (> 1 μM) increased the decay time of the e.p.ps and m.e.p.ps in a concentration-dependent manner until time course was 250–300% of control (Figure 5 and 6). Neither tacrine nor HP029 changed quantal content (Figures 5 and 6) or m.e.p.p. frequency, but occasionally, in the presence of higher concentrations of tacrine (10–30 μM), giant m.e.p.ps were observed, as previously reported (Thesleff *et al.*, 1990). 3,4-DAP had no effect on m.e.p.p. amplitude or e.p.p. time course (Figure 7). However, 3,4-DAP markedly increased e.p.p. amplitude and quantal content (Figure 7).

HP128 (1–10 μM) did not affect the decay time of e.p.ps or m.e.p.ps, or the quantal content of e.p.ps. However, higher concentrations of HP128 (30–100 μM) greatly reduced the amplitude of e.p.ps and m.e.p.ps.

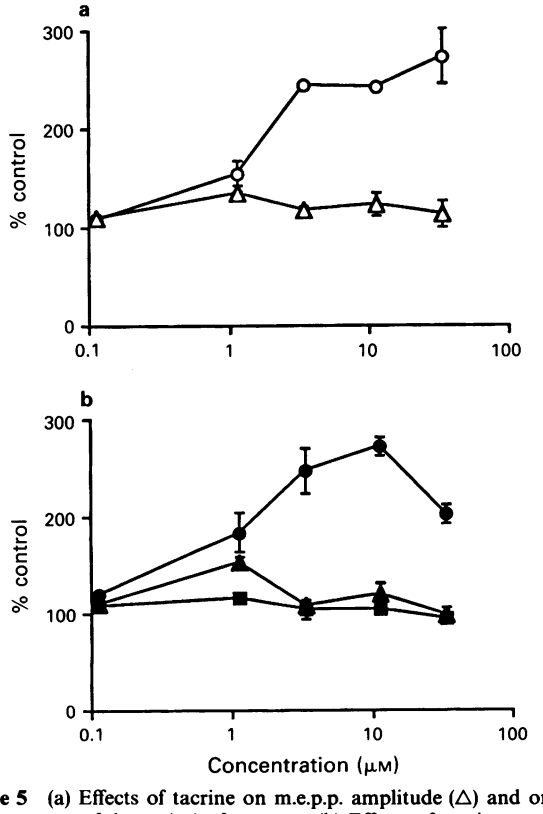

Figure 5 (a) Effects of tacrine on m.e.p.p. amplitude (△) and on the time constant of decay (○) of m.e.p.ps. (b) Effects of tacrine on e.p.p. amplitude (▲), on the time constant decay (●), and on the mean quantal content (■) of e.p.ps. Points are means of 3 experiments, and s.e. are indicated by the bars unless smaller than symbols.

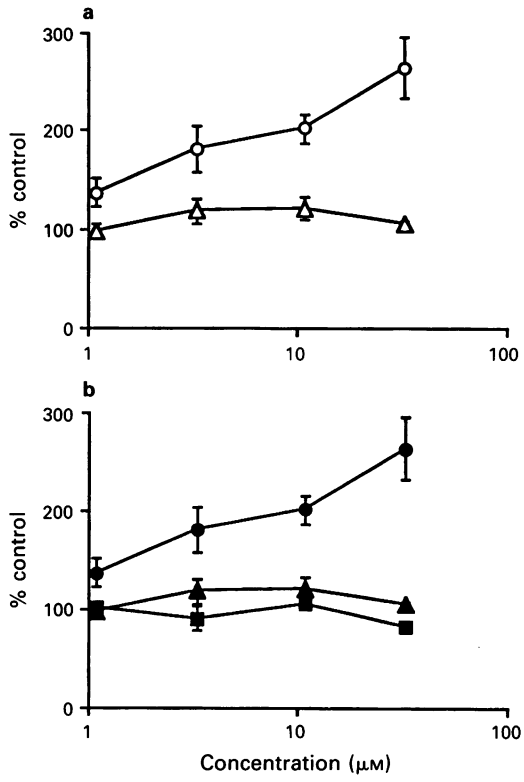

Figure 6 (a) Effects of HP029 on m.e.p.p. amplitude (△) and on the time constant of decay (○) of m.e.p.ps. (b) Effects of HP029 on e.p.p. amplitude (▲), on the time constant decay (●), and on the mean quantal content (■) of e.p.ps. Points are means of 3 experiments, and s.e. are indicated by the bars unless smaller than symbols.

Effects of tacrine, HP029, HP128, and 3,4-DAP on perineural waveforms

The perineural waveform is predominantly made up of two negative deflections, usually preceded by a small positivity as the action potential arrives near the nerve endings. The first

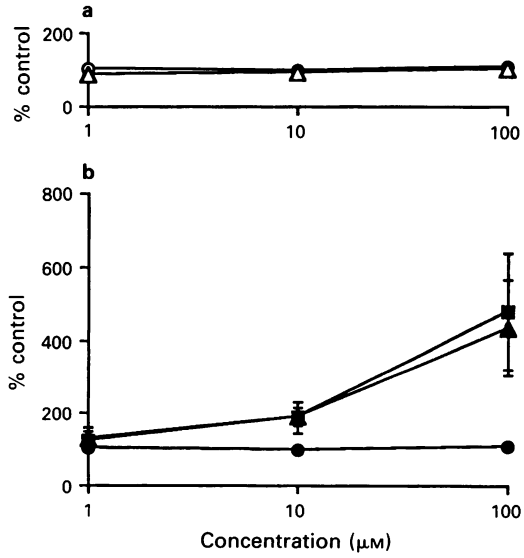

Figure 7 (a) Effects of 3,4-diaminopyridine (3,4-DAP) on m.e.p.p. amplitude (△) and on the time constant of decay (○) of m.e.p.ps. (b) Effects of 3,4-DAP on e.p.p. amplitude (▲), on the time constant decay (●), and on the mean quantal content (■) of e.p.ps. Points are means of 3 experiments, and s.e. are indicated by the bars unless smaller than symbols.

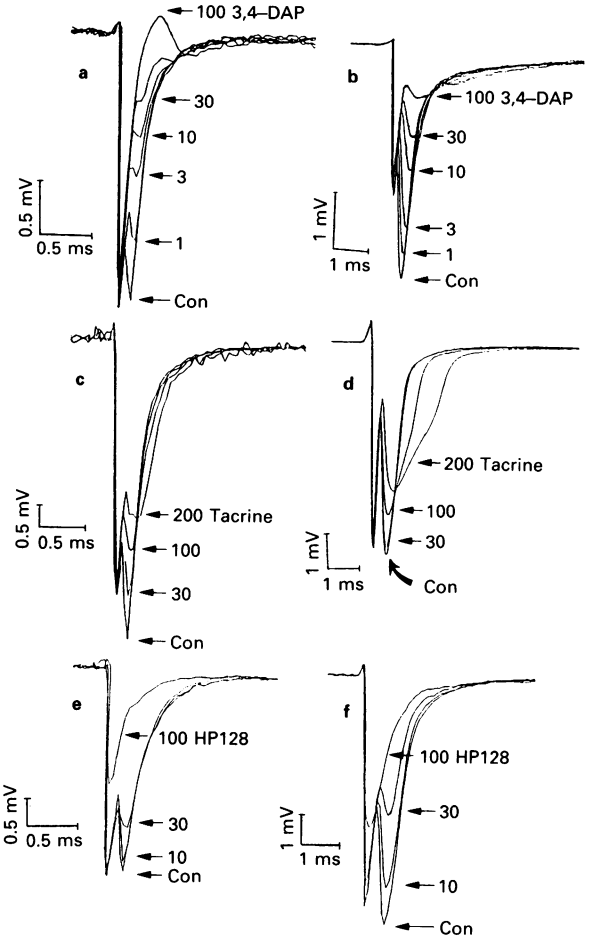

Figure 8 Effects of tacrine, HP128, and 3,4-diaminopyridine (3,4-DAP) on perineural waveforms recorded from mouse triangularis sterni preparations. (a) Superimposed recordings from the same site before (Con) and after the addition of 3,4-DAP (the numbers refer to the micromolar concentrations) at 36°C and (b) room temperature. (c) Effects of tacrine on perineural waveforms at 36°C and (d) room temperature. (e) Effects of HP128 on perineural waveforms at 36°C and (f) room temperature. Each record is an average of 20–25 waveforms. The stimulus artefacts have been omitted, for clarity.

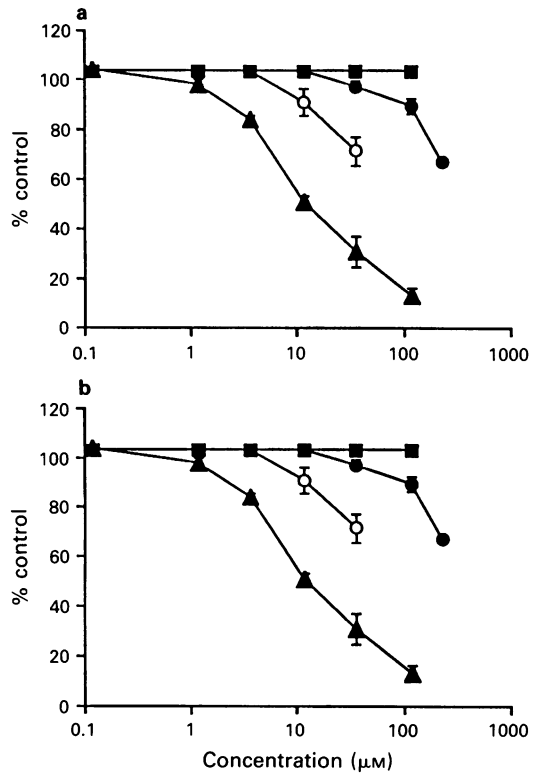

Figure 9 Effects of tacrine (●), HP029 (■), HP128 (○), and 3,4-DAP diaminopyridine (▲) on the second negative deflection of the perineural waveforms, which corresponds to the nerve terminal K^+ current. (a) Experiments carried out at room temperature; (b) experiments carried out at 36°C. Points are means of 3 experiments, and s.e. are indicated by the bars unless smaller than symbols.

negative deflection is associated with the movement of Na^+ ions (inward) at the nodes of Ranvier and heminode, and the second negative deflection is associated with the local circuit current that is generated by the movement of both K^+ (outward) and Ca^{2+} (inward) at the motor nerve terminals (see Brigant & Mallart, 1982; Mallart, 1985; Penner & Dreyer, 1986).

Effects on potassium and calcium currents Tacrine, HP128 and 3,4-DAP at room temperature and 36°C caused a concentration-dependent reduction in the second negative deflection of the perineural waveform (Figures 8 and 9), but did not affect the first negative spike. However, in the presence of higher concentrations of tacrine (300 μM) and HP128 (100 μM), there was a marked reduction on the Na^+-related component. Additionally, at room temperature, tacrine (100–200 μM) markedly prolonged the duration of the perineural waveform (Figure 8). Similar effects with tacrine and HP128 were also observed in the presence of Ca^{2+}-free salt solution (data not shown). However, 3,4-DAP, even at 100 μM, had no effect on the first negative deflection. HP029 (1–100 μM) did not affect the perineural waveform (Figure 9), and at higher concentrations, it blocked the perineural waveform completely.

Effects on sodium currents In order to investigate further if tacrine and HP128 had any effect on the Na^+ channel activity of motor nerves, perineural recordings were carried out in a low Ca^{2+} (0.5 mM) bathing solution and in the presence of maximally effective concentrations of 3,4-DAP (600 μM) and tetraethylammonium (15 mM) to block both voltage-operated and Ca^{2+}-activated K^+ currents in motor nerve terminals. Procaine (50 μM) was used to stop the spontaneous activity produced by the combination of 3,4-DAP and tetraethylammonium; this concentration of procaine had no effect on the first negative deflection. When tacrine (30–200 μM) or HP128 (10–100 μM) was added to the bath solution, the Na^+ spike was blocked in a concentration-dependent way (Figures 10 and 11).

Discussion

The results obtained with tacrine, HP029 and 3,4-DAP on the chick biventer cervicis and the mouse hemidiaphragm preparations show that these compounds can augment neuromuscular transmission. As previously demonstrated biochemically (Shutske *et al.*, 1989), tacrine and analogues have anticholinesterase activity, as they are able to increase the response to exogenously applied acetylcholine on chick biventer cervicis preparations. The anticholinesterase action of tacrine and HP029 explains the increase in m.e.p.p. and e.p.p. amplitude and time to half decay in the triangularis sterni preparation, since similar effects are observed with other anticholinesterase agents. Thus, the anticholinesterase activity of tacrine and HP029 could explain the twitch augmenting action seen in the chick biventer cervicis preparation and the facilitatory activity of these compounds under normal quantal content (reversal of tubocurarine blockade) and under impaired quantal content (reversal of low Ca^{2+}/high Mg^{2+} blockade) in the mouse hemidiaphragm preparation. HP128 did not augment twitch height, which may be a consequence of its anticholinesterase action being weaker than that of

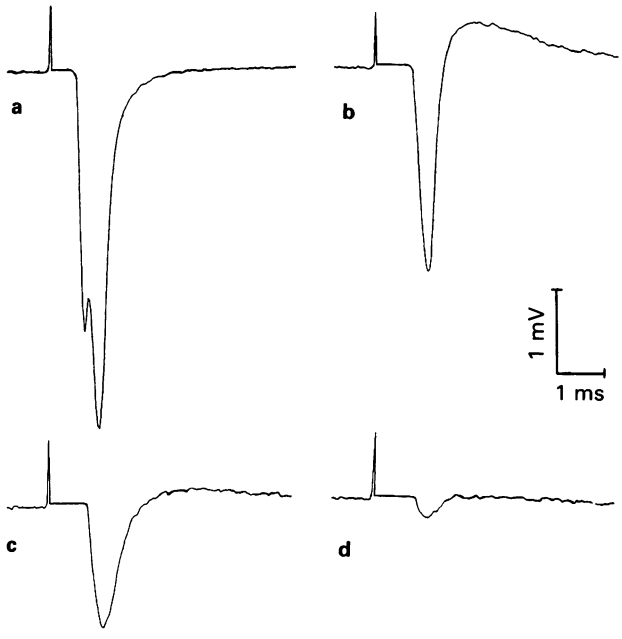

Figure 10 Effect of tacrine on the perineural waveform of a mouse triangularis sterni preparation in the presence of low Ca^{2+} (0.5 mM) bathing solution, 3,4-diaminopyridine (3,4-DAP), tetraethylammonium (TEA), and procaine. (a) Averaged control waveform in the presence of low Ca^{2+}; (b) after exposure to 3,4-DAP (600 μM), TEA (15 mM) and procaine (50 μM); (c) after tacrine 30 μM; and (d) after tacrine 200 μM. Each record is an average of 20–25 waveforms. The initial upward deflection is the stimulus artefact.

tacrine or HP029 (Shutske *et al.*, 1989), or HP128 may have additional blocking effects on neuromuscular transmission. HP128 reduced carbachol contractures of the chick biventer cervicis' preparation and reduced m.e.p.p. amplitude in the mouse triangularis sterni preparation, indicating a postjunctional blocking activity. 3,4-DAP did not increase the response to exogenously applied acetylcholine on the chick biventer cervicis preparation and did not alter the amplitude and time to half decay of the m.e.p.ps on the mouse triangularis sterni preparation, confirming its lack of effect on acetylcholinesterase activity.

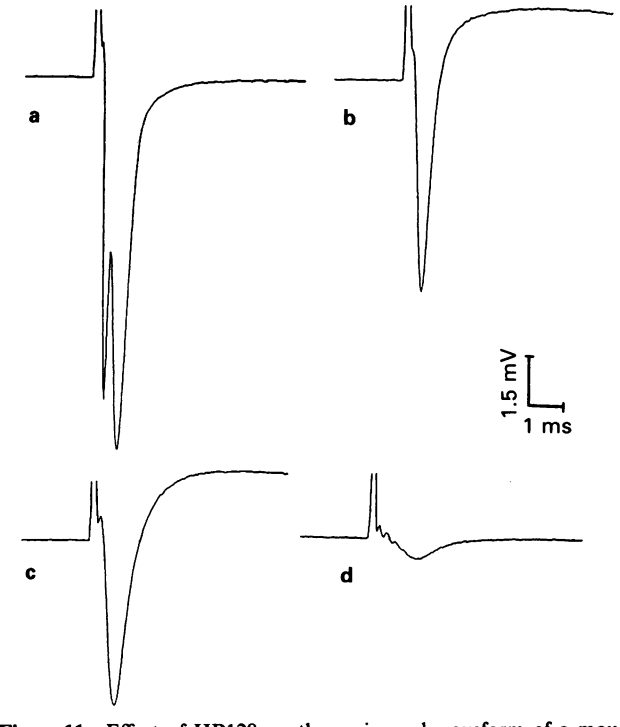

Figure 11 Effect of HP128 on the perineural waveform of a mouse triangularis sterni preparation in the presence of low Ca^{2+} (0.5 mM) bathing solution, 3,4-diaminopyridine (3,4-DAP), tetraethylammonium (TEA), and procaine. (a) Averaged control waveform in the presence of low Ca^{2+}; (b) after exposure to 3,4-DAP (600 nM), TEA (15 mM) and procaine (50 μM); (c) after HP128 10 μM; and (d) after HP128 100 μM. Each record is an average of 20–25 waveforms. The initial upward deflection is the stimulus artefact.

However, because of the structural similarity of tacrine and aminopyridines, which are classical K^+ blockers, and the previous demonstration that tacrine can increase quantal content and block K^+ channels in mouse neuromuscular preparations at room temperature (Harvey & Rowan, 1988; 1990), we wanted to investigate if tacrine and analogues were able to block K^+ channels and facilitate acetylcholine release in the triangularis sterni preparation of mouse at a more physiological temperature.

As expected (Harvey & Marshall, 1977a, b; Durant & Marshall, 1980; Molgo *et al.*, 1980; Harvey & Rowan, 1988; 1990), 3,4-DAP markedly increased quantal content, and this increase in acetylcholine release would explain the facilitatory activity and anti-curare actions seen in the twitch experiments. In contrast, tacrine and analogues had no demonstrable presynaptic facilitatory activity at 36°C. Perineural recording showed that 3,4-DAP reduced the second negative deflection, confirming its K^+ blocking activity at motor nerve terminals, as previously reported (Brigant & Mallart, 1982; Harvey & Rowan, 1988; 1990). Tacrine and HP128, but not HP029, were also able to block the K^+-dependent portion of the nerve terminal waveform. Since this action was also observed in Ca^{2+}-free solution, the effects of tacrine and HP128 on the second negative deflection of the perineural waveform were not via an effect on the Ca^{2+} current. Furthermore, as previous demonstrated (Harvey & Rowan, 1988), tacrine (100–200 μM) caused a marked prolongation of the perineural waveform at room temperature. However, at 36°C, this effect was very small, even at the highest concentrations used. These findings suggest that, at a more physiological temperature, the kinetics of the ion channels and their sensitivity to drugs may be different. This difference might be relevant to the control of transmitter release as tacrine was able to increase transmitter release at room temperature (Harvey & Rowan, 1990) but had no demonstrable facilitatory activity at 36°C.

Additionally, tacrine and HP128 are not very selective for nerve terminal K^+ currents. In the presence of maximally effective concentrations of 3,4-DAP and TEA in a low Ca^{2+} solution, tacrine and HP128 blocked the Na^+-dependent portion of the nerve terminal waveform. Similar findings with tacrine were seen earlier in unmyelinated invertebrate axons (Schauf & Sattin, 1987; Elinder *et al.*, 1989), although only K^+ channels seem to be affected in another invertebrate (Drukarch *et al.*, 1987). Tacrine also blocks A currents in hippocampal neurones but at high concentrations it blocks the slowly activating K^+ currents and the Na^+ current (Rogawski, 1987). Moreover, most of the effects of tacrine on neuronal K^+ currents require high concentrations of tacrine (30–500 μM), which are much higher than those measured in serum of patients treated with tacrine (Summers *et al.*, 1986).

However, tacrine and analogues may have another action that might be clinically relevant. For example, tacrine, HP029 and HP128 are able to block the neuronal uptake of noradrenaline, 5-hydroxytryptamine and dopamine (Drukarch *et al.*, 1987; Shutske *et al.*, 1989). Our results showed that tacrine and analogues have additional blocking effects on neuromuscular transmission, as HP128 reduced carbachol contractures in the chick biventer cervicis preparation, and tacrine, HP029 and HP128, at the highest concentrations used, reduced m.e.p.p. amplitude in the mouse triangularis sterni preparations, indicating a postjunctional blocking activity that could be masking any facilitatory activity of these compounds.

We thank Hoechst-Roussel Pharmaceuticals Inc for the gift of HP029 and HP128.

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(Received November 12, 1990
Revised December 17, 1990
Accepted January 3, 1991)

Br. J. Pharmacol. (1991), **102**, 916–918

Effects of helospectin I on insulin and glucagon secretion in the mouse

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1 The helospectins are peptides structurally related to helodermin, vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine (PHI) and secretin, which all potently stimulate glucagon secretion in the mouse. Therefore, the effects of helospectin I (0.1–0.8 nmol kg^{-1}) on insulin and glucagon secretion under basal conditions and after stimulation with glucose (2.8 mmol kg^{-1}) or the cholinoceptor agonist, carbachol (0.16 μmol kg^{-1}), were examined *in vivo* in the mouse.

2 Helospectin I potently increased plasma levels of glucagon after its intravenous injection in mice. The increase was observed after only 2 min, and was evident also after 6 min.

3 In contrast, plasma insulin levels were not altered by helospectin I after 2 min, but slightly increased after 6 min. Plasma glucose levels were not altered by the peptide.

4 Carbachol-induced glucagon secretion was markedly potentiated by helospectin I. In contrast, glucose- or carbachol-stimulated insulin secretion was not affected by the peptide.

5 In conclusion, helospectin I markedly stimulates glucagon secretion in the mouse whereas the peptide has no direct action on insulin secretion. This pattern of effect of helospectin I is similar to that previously reported for helodermin, VIP, PHI and secretin in the mouse, i.e., for all peptides belonging to this superfamily of peptides.

Keywords: Helospectin; glucagon; insulin secretion *in vivo* in mouse

Introduction

Helospectin I and helospectin II are two helodermin-like peptides isolated from the venom of the Gila monster (Parker *et al.* 1984). They show structural relationship with vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine (PHI), secretin, and glucagon, i.e., to peptides belonging to the glucagon peptide superfamily (Parker *et al.*, 1984; Robberecht *et al.*, 1985; Table 1). Furthermore, the helospectins and helodermin have been demonstrated to occur also in mammalian tissue (Robberecht *et al.*, 1985; Grunditz *et al.*, 1989; Bjartell *et al.*, 1989). The helospectins have previously been demonstrated to produce relaxation in rat isolated femoral arteries and to reduce blood pressure when injected intravenously in rats (Grundemar & Högestätt, 1990). Since similar effects were induced by helodermin and VIP, it was proposed that helospectin I and helospectin II act on the same receptors as these peptides (Grundemar & Högestätt, 1990). This raised the possibility that all the peptides belonging to the VIP/PHI/secretin/glucagon peptide family have the same profile of effects. A marked stimulation of glucagon secretion when helodermin, VIP, PHI or secretin was injected intravenously into mice was demonstrated previously, whereas insulin secretion was stimulated only weakly or not at all (Ahrén & Lundquist 1982a; 1986; 1988; Ahrén 1989). To discover whether the helospectins also potently stimulate glucagon secretion, the present study was carried out and the effects of helospectin I on basal and stimulated glucagon secretion in the mouse were studied. The cholinoceptor agonist carbachol was used as a glucagonotropic substance. The effects of helospectin I on glucose- and carbachol-stimulated insulin secretion were also studied.

Methods

Animals

Female mice of the NMRI strain (Anticimex, Stockholm, Sweden) weighing 25–30 g, were used throughout the experiments. The animals were given a standard pellet diet (Astra-

Table 1 Amino acid sequence of peptides belonging to the helodermin/vasoactive intestinal polypeptide (VIP)/peptide histidine isoleucine (PHI)/secretin peptide superfamily

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Helospectin I	His	Ser	Asp	Ala	Thr	Phe	Thr	Ala	Glu	Tyr	Ser	Lys	Leu	Leu	Ala	Lys	Leu	Ala	Leu
Helospectin II	His	Ser	Asp	Ala	Thr	Phe	Thr	Ala	Glu	Tyr	Ser	Lys	Leu	Leu	Ala	Lys	Leu	Ala	Leu
Helodermin	His	Ser	Asp	Ala	Ile	Phe	Thr	Gln	Glu	Tyr	Ser	Lys	Leu	Leu	Ala	Lys	Leu	Ala	Leu
VIP (rat)	His	Ser	Asp	Ala	Val	Phe	Thr	Asp	Asn	Tyr	Thr	Arg	Leu	Arg	Lys	Gln	Met	Ala	Val
PHI (rat)	His	Ala	Asp	Gly	Val	Phe	Thr	Ser	Asp	Tyr	Ser	Arg	Leu	Leu	Gly	Gln	Ile	Ser	Ala
Secretin (pig)	His	Ser	Asp	Gly	Thr	Phe	Thr	Ser	Glu	Leu	Ser	Arg	Leu	Arg	Asp	Ser	Ala	Arg	Leu
Glucagon (human)	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala

	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Halospectin I	Gln	Lys	Tyr	Leu	Glu	Ser	Ile	Leu	Gly	Ser	Ser	Thr	Ser	Pro	Arg	Pro	Pro	Ser	Ser
Helospectin II	Gln	Lys	Tyr	Leu	Glu	Ser	Ile	Leu	Gly	Ser	Ser	Thr	Ser	Pro	Arg	Pro	Pro	Ser	
Helodermin	Gln	Lys	Tyr	Leu	Ala	Ser	Ile	Leu	Gly	Ser	Arg	Thr	Ser	Pro	Pro	Pro			
VIP (rat)	Lys	Lys	Tyr	Leu	Asn	Ser	Ile	Leu	Asn										
PHI (rat)	Lys	Lys	Tyr	Leu	Glu	Ser	Leu	Ile											
Secretin (pig)	Gln	Arg	Leu	Leu	Gln	Gly	Leu	Val											
Glucagon (human)	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr									

Ewos, Södertälje, Sweden) and tap water *ad libitum* before and during the experiments.

Experiments

Unanaesthetized mice were injected intravenously in a tail vein with synthetic helospectin I (Peninsula Labs, Belmonte, Ca, U.S.A.; dissolved in saline + 0.1% gelatine) alone (0.1–0.8 nmol kg^{-1}) or in combination with D-glucose (British Drug Houses Ltd., Poole, England; 2.8 mmol kg^{-1}) or the cholinoceptor agonist, carbachol (British Drug Houses Ltd., Poole, England; 0.16 μmol kg^{-1}). The volume load was 10 μl g^{-1}. Controls were given saline/gelatine. Blood samples were taken from the retrobulbar plexus at 2 or 6 min after the intravenous injection. At 2 min, the maximal increases in plasma insulin levels after injection of glucose or carbachol and in plasma glucagon levels after intravenous injection of carbachol are seen (Ahrén & Lundquist 1981; 1986).

Determinations

Immediately after blood sampling, plasma was separated and stored at −20°C until analyses. Plasma levels of insulin and glucagon were determined radioimmunochemically (Herbert *et al.*, 1965; Ahrén & Lundquist, 1982b). For insulin radioimmunoassay, guinea-pig anti-porcine insulin antiserum (MILAB, Malmö, Sweden), ^{125}I-labelled porcine insulin and, as standard, porcine insulin (Novo Res, Bagsvaerd, Denmark) were used. For glucagon radioimmunoassay, a rabbit anti-porcine glucagon antiserum specific for pancreatic glucagon and ^{125}I-labelled porcine glucagon (MILAB, Malmö, Sweden) and, as standard, human glucagon were used. The insulin and glucagon antibodies do not cross-react with helospectin I. The separation of bound and free radioactivity was performed, in both assays, by the dextran-coated charcoal technique. In the insulin assay, a change of 0.3 μu/tube (= 3 μu ml^{-1}) is detected with 95% confidence, and 4 μu/tube (= 40 μu ml^{-1}) displaces 50% specific tracer activity. In the glucagon assay, a change of 0.75 pg/tube (= 7.5 pg ml^{-1}) is detected with 95% confidence, and 12.5 pg/tube (= 125 pg ml^{-1}) displaces 50% of specific tracer activity. Finally, plasma glucose levels were determined with the glucose oxidase technique.

Statistics

Means ± s.e.mean are given. Student's unpaired *t* test was used as a test of significance. When calculating the increase in plasma insulin and glucagon levels above basal, as demonstrated in the figures, the plasma insulin and glucagon levels in the saline-injected group were subtracted from those in the experimental group and the weighted s.e.mean was calculated, taking into account the s.e.mean of both groups. Therefore, saline-injected controls were included in all individual experiments.

Results

Basal levels of glucagon, insulin, and glucose

Basal plasma glucagon levels were significantly enhanced after the intravenous injection of helospectin I after 2 min (Figure 1). In contrast, plasma insulin of glucose levels were not altered. At 6 min after intravenous injection of helospectin I, plasma glucagon levels were markedly elevated, and, at this time, plasma insulin levels were also increased (Figure 2). In contrast, plasma glucose levels were, again, not altered.

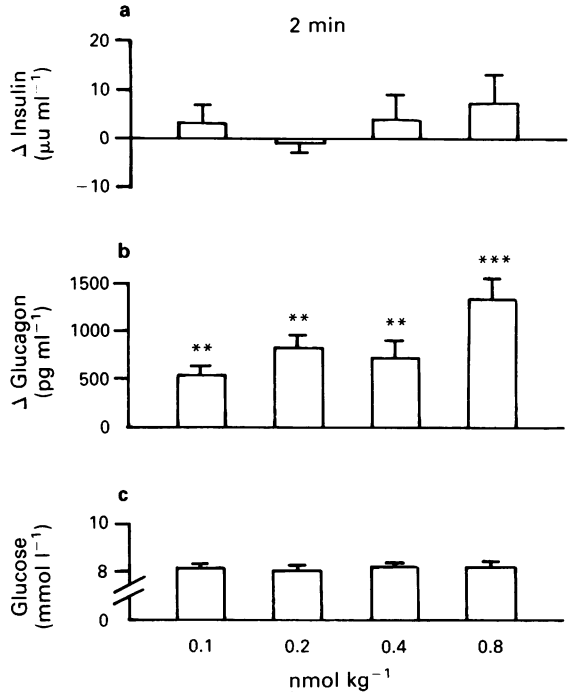

Figure 1 Changes in plasma levels of insulin (a), glucagon (b) and plasma levels of glucose (c) at 2 min after the intravenous injection of helospectin I at 0.1–0.8 nmol kg^{-1} in mice. Plasma levels in saline-injected controls were: insulin: 12 ± 3 μu ml^{-1}, glucagon 389 ± 86 pg ml^{-1}, glucose 8.0 ± 0.2 mmol l^{-1}. There were 20 animals in each group. Means are shown with s.e.mean indicated by vertical bars. Asterisks indicate the probability level of random difference versus the controls: ** $P < 0.01$; *** $P < 0.001$.

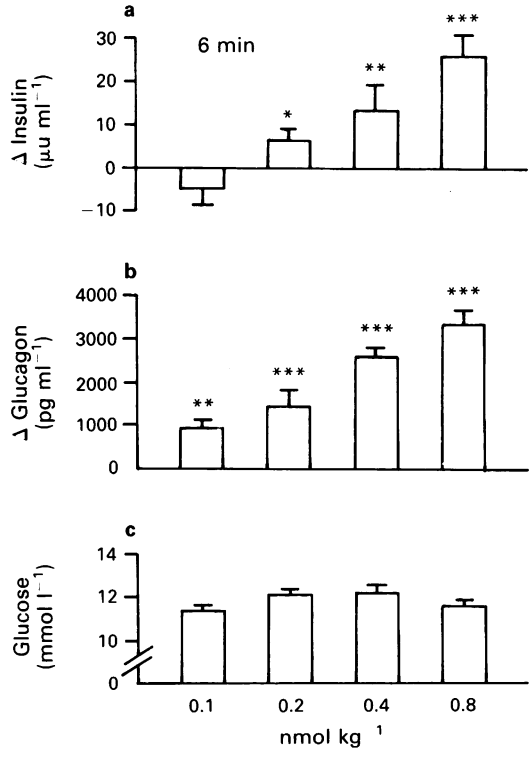

Figure 2 Changes in plasma levels of insulin (a), glucagon (b) and plasma levels of glucose (c) at 6 min after the intravenous injection of helospectin I at 0.1–0.8 nmol kg^{-1} in mice. Plasma levels in saline-injected controls were: insulin: 18 ± 4 μu ml^{-1}, glucagon 495 ± 106 pg ml^{-1}, glucose 10.2 ± 0.3 mmol l^{-1}. There were 20 animals in each group. Means are shown with s.e.mean indicated by vertical bars. Asterisks indicate the probability level of random difference versus the saline-injected controls: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

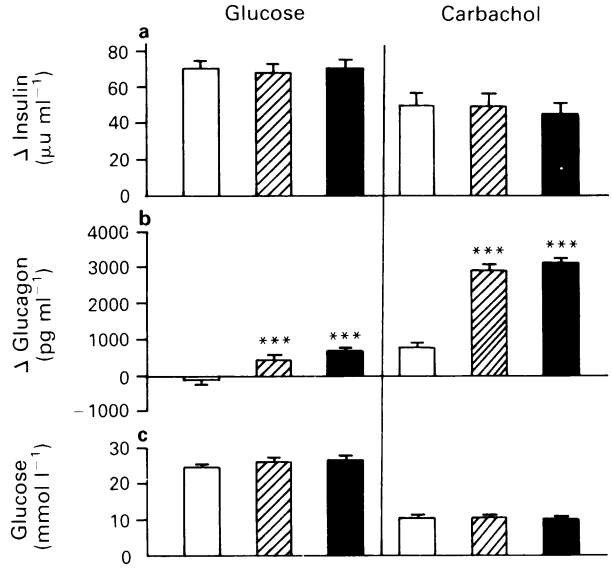

Figure 3 Changes in plasma levels of insulin (a), glucagon (b) and plasma levels of glucose (c) at 2 min after the intravenous injection of glucose (2.8 mmol kg^{-1}) or the cholinoceptor agonist, carbachol (0.16 μmol kg^{-1}) alone (control, open columns) or together with helospectin I, 0.2 (hatched columns) or 0.8 nmol kg^{-1} (solid columns) in mice. Plasma levels in saline-injected controls were: insulin 9 ± 2 μu ml^{-1}, glucagon 355 ± 46 pg ml^{-1}, and glucose 8.1 ± 0.2 mmol l^{-1}. Asterisks indicate the probability level of random difference versus the glucose- or carbachol-injected controls. *** $P < 0.001$.

Glucose- and carbachol-stimulated levels of glucagon, insulin, and glucose

Glucose and carbachol elevated plasma levels of insulin ($P < 0.001$). Helospectin I did not affect these increases (Figure 3a). Carbachol also increased plasma levels of glucagon ($P < 0.001$). Helospectin I, both at 0.2 and 0.8 nmol kg^{-1}, markedly potentiated this increase. Helospectin I also increased plasma glucagon levels after injection of glucose (Figure 3b). Plasma glucose levels after injection of glucose or carbachol were not altered by helospectin I (Figure 3c).

Discussion

Helospectin I was found to stimulate markedly basal glucagon secretion and to potentiate carbachol-stimulated glucagon secretion in mice. The marked stimulation of glucagon secretion is also evident from its potent stimulatory action during hyperglycaemia. In contrast, its stimulatory effect on insulin secretion was weak and evident only at a late time point when it is likely to be mediated by glucagon and not directly by helospectin I. Helospectin I is thus similar to VIP, secretin, PHI and helodermin in markedly stimulating glucagon secretion but affecting insulin secretion only marginally (Ahrén & Lundquist, 1981; 1986; 1988; Ahrén, 1989). This suggests that the peptides affect the same mechanisms in the islet A-cells. A similar identity of mechanism for the helospectins I and II, helodermin and VIP was recently suggested for their vascular effects (Grundemar & Högestätt, 1990). It is known that carbachol activates cholinoceptors which initiate phosphoinositide hydrolysis in the islets (Ahrén *et al.*, 1990). However, whether helospectin I and II, helodermin, and VIP augment this process is not known. Nonetheless, these peptides show a high degree of structural similarities (Table 1), which suggests that they activate the same receptors. Previously, both VIP and helodermin have been shown to activate exocrine pancreatic adenylate cyclase (Vandermeers *et al.*, 1984). Whether a similar action is exerted in the islets is not known. In any case, the present results suggest that helospectin I initiates the same islet mechanisms as the other peptides belonging to the helodermin/VIP/PHI/secretin peptide family with marked stimulation of glucagon secretion as the predominant action.

The author is grateful to Lena Kvist and Lilian Bengtsson for expert technical assistance. The study was supported by the Swedish Medical Research Council (Grants No 14x-6834 and 12x-712), Nordisk Insulinfond, Swedish Diabetes Association, Albert Påhlssons, Crafoordska and Magn Bergvalls Foundations, Sw Hoechst Diabetes Fund, and The Faculty of Medicine, Lund.

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(Received July 11, 1990
Revised November 20, 1990
Accepted November 26, 1990)

Br. J. Pharmacol. (1991), **102**, 919–925

Characteristics of 5-HT_3 binding sites in NG108-15, NCB-20 neuroblastoma cells and rat cerebral cortex using [^{3}H]-quipazine and [^{3}H]-GR65630 binding

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1 The biochemical and pharmacological properties of 5-HT_3 receptors in homogenates of NG108-15 and NCB-20 neuroblastoma cells and rat cerebral cortex have been ascertained by the use of [^{3}H]-quipazine and [^{3}H]-GR65630 binding.

2 In NG108-15 and NCB-20 cell homogenates, [^{3}H]-quipazine bound to a single class of high affinity (NG108-15: $K_d = 6.2 \pm 1.1$ nM, $n = 4$; NCB-20: $K_d = 3.0 \pm 0.9$ nM, $n = 4$; means ± s.e.means) saturable (NG108-15: $B_{max} = 1340 \pm 220$ fmol mg^{-1} protein; NCB-20: $B_{max} = 2300 \pm 200$ fmol mg^{-1} protein) binding sites. In rat cortical homogenates, [^{3}H]-quipazine bound to two populations of binding sites in the absence of the 5-hydroxytryptamine (5-HT) uptake inhibitor, paroxetine ($K_{d1} = 1.6 \pm 0.5$ nM, $B_{max1} = 75 \pm 14$ fmol mg^{-1} protein; $K_{d2} = 500 \pm 300$ nM, $B_{max2} = 1840 \pm 1040$ fmol mg^{-1} protein, $n = 3$), and to a single class of high affinity binding sites ($K_d = 2.0 \pm 0.5$ nM, $n = 3$; $B_{max} = 73 \pm 6$ fmol mg^{-1} protein) in the presence of paroxetine. The high affinity (nanomolar) component probably represented 5-HT_3 binding sites and the low affinity component represented 5-HT uptake sites.

3 [^{3}H]-paroxetine bound with high affinity ($K_d = 0.02 \pm 0.003$ nM, $n = 3$) to a site in rat cortical homogenates in a saturable ($B_{max} = 323 \pm 45$ fmol mg^{-1} protein, $n = 3$) and reversible manner. Binding to this site was potently inhibited by 5-HT uptake blockers such as paroxetine and fluoxetine (pK_is = 8.6–9.9), while 5-HT_3 receptor ligands exhibited only low affinity ($pK_i < 7$). No detectable specific [^{3}H]-paroxetine binding was observed in NG108-15 or NCB-20 cell homogenates.

4 [^{3}H]-quipazine binding to homogenates of NG108-15, NCB-20 cells and rat cortex (in the presence of 0.1 μM paroxetine) exhibited similar pharmacological characteristics. 5-HT_3 receptor antagonists competed for [^{3}H]-quipazine binding with high nanomolar affinities in the three preparations and the rank order of affinity was: (S)-zacopride > quarternized ICS 205-930 ≥ granisetron > ondansetron > ICS 205-209 ≥ (R)-zacopride > quipazine > renzapride > MDL-72222 > butanopride > metoclopramide.

5 [^{3}H]-GR65630 labelled a site in NCB-20 cell homogenates with an affinity ($K_d = 0.7 \pm 0.1$ nM, $n = 4$) and density ($B_{max} = 1800 \pm 1000$ fmol mg^{-1} protein) comparable to that observed with [^{3}H]-quipazine. Competition studies also indicated a good correlation between the pharmacology of 5-HT_3 binding sites when [^{3}H]-GR65630 and [^{3}H]-quipazine were used in these cells.

6 In conclusion, [^{3}H]-quipazine labelled 5-HT_3 receptor sites in homogenates of NG108-15 cells, NCB-20 cells and rat cerebral cortex. In rat cortical homogenates, [^{3}H]-quipazine also bound to 5-HT uptake sites, which could be blocked by 0.1 μM paroxetine. The pharmacological specificity of the 5-HT_3 receptor labelled by [^{3}H]-quipazine was similar in the neuroblastoma cells and rat cortex and was substantiated in NCB-20 cells by the binding profile of the selective 5-HT_3 receptor antagonist, [^{3}H]-GR65630.

Keywords: 5-HT_3 receptors; NCB-20 cells; NG108-15 cells; [^{3}H]-quipazine; [^{3}H]-GR65630

Introduction

Pharmacological studies have led to the differentiation and classification of 5-hydroxytryptamine (5-HT) receptors into at least three major classes, namely 5-HT_1, 5-HT_2, and 5-HT_3 (see Bradley *et al.*, 1986). 5-HT_1 receptors have been further subdivided into 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D} subtypes (Bradley *et al.*, 1986). Functional data obtained with 5-HT_3 antagonists (Round & Wallis, 1986; Fozard, 1984) have indicated the possible existence of multiple 5-HT_3 receptors although these may be attributed, in part, to species differences (Bradley *et al.*, 1986; Richardson & Engel, 1986; Butler *et al.*, 1990). *In vitro* or *in vivo* stimulation of 5-HT_3 receptors evokes depolarization (Neijt *et al.*, 1988a; Lambert *et al.*, 1989; Higgins *et al.*, 1989) and release of acetylcholine (Barnes *et al.*, 1989a) and dopamine (Blandina *et al.*, 1988). 5-HT_3 receptor antagonists act as potent antiemetics in animals and man (Cunningham *et al.*, 1987; Smith *et al.*, 1988a,b; Butler *et al.*, 1988; Higgins *et al.*, 1989), and as anxiolytics (Jones *et al.*, 1988), antipsychotics (Costall *et al.*, 1987) and inhibitors of withdrawal from drugs of abuse (Costall *et al.*, 1990) in animal models.

Radioligands such as [^{3}H]-ICS 205-930 (Hoyer & Neijt, 1988), [^{3}H]-quaternized ICS 205-930 (Watling *et al.*, 1988), [^{3}H]-GR65630 (Kilpatrick *et al.*, 1987), [^{3}H]-quipazine (Schmidt *et al.*, 1989; Milburn & Peroutka, 1989), [^{3}H]-zacopride (Barnes *et al.*, 1988; 1989b; Pinkus *et al.*, 1990), [^{3}H]-BRL43694 (Nelson & Thomas, 1989) and [^{3}H]-LY278584 (Wong *et al.*, 1989) have been used to label 5-HT_3 receptors in tissue homogenates (see above) and on tissue sections in autoradiographic studies (Waeber *et al.*, 1988; 1989; Higgins *et al.*, 1989; Kilpatrick *et al.*, 1989). While [^{3}H]-quipazine binds with high affinity to 5-HT_3 receptors and 5-HT uptake sites in the rat cerebral cortex (Peroutka, 1988; Milburn & Peroutka, 1989), [^{3}H]-GR65630 has been reported to exhibit selective binding to 5-HT_3 receptors (Kilpatrick *et al.*, 1987).

5-HT_3 binding sites in NG108-15 and N1E-115 neuroblastoma cells have been labelled with [^{3}H]-ICS 205-930 (Hoyer & Neijt, 1988; Neijt *et al.*, 1988a,b) and identified as functional 5-HT_3 receptors (Neijt *et al.*, 1988a,b; Lambert *et al.*, 1989). However, there is relative little information on the use of other

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radioligands for the study of 5-HT_3 receptors in these and other cells. In addition, the binding properties of 5-HT_3 receptors in the NCB-20 neuroblastoma cell-line have not been investigated to-date even though functional 5-HT_3 receptors have been characterized in these cells by electrophysiological techniques (Lambert *et al.*, 1989).

In the present study, the pharmacological properties of [^{3}H]-quipazine binding to 5-HT_3 receptor binding sites in the rat cerebral cortex have been examined in the presence or absence of the 5-HT uptake inhibitor, paroxetine. Furthermore, 5-HT_3 receptors on NG108-15 and NCB-20 neuroblastoma cells have been characterized by use of [^{3}H]-quipazine and [^{3}H]-GR65630. These studies have confirmed the previously known specificity of [^{3}H]-GR65630, and shown [^{3}H]-quipazine to label 5-HT_3 receptors and 5-HT uptake sites in the rat cortex, but only 5-HT_3 sites in neuroblastoma cells. These studies have also highlighted the high density of 5-HT_3 receptors on NG108-15 and NCB-20.

A preliminary account of the present studies has been published (Sharif *et al.*, 1990).

Methods

Cell culture

Mouse neuroblastoma—rat glioma hybridoma cells of the clone NG108-15 were grown in Dulbecco's modified Eagle's medium (DMEM). The media were supplemented with 10% foetal calf serum (Cellect) and HAT (hypoxanthine 0.1 mM, aminoterin 0.4 μM, and thymidine 16 μM). Cells were cultured in 150 cm^2 flasks at 37°C in 10% CO_2, fed every third day and subcultured every 7 days. The cells were grown to a density of 6×10^6 cells/flask and harvested by incubating with 0.25% trypsin for 1 min at 25°C.

Mouse neuroblastoma-hamster brain hybridoma cells of the clone NCB-20 cells were grown in high glucose DMEM medium containing 10% newborn calf serum and HAT supplement. Cells were cultured in 150 cm^2 flasks at 37°C in 10% CO_2, fed every third day and subcultured every 7 days. For experimental use the cells were grown to a density of 10^6 cells/flask and detached by incubation with 0.05% EDTA in phosphate buffered saline (PBS) for 1 min at 25°C.

Cell homogenate preparation

Harvested cells were centrifuged at 500 *g* for 6 min. The supernatant was discarded and the cell pellet was homogenized in 50 mM Tris HCl, 5 mM Na_2EDTA buffer (pH 7.4 at 4°C) in a polytron P10 tissue disrupter (setting 6; 2×10 s bursts). The homogenate was centrifuged at 48,000 *g* for 12 min. The pellets were washed, by resuspension and centrifugation, once in homogenizing buffer and twice in 50 mM Tris HCl, 0.5 mM EDTA buffer (pH 7.4 at 4°C). The homogenates were stored under liquid nitrogen (−70°C) until required.

Rat cerebral cortex homogenate preparation

Homogenates were prepared from the cerebral cortices of rat brains obtained from Pel-Freez. Tissues were homogenized in 50 mM Tris HCl, 5 mM Na_2EDTA (pH 7.4 at 4°C) in a polytron P10 tissue disrupter (setting 10, 2×10 s bursts). The homogenate was centrifuged at 48,000 *g* for 12 min and the pellet obtained was washed by resuspension and centrifugation, once in homogenizing buffer, and twice in resuspension buffer (50 mM Tris HCl, 0.5 mM EDTA, pH 7.4 at 4°C). Membranes were stored under liquid nitrogen until required.

Ligand binding studies

Binding assays for [^{3}H]-quipazine and [^{3}H]-GR65630 were conducted in Tris-Krebs (pH 7.4) assay buffer of the following composition (mM): NaCl 154, KCl 5.4, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.0, D-glucose 11, Tris 10. In all studies (S)-zacopride (1 μM) was used to define non-specific radioligand binding (NSB). For competition studies 5-HT_3 receptors present in rat cortical and cell homogenates were labelled with 0.7–1.5 nM [^{3}H]-GR65630 or [^{3}H]-quipazine. For [^{3}H]-quipazine binding to rat cortical homogenates, 0.1 μM paroxetine was added to the assay mixture to prevent [^{3}H]-quipazine binding to 5-HT uptake sites. Saturation studies on the three preparations were conducted with [^{3}H]-quipazine (0.08–72 nM) and [^{3}H]-GR65630 (0.04–10 nM). In rat cortex, paroxetine was included (19 fold greater than radioligand concentration to block binding to 5-HT uptake sites). Incubations (0.25 ml total vol.) were carried out for 45 min at 25°C and were terminated by vacuum filtration over Whatman GF/B glass fibre filters using a Brandel 48 well cell harvester. The filters were pretreated with 0.3% polyethyleneimine in order to reduce filter binding of the radioligand. After filtration the filters were washed for 8 s with ice-cold 0.1 M NaCl. Radioactivity retained on the filters was determined by liquid scintillation spectrometry.

Binding assays for [^{3}H]-paroxetine were conducted in 50 mM Tris-HCl containing 120 mM NaCl and 5 mM KCl (pH 7.4). Tissue homogenates were incubated at 23°C for 60 min with [^{3}H]-paroxetine (0.02–15 nM) and competing drugs in a total volume of 1.25 ml. Nonspecific binding was defined by use of 100 μM 5-HT (Marcusson *et al.*, 1988). The incubations were terminated and the filter bound radioactivity counted as described above for [^{3}H]-quipazine binding.

Protein analysis were performed with the Biorad colorimetrical method with bovine γ-globulin as the standard (Bradford, 1976).

Data analysis

All competition data were analyzed by iterative curve fitting procedures 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). Analyses of saturation data were performed with the 'LIGAND' computer programme (Munson & Rodbard, 1980). Statistical analyses were performed with an unpaired Student's *t* test.

Materials

NG108-15 cells were obtained from Dr R. Heller, Institute of Cancer and Developmental Biology, Syntex Research, Palo Alto, CA, U.S.A. NCB-20 cells were a generous gift from Dr T. Mansour, Stanford University, Palo Alto, CA, U.S.A. Frozen rat brains were purchased from Pel-Freez (Arkansas). [^{3}H]-quipazine (specific activity 55 Ci mmol^{-1}, Lot. No. 2389-249; 66 Ci mmol^{-1}, Lot No. 2604-123) and [^{3}H]-paroxetine (28.6 Ci mmol^{-1}) were purchased from DuPont NEN/Boston. The purity of [^{3}H]-quipazine on receipt from NEN was 98% and 94% for the two batches respectively (Dr S. Hurt, personal communication). [^{3}H]-GR65630 was a gift from Dr S. Hurt of DuPont NEN/Boston. Zacopride and its isomers, BRL 43684 (granisetron), BRL 24682 (renzapride), GR38032F (ondansetron), ICS 205-930, MDL 72222 and N-methyl-quipazine were synthesized by Dr R. Clark and co-workers, Institute of Organic Chemistry, Syntex Research, Palo Alto, CA, U.S.A. Paroxetine and fluoxetine were generous gifts from Beechams Pharmaceuticals (Harlow, UK) and Eli Lilly and Company (Indianapolis, USA) respectively. All other chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.) and Aldrich (U.S.A.).

Results

Saturation analysis

[^{3}H]-quipazine bound to homogenates of neuroblastoma cells (NG108-15 and NCB-20) and rat cortex in a saturable and reversible manner (Figures 1 and 2). [^{3}H]-quipazine interacted

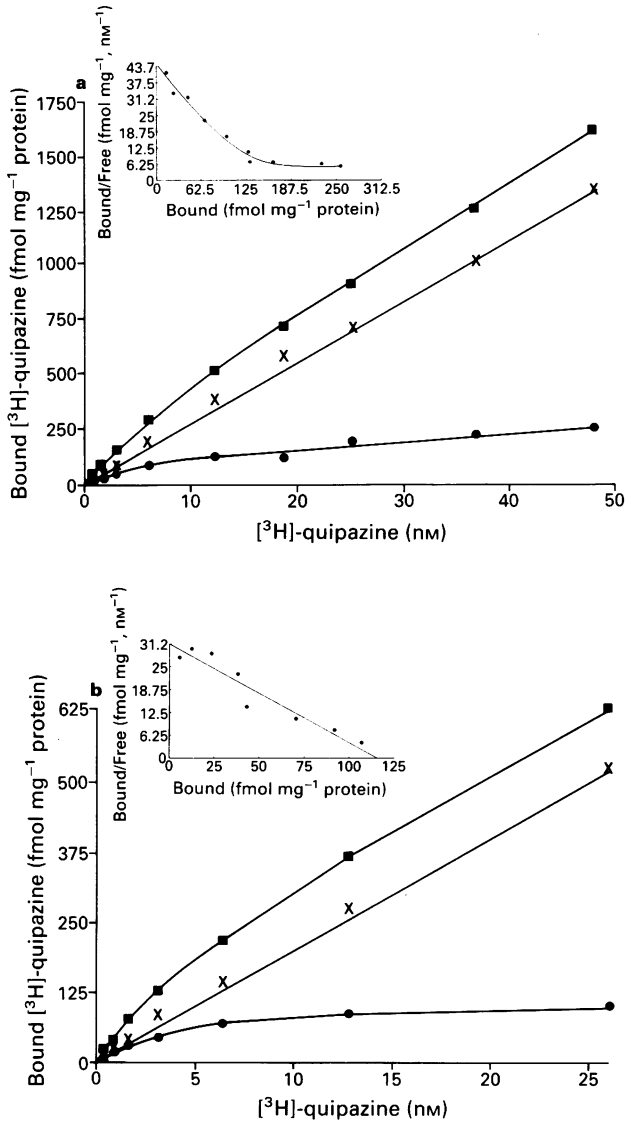

Figure 1 (a) Saturation analysis of [^{3}H]-quipazine binding to rat cortex homogenates in absence of paroxetine. Total (■), non-specific (×) and specific (●) binding from a representative experiment is shown. Inset: Scatchard analysis of the specific [^{3}H]-quipazine binding component. (b) Saturation analysis of [^{3}H]-quipazine binding to rat cortex homogenates in presence of paroxetine. Paroxetine was added to the [^{3}H]-quipazine and total (■), non-specific (×) and specific (●) binding determined as described in the Methods section. Inset: Scatchard analysis of the specific [^{3}H]-quipazine binding component.

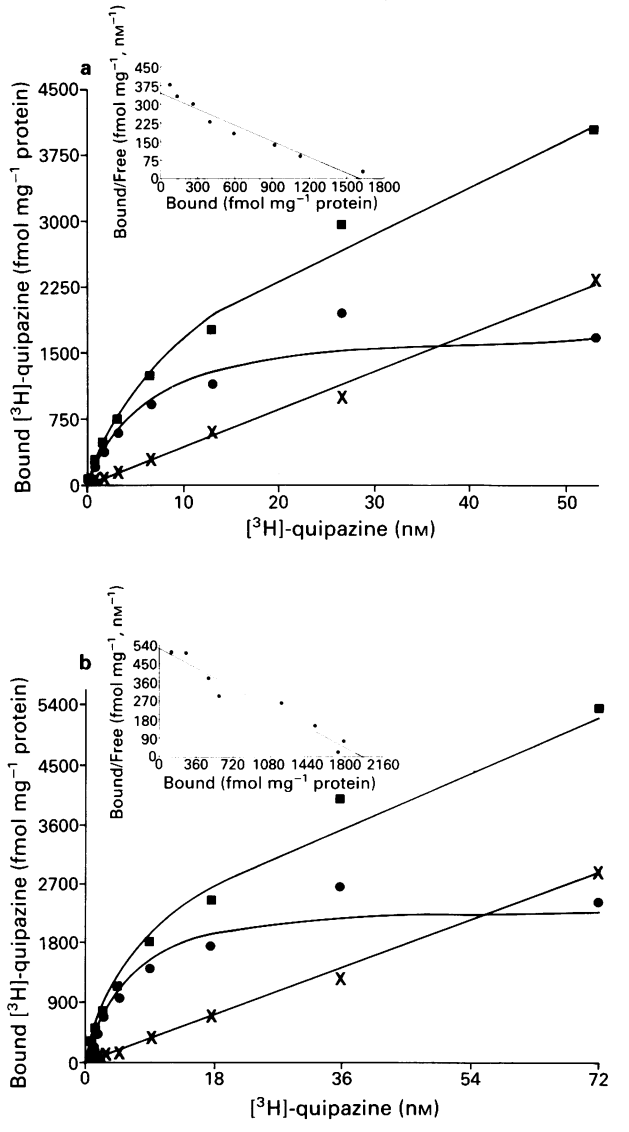

Figure 2 (a) Saturation analysis of [^{3}H]-quipazine binding to NG108-15 cell homogenates. Total (■), non-specific (×) and specific (●) binding is depicted from a sample experiment. Assays were conducted in the absence of any exogenous paroxetine. Inset: Scatchard analysis of specific [^{3}H]-quipazine binding to NG108-15 cell homogenates. (b) Example of saturation analysis of [^{3}H]-quipazine binding to NCB-20 cell homogenates in the absence of any exogenous paroxetine. Total (■), non-specific (×) and specific (●) binding from a sample assay is shown. Inset: Scatchard analysis of specific [^{3}H]-quipazine binding to NCB-20 cell homogenates.

with a single population of sites in the cultured cell homogenates but with two sites in the rat cortical homogenates (Figures 1 and 2; Table 1). In the presence of paroxetine, the biphasic Scatchard plot of [^{3}H]-quipazine binding in the rat cortex was rendered monophasic thereby indicating the presence of a single population of sites (Figure 1b).

[^{3}H]-quipazine binding to 5-HT$_3$ sites in the neuroblastoma cells and rat cortex exhibited a high affinity (2–6 nM) which was similar in all three preparations. The neuroblastoma cells have a greater B_{max} than the rat cortical homogenates (Table 1). The use of [^{3}H]-GR65630 to label 5-HT$_3$ receptors in NCB-20 cell membranes confirmed the identity and the density of 5-HT$_3$ sites in this cell-line (Table 1).

[^{3}H]-paroxetine bound with high affinity (K_d = 0.02 ± 0.003 nM, n = 3; means ± s.e.means) to 5-HT uptake sites in rat cortical homogenates in a saturable (B_{max} = 323 ± 45 fmol mg^{-1} protein) and reversible manner. No specific [^{3}H]-paroxetine binding was detected in either of the neuroblastoma cell-lines.

Competition studies

Specific [^{3}H]-quipazine (0.8–1.2 nM) binding in the rat cortex comprised 35 ± 1% (n = 10) of the total binding in the absence of 0.1 μM paroxetine. In the presence of this agent the specific binding was significantly ($P < 0.001$) increased to 44 ± 2% (n = 21). In contrast, specific [^{3}H]-quipazine binding, in the absence of paroxetine, was 95 ± 0.2% (n = 18) and 85 ± 1% (n = 15) of total binding in the NG108-15 and NCB-20 cells respectively. Specific [^{3}H]-GR65630 binding represented 88 ± 1% (n = 7) of the total binding in NCB-20 cell homogenates.

In rat cerebral cortical homogenates, 5-HT$_3$ receptor antagonists inhibited [^{3}H]-quipazine binding with shallow inhibition curves (Hill numbers < 1). The inclusion of 0.1 μM paroxetine made these curves steeper (Hill coefficient ≃ 1). (S)-zacopride exhibited a greater affinity than (R)-zacopride (Figure 3a, Table 3). The inclusion of 0.1 μM of (S)-zacopride but not paroxetine gave rise to a single high affinity [^{3}H]-

Table 1 Labelling of 5-HT_3 binding sites in rat cortex, NG108-15 and NCB-20 cell homogenates by [^{3}H]-quipazine and [^{3}H]-GR65630

Tissue	K_{d1} (nM)	K_{d2} (nM)	B_{max1} (fmol mg^{-1} protein)	B_{max2} (fmol mg^{-1} protein)
(a) [^{3}H]-quipazine				
Rat cortex (no paroxetine)	1.6 ± 0.5	500 ± 300	75 ± 14	1840 ± 1040
Rat cortex (+ paroxetine)	2.0 ± 0.5	—	73 ± 6	—
NG108-15 (no paroxetine)	6.2 ± 1.1	—	1340 ± 220	—
NCB-20 (no paroxetine)	3.0 ± 0.9	—	2300 ± 200	—
(b) [^{3}H]-GR65630				
NCB-20 (no paroxetine)	0.7 ± 0.1	—	1800 ± 1000	—

Data are means ± s.e.means from 3–4 experiments in each preparation using 0.4–72 nM [^{3}H]-quipazine and 0.04–10 nM [^{3}H]-GR65630.

quipazine binding site which showed high affinities for 5-HT uptake blockers (Table 2). These latter sites could also be labelled with [^{3}H]-paroxetine and represented labelling of 5-HT uptake sites. Conversely, the 5-HT_3 receptor antagonists tested exhibited low affinity for the [^{3}H]-paroxetine binding site (Table 2).

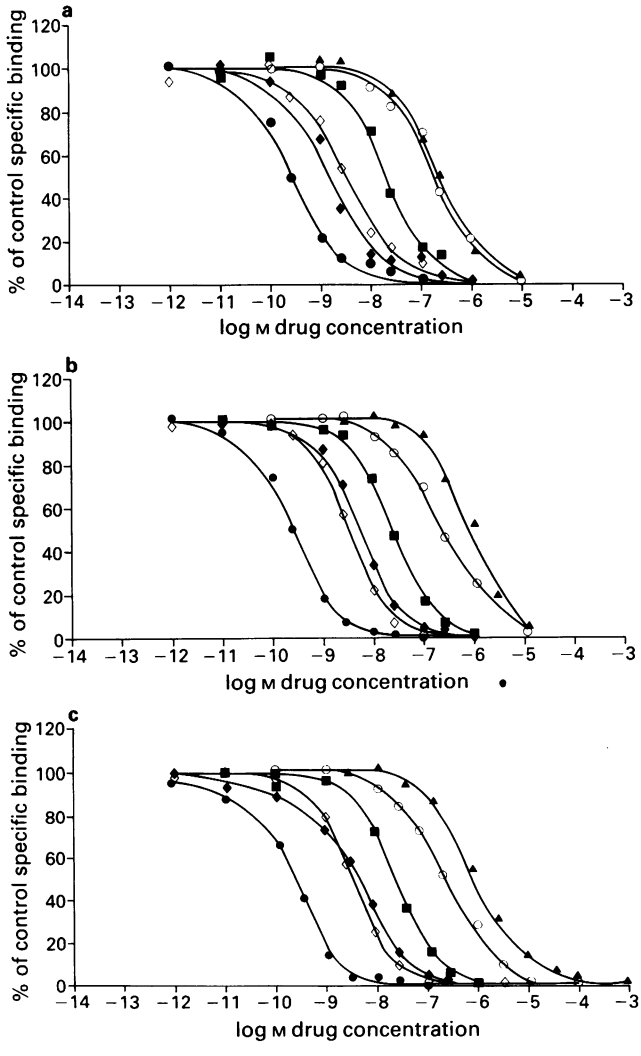

Figure 3 Inhibition [^{3}H]-quipazine binding to 5-HT_3 receptor binding sites in (a) homogenates from rat cortex, (b) NG108-15 cells and (c) NCB-20 cells, by (S)-zacopride (●), ondansetron (◆), (R)-zacopride (◇), MDL-72222 (■), metoclopramide (○), and 2-methyl-5-HT (▲).

In contrast to the situation in rat cortex, specific [^{3}H]-paroxetine binding was not detectable in the cell lines tested. [^{3}H]-quipazine binding in the cell membranes yielded good specific binding (see above) and was displaceable by 5-HT_3 receptor antagonists with high affinity and mass action profiles. Ligands which lack 5-HT_3 receptor affinity exhibited low affinities at these 5-HT_3 sites (Tables 3 and 4). [^{3}H]-GR65630 was shown to label a site in the NCB-20 cells with a pharmacological specificity very similar to that observed with [^{3}H]-quipazine (Table 4).

Comparisons were made between the rank order of potency of displacing ligands against [^{3}H]-quipazine binding in rat cortical homogenates (plus paroxetine) and [^{3}H]-quipazine binding to homogenates of NG108-15 and NCB-20 cells, and to [^{3}H]-GR65630 binding to NCB-20 cell homogenates. Significant correlations were observed for the pharmacology of [^{3}H]-quipazine binding in rat cortex and NG108-15 cells ($r = 0.98$, $P < 0.0001$), NCB-20 cells and NG108-15 cells ($r = 0.98$, $P < 0.001$), NCB-20 cells and rat cortex ($r = 0.97$, $P < 0.001$), and between [^{3}H]-quipazine and [^{3}H]-GR65630 binding in NCB-20 cells ($r = 0.97$, $P < 0.001$).

Discussion

The present studies demonstrated that [^{3}H]-quipazine binding to 5-HT_3 sites in homogenates of neuroblastoma cells (NG108-15, NCB-20) and rat cortex was saturable, reversible and of high affinity. Scatchard analysis revealed that [^{3}H]-quipazine also bound to low affinity sites in the rat cortex in contrast to the single high affinity population of sites observed in neuroblastoma cell homogenates. The present data are explicable in terms of the ability of [^{3}H]-quipazine to label the 5-HT uptake site in addition to the 5-HT_3 binding site. Labelling of the 5-HT uptake site was substantiated further by the high affinity of standard 5-HT uptake blockers for [^{3}H]-quipazine and [^{3}H]-paroxetine binding in rat cortex (this study and Milburn & Peroutka, 1989). The high density of [^{3}H]-paroxetine binding sites (B_{max} = 323 ± 45 fmol mg^{-1} protein) in the rat cortex is consistent with the existence of a high density of 5-hydroxytryptaminergic terminals in this tissue (Marcusson *et al.*, 1988). The high proportion of these uptake sites relative to the low number of 5-HT_3 receptors in this tissue gave rise to the resultant curvilinear Scatchard plots. Inclusion of 100 nM paroxetine to exclude the uptake component allowed the selective labelling of 5-HT_3 receptor binding sites. The superior profile of [^{3}H]-quipazine and [^{3}H]-GR65630 in neuroblastoma cell lines compared to that found in rat cortex can be explained by the greater density of 5-HT_3 binding sites and the apparent lack of 5-HT uptake sites as shown by the absence of [^{3}H]-paroxetine binding.

A limited number of studies have been performed to identify and characterize 5-HT_3 receptors on neuroblastoma cell lines

Table 2 Affinity of 5-HT uptake blockers and 5-HT$_3$ receptor antagonists for 5-HT uptake sites

	[^{3}H]-paroxetine		[^{3}H]-quipazine	
Compound	pK$_i$	nH	pK$_i$	nH
5-HT uptake blockers				
Paroxetine	9.9 ± 0.1	1.2 ± 0.1	8.5 ± 0.2	1.2 ± 0.3
Sertraline	9.2 ± 0.1	1.0 ± 0.1	8.6 ± 0.2	1.2 ± 0.1
Chlorimipramine	9.1 ± 0.1	1.0 ± 0.1	8.2 ± 0.1	0.9 ± 0.1
Fluoxetine	8.6 ± 0.1	1.0 ± 0.1	8.2 ± 0.2	1.0 ± 0.1
5-HT$_3$ receptor ligands				
Quipazine	7.6 ± 0.1	1.1 ± 0.1		
Bufotenine	6.6 ± 0.1	1.1 ± 0.2		
ICS 205-930	6.4 ± 0.1	1.1 ± 0.1		
MDL 72222	6.0 ± 0.1	1.1 ± 0.1		
2-methyl-5-HT	5.1 ± 0.1	1.5 ± 0.2		
Granisetron	5.2 ± 0.1	1.2 ± 0.1		
(S)-zacopride	4.9 ± 0.1	1.3 ± 0.1		
Ondansetron	4.8 ± 0.1	1.3 ± 0.2		
Phenylbiguanide	<4	—		

Data are means ± s.e.means from 3–4 experiments. The [^{3}H]-quipazine binding experiments were conducted in the presence of 0.1 μM zacopride to prevent [^{3}H]-quipazine binding to 5-HT$_3$ sites. The Hill coefficients were not statistically significantly different from unity.

(Hoyer & Neijt, 1988; Neijt *et al.*, 1988a,b). The present study has highlighted the importance of these cells, using two different radioligands, as a model system for 5-HT$_3$ receptor research. The problem of a low density of 5-HT$_3$ receptors in the mammalian brain (Kilpatrick *et al.*, 1987; 1989; Barnes *et al.*, 1989b; Wong *et al.*, 1989; Nelson & Thomas, 1989; Milburn & Peroutka, 1989; this study) has hampered pharmacological and biochemical investigations into issues of receptor regulation, receptor-effector mechanisms, biophysical characterization (Lummis *et al.*, 1990) and receptor purification. Hence the neuroblastoma cells represent a very good source of 5-HT$_3$ receptors for studies of this nature.

A question of the specificity of [^{3}H]-quipazine as a 5-HT$_3$ receptor radioligand was raised as a result of a report by Schmidt *et al.* (1989) on a [^{3}H]-quipazine-degradation-product with high affinity for 5-HT uptake sites. We believe the data obtained in the present study are not explicable in terms of the presence of such an impurity in our radioligand

Table 3 Dissociation constants of different pharmacological agents at the 5-HT$_3$ binding sites in the rat cortex and NG108-15 cell homogenates

	Receptor affinities					
	Cortex 5-HT$_3$ sites			*NG108-15 5-HT$_3$ sites*		
Compound	pK$_i$	nH	*n*	pK$_i$	nH	*n*
5-HT$_3$ antagonists						
(S)-zacopride	9.6 ± 0.1	0.9 ± 0.2	3	9.5 ± 0.1	1.1 ± 0.2	3
Q-ICS 205-930	9.1 ± 0.2	0.6 ± 0.1*	3	8.8 ± 0.2	1.0 ± 0.1	3
Granisetron	9.1 ± 0.1	1.2 ± 0.2	5	8.8 ± 0.1	1.3 ± 0.2	3
N-methyl quipazine	9.1 ± 0.1	0.9 ± 0.1	3	8.9 ± 0.1	1.0 ± 0.1	3
(R/S)-zacopride	8.9 ± 0.1	0.8 ± 0.1	8	9.4 ± 0.2	0.9 ± 0.2	4
Ondansetron	8.9 ± 0.1	0.8 ± 0.1	6	8.4 ± 0.1	1.0 ± 0.1	3
BRL 24682	8.8 ± 0.1	1.0 ± 0.1	3	8.8 ± 0.1	1.1 ± 0.1	3
ICS 205-930	8.7 ± 0.2	1.1 ± 0.1	5	8.3 ± 0.4	0.9 ± 0.2	3
Quipazine	8.7 ± 0.2	1.3 ± 0.3	6	8.1 ± 0.2	0.8 ± 0.2	4
(R)-zacopride	8.7 ± 0.2	1.2 ± 0.3	3	8.5 ± 0.1	1.3 ± 0.1	3
Renzapride	8.3 ± 0.1	1.1 ± 0.1	3	8.4 ± 0.1	1.0 ± 0.1	3
MDL-72222	7.4 ± 0.1	1.3 ± 0.2	9	7.6 ± 0.1	1.1 ± 0.1	3
Butanopride	7.1 ± 0.1	1.1 ± 0.1	3	7.2 ± 0.1	1.2 ± 0.1	3
Metoclopramide	6.7 ± 0.1	1.2 ± 0.1	4	6.7 ± 0.1	1.0 ± 0.1	3
5-HT$_3$ agonists						
Bufotenine	7.3 ± 0.1	1.2 ± 0.1	3	7.2 ± 0.1	0.8 ± 0.1	3
Phenylbiguanide	7.2 ± 0.1	1.4 ± 0.1	3	6.4 ± 0.1	1.2 ± 0.2	3
2-methyl-5-HT	6.8 ± 0.1	1.2 ± 0.1	3	6.3 ± 0.1	0.9 ± 0.1	3
5-HT	6.3 ± 0.1	1.1 ± 0.1	3	6.3 ± 0.3	1.3 ± 0.2	4
5-Methoxytryptamine	5.4 ± 0.2	1.6 ± 0.1*	3	4.8 ± 0.1	1.0 ± 0.1	3
Other compounds						
RU 24969	5.9 ± 0.1	1.1 ± 0.2	3	5.1 ± 0.5	1.3 ± 0.2	3
Ritanserin	5.9 ± 0.1	1.2 ± 0.1	3	5.0 ± 0.3	1.4 ± 0.3	3
SCH 23390	5.8 ± 0.1	1.0 ± 0.1	3	5.3 ± 0.1	1.2 ± 0.1	3
Ketanserin	5.7 ± 0.4	1.1 ± 0.2	6	5.4 ± 0.4	0.9 ± 0.1	3
5-methyl-Tryptamine	5.6 ± 0.3	1.2 ± 0.1	3	5.1 ± 0.1	1.2 ± 0.1	3
(−)-Propanolol	5.5 ± 0.1	0.8 ± 0.1	3	4.9 ± 0.1	1.2 ± 0.1	3
8-OH-DPAT	5.4 ± 0.1	0.9 ± 0.1	3	5.3 ± 0.1	1.0 ± 0.2	4
(S)-Sulpiride	5.4 ± 0.4	1.2 ± 0.2	3	5.2 ± 0.4	1.0 ± 0.1	3
Methiothepin	5.4 ± 0.2	1.1 ± 0.1	6	5.9 ± 0.1	1.4 ± 0.2	3
Sumatriptan	5.0 ± 0.1	1.1 ± 0.2	3	4.3 ± 0.3	0.7 ± 0.1	4

Data are means ± s.e.means from the indicated number (*n*) of experiments for each tissue.
Q-ICS 205-930 = quaternized ICS 205-930.
* Statistically significantly different from unity, $P < 0.05$, by Student's unpaired *t* test.

Table 4 Inhibition of [^{3}H]-quipazine and [^{3}H]-GR65630 binding in NCB-20 cell homogenates

Compound	[^{3}H]-quipazine		[^{3}H]-GR65630	
	pK_i	nH	pK_i	nH
5-HT$_3$ receptor antagonists				
(S)-zacopride	10.0 ± 0.1	1.4 ± 0.2*	9.9 ± 0.1	1.1 ± 0.1
ICS 205-930	9.1 ± 0.1	0.9 ± 0.1	9.2 ± 0.1	1.2 ± 0.1
Granisetron	9.0 ± 1.2	1.2 ± 0.1	8.9 ± 0.1	1.0 ± 0.1
(R)-zacopride	8.4 ± 0.1	1.0 ± 0.1	8.6 ± 0.1	1.1 ± 0.1
Renzapride	8.4 ± 0.1	0.7 ± 0.1	8.3 ± 0.1	0.9 ± 0.1
Ondansetron	8.3 ± 0.1	0.9 ± 0.1	8.5 ± 0.1	1.0 ± 0.1
MDL 72222	7.6 ± 0.2	1.3 ± 0.3	8.1 ± 0.1	0.9 ± 0.1
Butanopride	7.2 ± 0.1	1.0 ± 0.1	7.2 ± 0.1	1.1 ± 0.1
(+)-Tubocurarine	7.0 ± 0.1	0.9 ± 0.1	7.4 ± 0.1	0.8 ± 0.1
Metoclopramide	6.4 ± 0.2	0.9 ± 0.1	6.9 ± 0.1	1.0 ± 0.1
5-HT$_3$ receptor agonists				
Bufotenine	7.2 ± 0.1	0.8 ± 0.1	7.3 ± 0.1	1.0 ± 0.1
5-HT	7.1 ± 0.1	1.1 ± 0.1	6.8 ± 0.1	1.2 ± 0.1
2-methyl-5-HT	6.6 ± 0.1	1.0 ± 0.1	6.1 ± 0.1	0.9 ± 0.1
Phenylbiguanide	6.4 ± 0.1	0.9 ± 0.1	6.3 ± 0.1	0.9 ± 0.1

Data are means ± s.e.means for the inhibition constants (pK_i) and Hill coefficients (nH) from at least three separate experiments.
* Statistically significantly different from unity, $P < 0.05$ by Student's unpaired t test.

because of the following reasons: (1) the batches of radioligands employed in the present study were different from that used by Schmidt *et al.* (1989) (Lot No. 2389-205); (2) the batches of [^{3}H]-quipazine used here were 94–98% pure (see Materials section); (3) contrary to previous reports (Milburn & Peroutka, 1989; Schmidt *et al.*, 1989; Perry, 1990) any possibility of labelling the 5-HT uptake sites has been eliminated by the inclusion of paroxetine in the assay buffer; (4) (S)-zacopride was used to define the nonspecific binding to ensure exclusive labelling of 5-HT$_3$ receptor sites under our experimental conditions. These data therefore indicated that the nanomolar affinity of [^{3}H]-quipazine binding in rat cortical homogenates reflected radiolabelling of 5-HT$_3$ binding sites.

The hypothesis that [^{3}H]-quipazine labelled 5-HT$_3$ binding sites with characteristics of 5-HT$_3$ receptors in both the rat cortex and neuroblastoma cells was confirmed by the pharmacological characteristics of [^{3}H]-quipazine and [^{3}H]-GR65630 binding. Thus, potent 5-HT$_3$ receptor antagonists such as zacopride (Smith *et al.*, 1988a,b), ICS 205-930 (Richardson *et al.*, 1985), granisetron, renzapride and BRL 24682 (Sanger, 1987; Fake *et al.*, 1987), ondansetron (Ireland & Tyers, 1987; Butler *et al.*, 1988) and quipazine (Ireland & Tyers, 1987) exhibited high affinity for sites in rat cortical and NG108-15 and NCB-20 cell homogenates. Other 5-HT$_3$ antagonists such as metoclopramide (Ireland & Tyers, 1987) and MDL 72222 (Fozard, 1984) also inhibited [^{3}H]-quipazine binding in these preparations but with lower affinity than the above-mentioned antagonists. 5-HT$_3$ receptor agonists such as 5-HT, 2-methyl-5-HT, bufotenine and phenylbiguanide (Richardson *et al.*, 1985; Ireland & Tyers, 1987; Fozard, 1984) competed for [^{3}H]-quipazine binding in rat cortical and NG108-15 and NCB-20 cell homogenates with a relatively low affinity. Although the functional significance of the potent displacement of [^{3}H]-quipazine and [^{3}H]-GR65630 binding in NCB-20 cell homogenates by (+)-tubocurarine remains to be determined, the high affinity of this nicotinic antagonist for 5-HT$_3$ binding sites correlated well with its ability to block 5-HT-induced inward currents in N1E-115 (Peters *et al.*, 1990) and NG108-15 (Yakel & Jackson, 1988) cells. However, the effect of (+)-tubocurarine on 5-HT$_3$ receptor function in NCB-20 cells has not been investigated so far.

In general, the inhibition of [^{3}H]-quipazine binding to rat cortical and neuroblastoma cell homogenates by 5-HT$_3$ antagonists was consistent with the recognition of a single population of sites, as indicated by the Hill coefficients being close to unity. However, it is of interest that quaternized-ICS 205-930 apparently inhibited cortical 5-HT$_3$ binding with a Hill coefficient less than unity suggesting interaction with more than one population of sites. In contrast, quaternized ICS 205-930 gave mass action inhibition curves in NG108-15 cells. It is not clear at present whether the compound was recognizing different affinity states or subtypes of 5-HT$_3$ receptors in the rat cortex. The pharmacological relevance of this observation requires further study. Displacement of [^{3}H]-quipazine binding by zacopride from 5-HT$_3$ sites in the rat cortex and neuroblastoma cells exhibited a similar degree of stereoselectivity. The (S)-isomer of zacopride was markedly more potent than the (R)-isomer. These observations were consistent with previous reports for other tissues in which [^{3}H]-(S)-zacopride was used as the radioligand (Pinkus *et al.*, 1990). The overall pharmacological specificity of 5-HT$_3$ binding sites observed in the rat cortex and neuroblastoma cells was consistent with that of 5-HT$_3$ receptors previously identified from functional data obtained in peripheral tissues (Richardson & Engel, 1986; Bradley *et al.*, 1986; Butler *et al.*, 1990).

[^{3}H]-GR65630 is a selective, high affinity ligand that has been previously employed to label 5-HT$_3$ binding sites in rodent tissue homogenates and sections including rat cerebral cortex (Kilpatrick *et al.*, 1987; 1989). In order to verify that [^{3}H]-quipazine interacted with 5-HT$_3$ receptors in the neuroblastoma cells, [^{3}H]-GR65630 binding was tested in NCB-20 cell homogenates. The excellent correlation between the pharmacological specificity of [^{3}H]-quipazine and [^{3}H]-GR65630 binding in these cells, as well as the similar stereoselectivity obtained with isomers of zacopride, confirmed that [^{3}H]-quipazine labelled 5-HT$_3$ binding sites in the neuroblastoma cells. The (S)-isomer of zacopride exhibited a much greater affinity for [^{3}H]-GR65630 binding in NCB-20 cell homogenates than the (R)-isomer. These data demonstrated an appropriate degree of stereoselectivity of the 5-HT$_3$ binding sites in these cells and confirmed the results obtained with [^{3}H]-quipazine.

In conclusion, [^{3}H]-quipazine labelled 5-HT$_3$ receptors under experimental conditions when its affinity for the 5-HT uptake sites was eliminated. NG108-15 and NCB-20 cell homogenates exhibited a high density of 5-HT$_3$ receptors as determined by [^{3}H]-quipazine and [^{3}H]-GR65630 binding. These cell lines constitute a source with a high density of 5-HT$_3$ receptors suitable for future biochemical and molecular biological investigations.

The authors thank M.L. Burman and R.E. Delmendo for excellent technical assistance and Santos Sanchez and Judi Galyardt for skilful typographic assistance.

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(Received September 10, 1990
Revised November 23, 1990
Accepted November 28, 1990)

Br. J. Pharmacol. (1991), **102**, 926–930

Characterization of Gaddum's substance R

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1 When the isolated small intestine of the rat is perfused via the mesenteric artery, an oxytocic principle (Gaddum's substance R) is released which is detectable in the perfusate after 30 min and is present in samples collected 8 h later.

2 The oxytocic activity of substance R is lost after boiling but is unaffected by treatment with thioglycolate. Furthermore, atropine, methysergide and indomethacin failed to antagonize uterine contractions to substance R.

3 Neither substance R nor urinary kallikrein alone induce a contraction of the guinea-pig isolated ileum. However, in the presence of kininogen both substance R and urinary kallikrein produce a slow and prolonged contraction of the guinea-pig ileum.

4 The oxytocic and kininogenase properties of both substance R and urinary kallikrein are inhibited by Trasylol.

5 Soy bean trypsin inhibitor (SBTI) selectively inhibited both the oxytocic and the kininogenase activities of substance R but not those of urinary kallikrein.

6 Gel filtration of substance R resolved a single peak of oxytocic activity with an estimated molecular weight of 40 kDa.

7 We conclude that substance R is a kininogenase enzyme which may be distinguished from plasma kallikrein by its molecular weight and from urinary kallikrein by its susceptibility to SBTI. The exact nature of this enzyme remains to be elucidated.

Keywords: Substance R; kallikrein; perfused small intestine; kininogenase

Introduction

In 1953 Gaddum published a highly cited paper in which he described for the first time, the superfusion technique. Less well-known is that in this paper Gaddum also reported the production by the perfused rat intestine of a factor with oxytocic activity. The results he obtained suggested that this factor was a protein. We have recently confirmed these observations (Douglas *et al.*, 1988). Furthermore, we observed that the spectrum of pharmacological actions of 'substance R' was similar, but not identical, to those of a tissue kallikrein.

Methods

Collection of substance R

Male Wistar rats (200–300 g) were first anaesthetized with ether, and heparin (1000 u) was administered by a tail vein to prevent clotting during the perfusion. Following laparotomy the small intestine was ligated at the duodenal level, distal to the pancreas and also at the ileo-caecal junction. The superior mesenteric artery was cannulated and all other vessels, with the exception of the hepatic portal vein, were tied off. The intestine was sectioned anterior to the duodenal ligature and distal to the caecal ligature. The intact mesenteric bed was then removed, care being taken to exclude all pancreatic tissue, suspended in a heated jacket and perfused with oxygenated Tyrode solution (37°C, 0.4 ml min^{-1}) through the mesenteric artery. Effluent from the hepatic portal vein was either collected for consecutive 20 min periods over 6 h and aliquots of each fraction were assayed for oxytocic activity, or, after a 30 min equilibration period, perfusate, collected over 6 h, was pooled, lyophylized and stored at −20°C. For preparative purposes pooled perfusates from three experiments were used.

The pooled perfusates from three such preparations were then dialysed overnight against 10 volumes of ammonium bicarbonate (50 mM, pH 7.8). The high molecular weight residue was freeze-dried and stored at −20°C. The lyophylized perfusate from nine intestinal preparations was redissolved in 6 ml of Trisbuffer (50 mM, pH 7.5) and filtered before application to the ion-exchange column.

Assay of oxytocic activity

Segments of stilboestrol-primed rat uterus superfused with de Jalon's solution (30°C, 5 ml min^{-1}) were used for the assay of oxytocic activity of samples. Volumes of between 10 and 150 μl were injected into the stream of superfusion fluid above the tissue.

Assay of kinin-releasing activity

The kinin-releasing activity of substance R and kallikrein was assayed on the guinea-pig isolated ileum. In these experiments a 2 cm length of ileum was suspended in an 8 ml organ bath and bathed in oxygenated Tyrode solution containing captopril (100 μg ml^{-1} at 37°C). Bradykinin was used as a standard.

Ion-exchange chromatography

Ion-exchange chromatography of the soluble intestinal extract was performed by use of DEAE-Sepharose. The sample was applied to the column (2.6 × 30 cm), equilibrated in Tris buffer (50 mM, pH 7.5). Fractions of 5 ml were eluted at a flow rate of 0.4 ml min^{-1} by a linear salt gradient of 0–1 M sodium chloride and assayed for oxytocic activity before the absorbance at 280 nm was measured. Fractions containing oxytocic activity were then pooled, dialysed against 50 volumes of ammonium bicarbonate (50 mM, pH 7.8) for 12–18 h and lyophilized.

Gel filtration

Gel filtration of the oxytocic principle was carried out routinely on a calibrated Sephacryl S-300 column (2.6 × 90 cm). The column was equilibrated with phosphate-buffered saline

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(50 mM, pH 7.5 (PBS) and 5 ml of soluble intestinal extract was applied to the column. The sample was eluted at a flow rate of 0.4 ml min^{-1} and 5 ml fractions were collected. Fractions were then assayed for oxytocic activity and the absorbance at 280 nm measured.

Estimation of the molecular weight of the oxytocic principle was made on a Sephacryl S-200 column (2.6 × 90 cm), calibrated with molecular weight markers, under the conditions described above and the fraction containing the peak oxytocic activity was determined by assay on the superfused isolated uterus of the rat.

Urinary kallikrein

Initially, crude preparations of rat urinary kallikrein were made by desalting rat urine on a Sephadex G-25 (PD-10) column equilibrated with PBS. Later, for comparative purposes, rat urinary kallikrein was prepared from urine collected overnight from male Wistar rats. The urine from individual rats was pooled and filtered before an overnight dialysis against PBS. The dialysed sample was then stored at −20°C. A partially purified preparation or urinary kallikrein was obtained by the same preparative and assay techniques employed in the isolation of substance R.

Materials

Reagents and drugs were obtained from the following sources: aprotinin (Trasylol), (Bayer UK Ltd); H-D-Val-L-Leu-L-Arg-*p*-nitroanilide dichloride (S-2266), (Kabi Vitrum), D-Arg-[Hyp^3, $Thi^{5,8}$, D-Phe^7]-bradykinin, (Novabiochem); PD-10 columns, Sephacryl S-200, Sephacryl S-300 and DEAE-Sepharose, (Pharmacia Ltd); methysergide hydrogen maleate, (Sandoz UK Ltd), atropine sulphate, bradykinin, carbamylcholine, α- and δ-chymotrypsin, diethylstilboestrol, heparin sulphate, indomethacin, soy bean trypsin inhibitor (SBTI), thioglycolic acid, Trizma base, Trizma hydrochloride, and trypsin, (Sigma); captopril, (Squibb). Human plasma kininogen was kindly donated by Dr K.D. Bhoola, University of Bristol.

Statistical analysis

Data are expressed as mean ± s.e.mean. Statistical significance was computed by the paired *t* test or analysis of variance (ANOVAR) where appropriate.

Results

The effluent from perfused rat small intestines was found to contract the superfused rat uterus in a dose-dependent manner. Contractions were delayed in onset (30 s) when compared to those produced by carbachol or bradykinin. When consecutive fractions of perfusate were collected, at least 30 min elapsed before oxytocic activity was detectable (Figure 1). However, oxytocic activity was released in increasing amounts thereafter, reaching a maximum concentration at 3–4 h, which was sustained for a further 2–3 h. Protein concentration, estimated by the Lowry-Folin method (Lowry *et al.*, 1951), remained between 1–2 mg ml^{-1} throughout the period of perfusion with no correlation between protein concentration and oxytocic activity.

The characteristics of the oxytocic principle isolated are very similar to those described by Gaddum (1953) for substance R. As Gaddum himself observed, these properties are similar to those of (then recently described) kallikrein. To compare the activities of the two substances further, we used crude preparations of rat urinary kallikrein as a source of tissue kallikrein, and compared its properties with those of substance R.

The oxytocic principle from the intestinal perfusate was non-dialysable (mol. wt. cut off 10–15 kDa) and oxytocic activity was not inhibited by addition of atropine (10^{-6} M), methysergide (5.7×10^{-7} M) or indomethacin (1×10^{-5} M) to the superfusate. Samples of perfusate possessing substantial oxytocic activity were inactive when tested on the isolated superfused guinea-pig ileum or rat fundic strip preparations. The perfusate lost oxytocic activity on heating but was unaffected by treatment with thioglycolate which inactivates oxytocin (Ames *et al.*, 1950). The material was stable when frozen at −20°C for at least 8 weeks.

Trypsin and chymotrypsin contract the superfused rat uterus in a similar way to substance R (Gaddum, 1953). Gaddum (1953) found that a preparation of substance R was more active, weight for weight, on the rat uterus than crystalline preparations of trypsin or chymotrypsin. It has since been shown that both trypsin and chymotrypsin sensitize certain smooth muscle preparations to bradykinin (Aarsen, 1968; Edery & Grumfeld, 1969). In the present study it was found that trypsin, α-chymotrypsin and δ-chymotrypsin induced sensitization to bradykinin of both the superfused rat uterus and guinea-pig ileum. However, in each of three experiments, substance R and urinary kallikrein failed to induce sensitization to bradykinin in either of these preparations.

To investigate further the similarity between substance R and tissue kallikrein, the effect of inhibitors of kallikrein on the oxytocic activities of substance R and urinary kallikrein were studied. Addition of the dual plasma and tissue kallikrein inhibitor aprotinin (Trasylol, 50–100 Kiu ml^{-1}), to the fluid superfusing the isolated uterus completely inhibited the activity of both substance R and urinary kallikrein in six experiments (Figure 2). However, the plasma kallikrein inhibitor, soy bean trypsin inhibitor (SBTI, 100 μg ml^{-1}), completely inhibited the action of substance R on the isolated uterus (99.4 ± 0.3%) whereas the oxytocic activity of urinary kallikrein was only reduced by 12.6 ± 2.5% ($n = 6$) (Figure 3). A reduction of 9.9 ± 1.8% and 3.0 ± 0.8% was seen in response to bradykinin and carbachol respectively ($n = 4$). The inhibition of the oxytocic activity of substance R by SBTI was significant ($P < 0.01$, ANOVAR).

Both urinary kallikrein and substance R were found to contract the guinea-pig isolated ileum in the presence of crude human plasma kininogen. Use of the selective bradykinin receptor antagonist, D-Arg-[Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin (Regoli *et al.*, 1986; Schachter *et al.*, 1987) inhibited the contractions induced by substance R plus kininogen indicating that the generated factor was a kinin. The kinin releasing activity was assayed on the guinea-pig isolated ileum in the

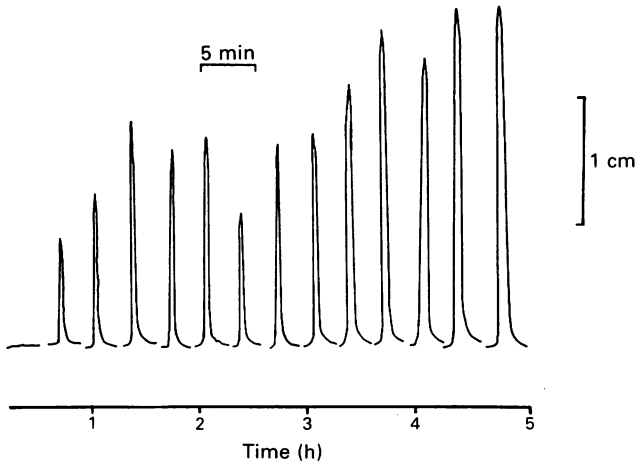

Figure 1 Appearance of oxytocic activity in perfusate from the mesenteric bed. The contractions of the superfused rat uterus induced by 100 μl aliquots of intestinal perfusate from samples collected over consecutive 20 min periods. No oxytocic activity was detectable during the first 20 min of perfusion, but thereafter the samples contained increasing amounts of activity, reaching a maximum concentration at 3–4 h. The lower time scale refers to the time of collection of the perfusate.

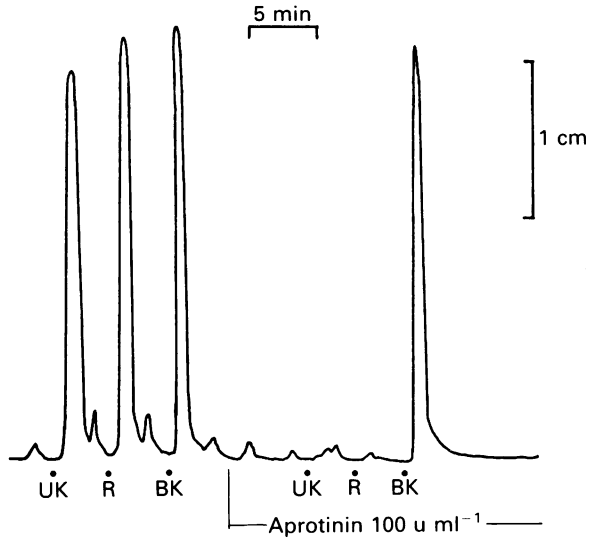

Figure 2 The effect of aprotinin on the oxytocic activities of urinary kallikrein (UK), substance R (R) and bradykinin (BK). Aprotinin completely inhibited uterine contraction to both kallikrein and substance R but did not affect responses to bradykinin.

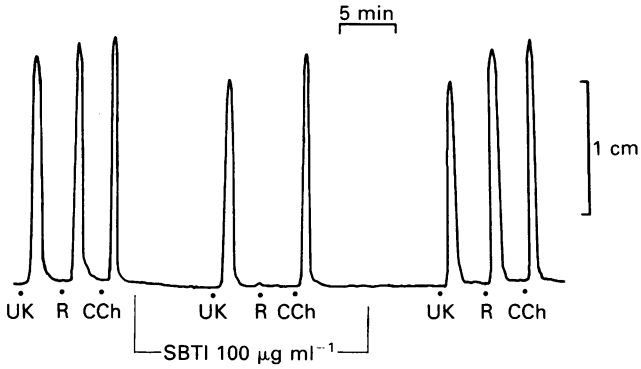

Figure 3 The effect of soy bean trypsin inhibitor (SBTI) on the oxytocic activities of urinary kallikrein (UK), substance R (R) and carbachol (CCh). SBTI reversibly inhibited the response to substance R without a significant reduction in the response to kallikrein or carbachol.

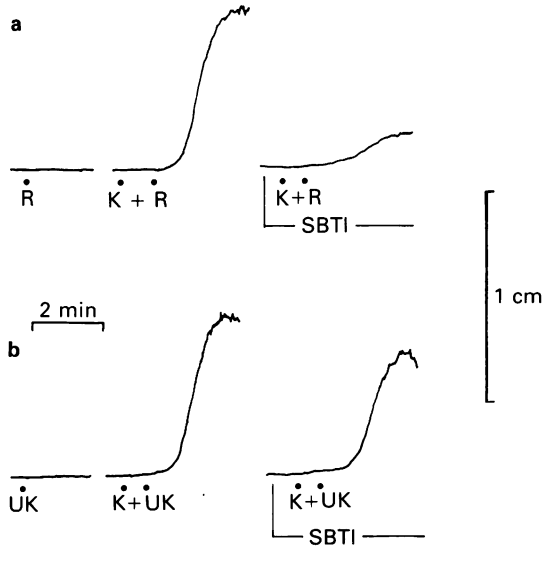

Figure 4 The kininogenase activity of substance R. (a) Addition of substance R (R) alone to guinea-pig isolated ileum elicited no response. However, when kininogen (K) was added, followed by substance R, a slow contraction was seen which was blocked by the presence of soy bean trypsin inhibitor (SBTI). (b) Urinary kallikrein (UK) induced no effect unless kininogen was also present. The contraction induced by kininogen plus urinary kallikrein was not markedly inhibited by SBTI.

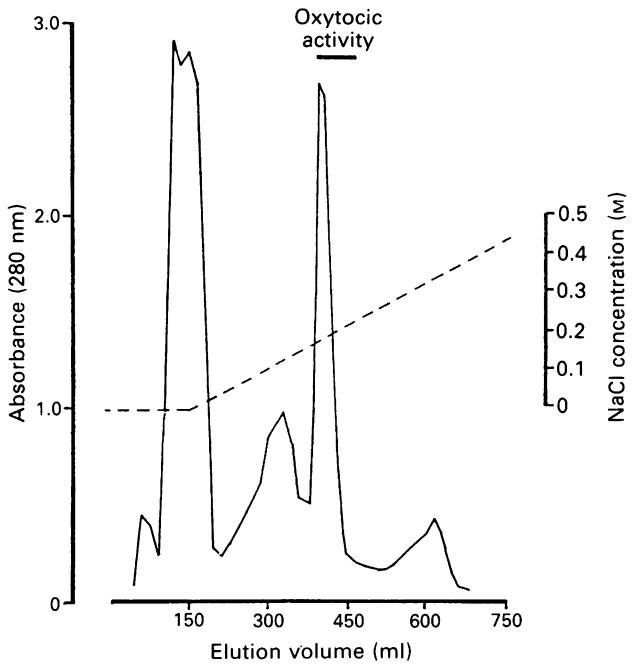

Figure 5 Ion-exchange chromatography of crude intestinal perfusate on DEAE-Sepharose. The sample was applied at V = 0 and was washed with 100 ml Tris buffer (50 mM, pH 7.5). Substance R was eluted with a linear salt gradient at approximately 0.2 M NaCl indicated by the solid bar above the elution profile. The absorbance of fractions was measured at 280 nm and oxytocic activity determined on the superfused rat uterus.

presence of captopril (4.6×10^{-4} M). The ileum was unresponsive to either kallikrein, substance R, or kininogen alone (Figure 4a and b). However, in the presence of kininogen, addition of kallikrein or substance R to the bath resulted in kinin liberation (Figure 4a). Aprotinin (100 Kiu ml^{-1}) completely inhibited the kinin-releasing activity of both urinary kallikrein and substance R ($n = 3$). In contrast, SBTI (100 $\mu g\, ml^{-1}$) produced a small inhibition of the kinin liberation by urinary kallikrein of $13.2 \pm 6.3\%$ but significantly ($P < 0.001$, paired t test) reduced the release of kinin by substance R by $80.4 \pm 5.0\%$ ($n = 6$) (Figure 4b).

When the selective BK_2 bradykinin receptor antagonist D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin was added to the superfusate (1×10^{-7} M), oxytocic responses to bradykinin, kallikrein and substance R were reduced by $63.8 \pm 10.7\%$, $69.5 \pm 11.7\%$, $85.9 \pm 8.6\%$, respectively ($P < 0.01$, ANOVAR) ($n = 8$). Responses to carbachol were not reduced in the presence of the antagonist.

Chromatography of crude intestinal perfusate preparations on DEAE-sepharose resulted in elution of a single peak of activity with approximately 0.2 M NaCl (Figure 5). When the active ion-exchange fractions were applied to the Sephacryl S-200 column, a single peak of oxytocic activity was resolved with a molecular weight of approximately 40 kDa (Figure 6). There was no detectable oxytocic activity in fractions either side of this peak. Preliminary studies with SDS-PAGE confirm this estimate of molecular weight. Urinary kallikrein was found to have very similar chromatographic properties to substance R.

The kininogenase activity of fractions eluted from the Sephacryl column was determined by hydrolysis of the chromogenic substrate S-2266 using the endpoint method. A single peak of kininogenase activity was found which coincided with that of oxytocic activity.

Discussion

Perfusion of the rat isolated small intestine resulted in the liberation of a non-dialysable oxytocic principle which Gaddum

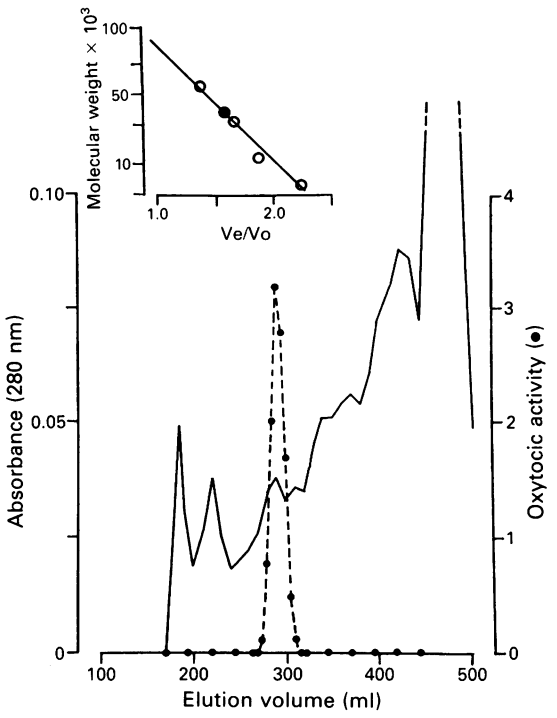

Figure 6 Gel filtration on Sephacryl S-200 of pooled oxytocic fractions previously subjected to ion-exchange chromatography. The sample was applied at V = 0 and was eluted with 50 mM PBS, pH 7.5. The absorbance of fractions was measured at 280 nm and the elution profile is shown by the solid line. Oxytocic activity of the eluted fractions was determined on the isolated superfused uterus of the rat, indicated by the dashed line, and is expressed as % maximum contraction per μl eluate. The molecular weight of the substance R peak (●) corresponded to 40 kD when compared to calibrating proteins (○).

(1953) named substance R. Release of substance R from the perfused intestine increased with time from 20 min to approximately 3 h after which the oxytocic activity of samples collected remained high and fairly constant. Contraction of the rat uterus by substance R was unaffected by atropine, methysergide or by treatment with thioglycolate. However, boiling samples of substance R for 3–4 min resulted in a substantial loss of activity. These results are in agreement with the observations of Gaddum (1953).

Substance R and tissue kallikrein share a number of properties, as Gaddum himself remarked (1953). Our results with the guinea-pig isolated ileum support this, as kallikrein and substance R only induce contraction of the ileum when kininogen is also present, resulting in the generation of a kinin.

The polyvalent protease inhibitor, aprotonin (Trautschold *et al.*, 1967) was found to inhibit the oxytocic responses of the rat uterus to both substance R and urinary kallikrein. However, SBTI, which selectively inhibits plasma kallikrein (Werle & Maier, 1952; Back & Steger, 1968) blocked responses of the uterus to substance R without affecting those to urinary kallikrein. The release of kinin from kininogen by substance R is likewise inhibited by SBTI and aprotinin, whereas kinin release by urinary kallikrein was inhibited by the latter only. These results suggest that substance R possesses kininogenase activity but is unlikely to be a tissue kallikrein. An attempt was therefore made to purify and further characterise substance R.

Gel filtration chromatography of the oxytocic principle obtained from ion exchange chromatography of crude intestinal perfusate resolved a single peak of biological activity with a molecular weight corresponding to approximately 40 kDa. Preliminary experiments with SDS-PAGE confirm this estimate. As plasma kallikreins have been reported to possess a molecular mass of over 100 kDa (see Movat, 1979; Schachter, 1980) it is unlikely that substance R is a plasma kallikrein. The site of release of substance R from the perfused intestine is at present unknown. The presence of kininogenase in rat vascular tissues has previously been reported (Nolly & Lama, 1982; Nolly *et al.*, 1985; 1986). However, when the arterial vasculature of the mesentery alone was perfused, none of the 20 min fractions collected over 6 h contained oxytocic activity. The kininogenase reported by Nolly and colleagues was not inhibited by SBTI. It thus seems probable that substance R is released from the intestinal tissue during perfusion, rather than the arterial vasculature (unpublished data). The physiological significance of substance R in the gut is not fully understood but is the subject of further investigation.

It has recently been demonstrated that contraction of the rat isolated uterus by glandular kallikrein is dependent on the presence of kininogen within the uterine tissue (Figueiredo *et al.*, 1990). This supports the evidence presented by Orce *et al.* (1989) who suggested that the oxytocic activity of glandular kallikrein is mediated, at least in part, by kinins generated from uterine kininogen. In the present study uterine contractile responses to both urinary kallikrein and substance R were inhibited by the kinin antagonist D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin. There was no significant difference between the inhibition of responses to bradykinin, urinary kallikrein or substance R by this antagonist, suggesting that the oxytocic activity of each is mediated by the BK_2 kinin receptor. It is proposed that substance R shares with glandular kallikrein the same mechanism in the contraction of the rat isolated uterus.

We conclude that substance R is a proteolytic enzyme with a molecular weight of approximately 40 kDa which is biochemically related to the tissue kallikrein family of enzymes but has an anomalous sensitivity to SBTI. The reasons for this discrepancy require further elucidation.

G.J.D. is a SERC student.

We thank Dr M. Schachter for valuable advice, Dr Julie Chao for helpful discussion and Mrs Helen Lynch for secretarial assistance.

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(Received February 22, 1990
Revised October 2, 1990
Accepted November 11, 1990)

Br. J. Pharmacol. (1991), **102**, 931–937

Effect of hyperosmolarity on human isolated central airways

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1 We studied the effect of hyperosmolarity on human isolated airways because a better understanding of the effect of hyperosmolarity on the human airway wall may improve insight into the pathophysiology of hyperosmolarity-induced bronchoconstriction in asthma.

2 In cartilaginous bronchial rings dissected from fresh human lung tissue, hyperosmolar Krebs-Henseleit buffer (450 mosM, extra sodium chloride added) evoked a biphasic response: a rapid relaxation phase (peak after 5.0 ± 0.3 min) followed by a slow contraction phase (peak after 25.4 ± 0.8 min).

3 With the histamine (H_1) receptor antagonist mepyramine, the contraction phase was reduced to 41.2% of the control value ($P < 0.001$), with atropine to 50.0% ($P < 0.01$), with the local anaesthetic lignocaine to 48.7% ($P < 0.05$) and with mepyramine together with atropine to 19.2% ($P < 0.001$).

4 With the inhibitor of neutral metalloendopeptidase, phosphoramidon, the contraction phase increased to 128.0% of the control value ($P < 0.05$) and after removal of the epithelium to 131.8% ($P < 0.05$).

5 Indomethacin, the leukotriene C_4/D_4 (LTC_4/D_4) antagonist FPL 55712 or the blocker of nerve conduction, tetrodotoxin, had no effect on the contractile phase.

6 The relaxation phase was not altered by any of these drugs nor by epithelial denudation. The relaxation phase was also unchanged in the presence of α-chymotrypsin, which degrades muscle relaxing peptides such as vasoactive intestinal peptide.

7 Hyperosmolar buffer slightly increased the sensitivity and maximal response to methacholine as well as the cholinergic twitch to electric field stimulation.

8 We conclude that hyperosmolarity releases acetylcholine, histamine and neuropeptides in the human airway wall in sufficient quantities to contract airway smooth muscle. This release itself or its effect on airway muscle is modulated by the airway epithelium. The mechanism of the relaxation phase may be an unknown smooth muscle relaxing substance or a direct effect on the airway muscle, related to ion fluxes.

Keywords: Human isolated bronchi; hypertonicity; hyperosmolarity; bronchial smooth muscle; airway smooth muscle; methacholine; cholinergic responsiveness

Introduction

In asthmatics inhalation of nebulized hypertonic saline leads to bronchoconstriction (Schoeffel *et al.*, 1987). The mechanism behind this phenomenon is unknown. *In vivo*, the maximal fall in FEV_1 after inhalation of hyperosmolar saline is reduced after pretreatment with the anticholinoceptor drug, ipratropium bromide (Boulet *et al.*, 1989) or the H_1-receptor antagonist, terfenadine (Finnerty *et al.* 1989; Finney *et al.*, 1990) whereas flurbiprofen, a potent inhibitor of cyclo-oxygenase, gives minor protection (Finnerty *et al.*, 1989). These data suggest that in asthmatics the release of histamine and a vagal reflex component contribute to hyperosmolarity-induced bronchoconstriction.

Because a better understanding of the effect of hyperosmolarity on the human airway wall may improve our insight into the pathophysiology of hyperosmolarity-induced bronchoconstriction in asthma, we evaluated the role of histamine, acetylcholine, metabolites of arachidonic acid, neuropeptides and airway epithelium in the response of fresh human airways to a hyperosmolar stimulus. In addition, we investigated the influence of hyperosmolarity on the responses to methacholine, an analogue of acetylcholine, and on the effect of electric field stimulation (EFS), which selectively activates postganglionic nerves in the bronchial wall.

Patients and airway preparations

Lung tissue specimens were obtained from 35 patients, 32 male and 3 female, with a mean age of 65.6 years (range 50–80 years). All were operated because of bronchial carcinoma. None had a history of asthma, 29 were smokers and 19 had chronic obstructive pulmonary disease according to the criteria of the American Thoracic Society (1987). Preoperative lung function showed mean values for Inspiratory Vital Capacity (IVC) of 95.6 ± 3.5% of the predicted value and forced expiratory volume in 1 s as a percentage of IVC of 60.2 ± 1.2% of the predicted value. No provocation tests were done on these patients so that information about their airway reactivity was not available. Medication during anaesthesia was the same for all patients: atropine, thiopentone, fentanyl, O_2/N_2O, halothane and pancuronium. Fourteen patients received steroids and/or theophylline before and during the operation. Thirty to 60 min after surgical resection, a macroscopically normal part of the resected tissue was immersed in Krebs-Henseleit buffer of room temperature (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 5.55), which had been gassed with carbogen (95% O_2, 5% CO_2) to produce a pH of 7.35, a P_{CO_2} of 4.7 kPa and a P_{O_2} of 71.8 kPa. The tissue remained in fresh aerated buffer throughout the dissection procedure and the experiments. On the cut surface cartilaginous bronchi with a diameter of 3–5 mm (3rd–7th generation) were identified, cannulated, taken out and dissected free from parenchyma and vessels under a 20× magnification stereo microscope using iris-scissors and forceps. The cleaned airways were cut into segments 3–4 mm in length which were mounted between two small hooks made of polished stainless steel (diameter 0.3 mm) in a double jacketed 10 ml organ bath and fixed to a glass hook at the bottom of the bath and a high precision isotonic angular position transducer (Penny and Giles 3810/60, Great Britain) which was connected to a digital voltmeter (Fluke 73 multimeter, U.S.A.) and a pen-recorder (Kipp BD 40, The Netherlands). This method has been described in detail pre-

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viously (De Jongste et al., 1985; Jongejan et al., 1988). To prevent adhesion of peptides the organ baths were siliconized (dimethyl-dichlorosilane, BDH, United Kingdom). The bronchi contracted against an isotonic load of 500 mg which was found to be optimal in preliminary studies. During an equilibration period of 2 h at 37°C, the preparations were contracted twice with methacholine (10^{-5} M, 10^{-4} M), to assess their contractile function. Between stimulations the preparations were washed 4 times with intervals of 2 min and every 15 min afterwards until stabilization of resting length.

Effect of hyperosmolarity and its mechanism

A first osmotic challenge was carried out by raising the osmolarity of the organ bath fluid to 450 mosM with 180 μl of a 3.84 M NaCl solution. This stimulus was chosen because we had previously found that 450 mosM was the highest osmolarity that would not cause osmotic shrinkage (Jongejan et al., 1990). Furthermore, the osmolarity of the airway lining fluid has been estimated to surpass 640 mosM in the first 10 airway generations within 5 min after the initiation of a standard challenge with nebulized saline of 1330 mosM (Anderson & Smith, 1989). Hence, we expected buffer of 450 mosM to be a stimulus that is relevant to the *in vivo* situation, without causing osmotic shrinkage *in vitro*. The response to hyperosmolar buffer consisted of a rapid deflection below baseline followed by a slow contractile phase (Figure 1). After a maximum response was reached the hypertonic buffer was replaced by isotonic buffer and 30 min later, the segments were incubated with one of the following drugs (De Jongste et al., 1987b): the cyclo-oxygenase inhibitor indomethacin (6×10^{-6} M); the leukotriene C_4/D_4 (LTC_4/D_4) receptor antagonist FPL 55712 (11.5×10^{-6} M); the histamine H_1 receptor antagonist mepyramine (5×10^{-6} M); the muscarine receptor antagonist atropine (1.2×10^{-6} M); a combination of mepyramine (5×10^{-6} M) and atropine (1.2×10^{-6} M); the local anaesthetic lignocaine (10^{-5} M); or the blocker of nerve conduction, tetrodotoxin, (TTX, 9.4×10^{-6} M). The role of neuropeptides in the bronchial response to hyperosmolarity was studied by incubating the airway with drugs which inhibit the degradation of these peptides, such as the inhibitor of neutral metalloendopeptidase (NEP) phosphoramidon (5×10^{-6} M) or a combination of phosphoramidon (5×10^{-6} M), captopril (10^{-5} M) the inhibitor of angiotensin converting enzyme which also has enkephalinase activity, and the aminopeptidase inhibitor bacitracin (10^{-5} M) (Johnson et al., 1985). To examine the possible role of airway smooth muscle relaxing peptides such as vasoactive intestinal polypeptide (VIP) and peptide histidine isoleucine (PHI) (Palmer et al., 1986) segments were incubated with α-chymotrypsin (2 u ml^{-1}). This enzyme cleaves aromatic amino acyl bonds and abolishes relaxations to VIP and PHI in guinea-pig airways (Ellis & Farmer, 1989). After 20 min of incubation with these drugs the osmolarity was raised to 450 mosM. Time-parallel control preparations from the same lung tissue specimens were challenged with hyperosmolar buffer twice, but without adding drugs.

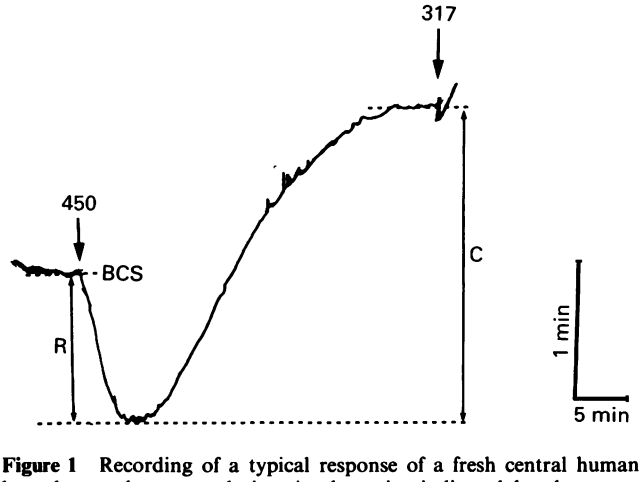

Figure 1 Recording of a typical response of a fresh central human bronchus to hyperosmolarity. At the point indicated by the arrow (450) the osmolarity of the Krebs-Henseleit buffer in the organ bath was raised from 317 mosM to 450 mosM by adding extra NaCl. BCS indicates the baseline contractile state. R indicates the relaxation phase and C the contraction phase.

To study the role of airway epithelium in the response to hyperosmolarity, bronchial rings from which the epithelium had been removed by gentle rubbing with a forceps were challenged with hyperosmolar buffer of 450 mosM. The responses were compared to those of paired bronchial rings with intact epithelium from the same lung tissue specimen. The absence of epithelium was confirmed histologically.

Since lignocaine has been reported to bind to muscarinic receptors (Murlas et al., 1982) we evaluated its anticholinoceptor properties in separate experiments by obtaining cumulative concentration-response curves (CCRC) to methacholine (10^{-8} M–10^{-4} M) in the presence or absence of lignocaine (10^{-5} M).

Hyperosmolarity and the cholinergic responsiveness of the airways

To investigate the influence of hyperosmolarity on the cholinergic responsiveness of the airway preparations, first a CCRC to methacholine (10^{-8} M–10^{-4} M) was obtained and, after washout and return to baseline, hyperosmolar buffer of 450 mosM was added to the organ bath. Once the contractile response to hyperosmolar buffer had reached a plateau (after about 30 min) a second CCRC to methacholine was obtained in the presence of hyperosmolar buffer and, after washout, a third CCRC again at 317 mosM. In time-parallel control preparations three consecutive CCRC to methacholine were obtained. Here, methacholine was added to the organ bath before the second CCRC to match the increase in BCS caused by hyperosmolar buffer in the treated segment.

Hyperosmolarity and airway responses to electric field stimulation

Electric field stimulation (EFS) was applied with a custom-made tissue stimulator that produced voltage-constant rectangular pulses of alternating polarity, via platinum plate electrodes positioned parallel to the preparation. Tetani (30 s) of supramaximal voltage (50 V), a short pulse duration (0.3 ms) and a frequency of 30 Hz were used (De Jongste et al., 1987b). We have previously shown that these stimuli elicit a triphasic contraction-relaxation-contraction response of the airway muscle. The first contractile phase results from activation of cholinergic excitatory nerves. The relaxation phase is non-adrenergic and partially inhibited by the neurotoxin tetrodotoxin (TTX) (De Jongste et al., 1987b,c). In addition, EFS gives rise to tetrodotoxin-insensitive slow contractile responses which result from the release of metabolites of arachidonic acid in the bronchial wall (De Jongste et al., 1987b). A typical response is shown in Figure 2.

First, a control response to EFS was obtained in each of two airway segments. After washout, 30 min later, the osmolarity was raised to 450 mosM in one organ bath. Since earlier findings had indicated that the response to EFS depended on baseline airway tone (De Jongste et al., 1987a) the control preparation received methacholine to match the increase in tone caused by hyperosmolarity. The second response to EFS was obtained at the peak of the contraction to methacholine or hyperosmolar buffer. After washout 30 min later a third response to EFS was obtained after contracting both airways with methacholine to the same level as before the second EFS-stimulation.

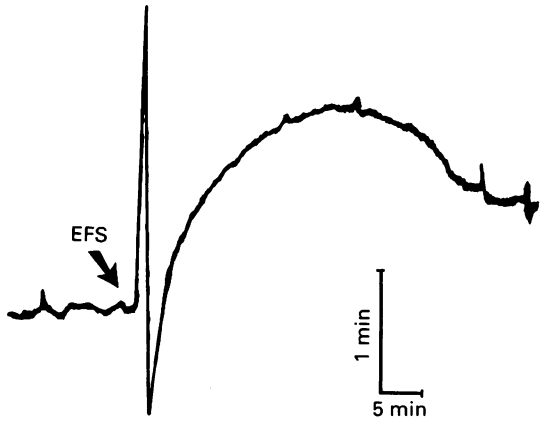

Figure 2 Tracing of a typical response of a fresh central human bronchus to electric field stimulation (EFS) *in vitro*. At the point indicated by the arrow a 30 s tetanus of supramaximal voltage (50 V), short pulse duration (0.3 ms) and a frequency of 30 Hz was given. A cholinergic contraction is followed by a deflection below baseline (non-adrenergic relaxation) and a slow contraction (De Jongste *et al.*, 1987b).

Data analysis

The spontaneous position of a preparation under a 500 mg load is defined as the baseline contractile state (BCS). BCS is expressed on a 0–100% functional scale which defines the maximal active contractile range (% MACR) (Mansour & Daniel, 1986; Jongejan *et al.*, 1988; 1990). The second maximal response to 10^{-4} M methacholine (see: patients and airway preparations) was taken as 100% MACR and the maximal relaxation to isoprenaline (10^{-4} M) in calcium-free buffer with EDTA (1.5×10^{-3} M), which was determined at the end of each experiment, was taken as 0% MACR. Since BCS before the first (BCS_1) and the second (BCS_2) response were different it was important to know if the relaxation and contraction phase were dependent on BCS. Therefore the relation between BCS_1, the relaxation and the contraction phase was analyzed for the first responses of all the bronchial rings using a standard analysis of covariance with lungs as groups and BCS_1 as the covariable (SPSSx, 1983). The analysis revealed that the relaxation phase depended on BCS_1 (range of BCS_1 8.9–79.8% MACR) but only for values of BCS_1 below 30% MACR (regression coefficient for covariate $B = -0.137$, $P < 0.002$). The contraction phase was independent of BCS_1 ($B = -0.056$, $P > 0.45$). The analysis also showed significant differences in the mean relaxation and contraction phase between rings from different lung tissue specimens ($P < 0.001$). Therefore the contraction and relaxation phase of the second response are expressed as a percentage of the contraction and relaxation phase of the first response (% first). Since pairs of rings with and without epithelium were stimulated with hyperosmolar buffer only once, the relaxation and contraction phase were expressed as % MACR. The sensitivity to methacholine is expressed as $-\log EC_{50}$. The maximal effect (E_{max}) of methacholine and the different phases of the responses to EFS are expressed as % MACR. Values of the contraction and relaxation phase as well as differences between sequential responses to EFS were compared for treated preparations and controls from the same tissue specimens using paired Student's t tests (after a logtransformation if appropriate). P values <0.05 (two-tailed) were considered significant. Data are expressed as means ± s.e.mean.

Drugs

Indomethacin (Duchefa, The Netherlands) and 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid (FPL 55712, a gift from Fisons, United Kingdom) were dissolved in methanol. Mepyramine (Rhône-Poulenc, France), atropine (Brocacef, The Netherlands), lignocaine (Astra, Sweden), tetrodotoxin (Sigma, U.S.A.), N-(α-rhamnopyranosyloxy-hydrophosphinyl)-Leu-Trp (phosphoramidon, Sigma, U.S.A.), bacitracin (Sigma, U.S.A.), captopril (Squibb, U.S.A.), α-chymotrypsin type I-S from bovine pancreas (Sigma, U.S.A.), methacholine hydrobromide (Janssen Pharmaceuticals, Belgium) and ethylenediamine tetra-acetic acid disodium salt (EDTA) (Sigma, U.S.A.) were dissolved in saline. (−)-Isoprenaline sulphate (Janssen Pharmaceutical, Belgium) was dissolved in water containing ascorbic acid (88 mg l^{-1}). Preliminary experiments showed that ascorbate and methanol in these concentrations have no effect on airway muscle function.

Results

Effect of hyperosmolarity and its mechanism

Addition of hyperosmolar buffer caused a rapid relaxation, which was maximal after 5.0 ± 0.3 min, followed by a slow contraction, which was maximal after 25.4 ± 0.8 min. BCS before the first response was higher than BCS before the second response (41.3 ± 2.3% MACR vs. 31.2 ± 2.3% MACR, $P < 0.001$). The relaxation and contraction phase of the first and second response did not differ significantly (13.9 ± 1.2% MACR vs. 12.2 ± 1.3% MACR and 26.0 ± 1.8% MACR vs. 29.5 ± 2.0% MACR, both NS).

Table 1 shows the effect of several drugs on BCS, the relaxation and the contraction phase. With indomethacin, mean BCS_2 was significantly above the control value (+12.3% MACR, $P < 0.05$) and with atropine plus mepyramine mean BCS_2 was significantly lower than the control value (−15.4% MACR, $P < 0.05$). In the groups where mean BCS_2 was lowest, i.e. the preparations treated with mepyramine, mepyramine plus atropine and lignocaine, the relaxation phase was also significantly lower, suggesting that this reduction of the relaxation phase was non-specific and related to the low BCS. With mepyramine the contraction phase was 41.2% of the control-value ($P < 0.001$), with atropine 50.0% ($P < 0.01$) and with lignocaine 48.7% ($P < 0.05$). The combination of mepyramine and atropine reduced the contraction phase to 19.2% of the control value ($P < 0.001$), and this reduction was significantly larger than the reductions caused by mepyramine or atropine alone (unpaired t test, $P < 0.001$ for both comparisons). The effect of mepyramine plus atropine on BCS, the relaxation and the contraction phase is illustrated by the tracings shown in Figure 3. Indomethacin, FPL 55712 or TTX had no effect on the response to hyperosmolar buffer.

Table 2 shows that the presence of phosphoramidon, phosphoramidon plus captopril and bacitracin, α-chymotrypsin or the absence of epithelium did not alter BCS_2 or the relaxation phase. With phosphoramidon the contraction phase increased to 128.0% of the control value ($P < 0.05$) and with phosphoramidon plus captopril and bacitracin to 131.0% ($P < 0.01$). In the absence of epithelium the contraction phase increased to 131.8% of the control value ($P < 0.05$). The effect of phosphoramidon on the contraction phase is exemplified by the recording shown in Figure 3.

Ligocaine did not affect the dose-response curves to methacholine indicating that lignocaine does not block muscarinic receptors. The mean $-\log EC_{50}$ in the absence and presence of lignocaine was 5.73 ± 0.18 and 5.77 ± 0.14 respectively (NS, $n = 5$).

Hyperosmolarity and the cholinergic responsiveness of the airways

Table 3 shows the mean $-\log EC_{50}$ and E_{max} values of methacholine in buffer of 317 and 450 mosM. In buffer of 450 mosM, the CCRC shifted leftwards and $-\log EC_{50}$ increased 0.22 ± 0.05. This was significant compared to a difference of

Table 1 Values of BCS_2, the relaxation and the contraction phase in the absence and presence of several drugs

		BCS_2 (% MACR)	*Relaxation phase* (% first)	*Contraction phase* (% first)	n
Indomethacin	−	32.8 ± 5.2	88.0 ± 10.6	127.3 ± 9.6	
	+	45.1 ± 3.5*	70.9 ± 22.1	108.9 ± 17.6	7
FPL 55712	−	24.0 ± 5.5	62.9 ± 11.6	112.9 ± 4.1	
	+	23.3 ± 5.1	77.3 ± 16.6	149.3 ± 20.2	6
Mepyramine	−	28.5 ± 4.1	101.8 ± 14.4	128.4 ± 11.3	
	+	21.0 ± 7.2	57.7 ± 14.1**	52.9 ± 14.2**	9
Atropine	−	35.1 ± 8.3	91.9 ± 16.8	115.9 ± 11.8	
	+	29.5 ± 5.1	123.9 ± 16.5	58.0 ± 11.7**	7
Mepyramine plus atropine	−	31.1 ± 3.7	72.1 ± 9.3	138.9 ± 11.5	
	+	15.7 ± 3.6*	38.0 ± 10.5*	26.6 ± 10.5***	8
Lignocaine	−	24.3 ± 5.0	74.2 ± 13.2	116.6 ± 5.3	
	+	16.8 ± 2.5	36.9 ± 14.5*	56.8 ± 14.9*	9
Tetrodotoxin	−	35.2 ± 5.8	103.7 ± 17.8	110.8 ± 12.5	
	+	35.7 ± 7.1	105.5 ± 24.2	126.3 ± 14.6	8

Abbreviations: BCS_2: the baseline contractile state before the second response to hyperosmolar buffer expressed as a percentage of the maximum active contractile range (% MACR), which is the difference between the maximal contraction to methacholine (10^{-4} M) and the maximal relaxation to isoprenaline (10^{-4} M) in calcium-free buffer with EDTA (1.5×10^{-3} M).
The relaxation and contraction phase are expressed as a percentage of the first response on the same preparation.
Statistical significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ for comparison of responses in the absence (−) and presence (+) of a drug.

0.01 ± 0.03 between the first and the second curves in control preparations kept in 317 mosM ($P < 0.001$). This effect was reversible so that after re-establishing iso-osmolarity $-\log EC_{50}$ was no longer significantly different from the initial values. E_{max} increased by 8.2 ± 1.7% MACR in buffer of 450 mosM, and this was significant compared to a decrease of 0.9 ± 2.4% MACR in controls ($P < 0.001$).

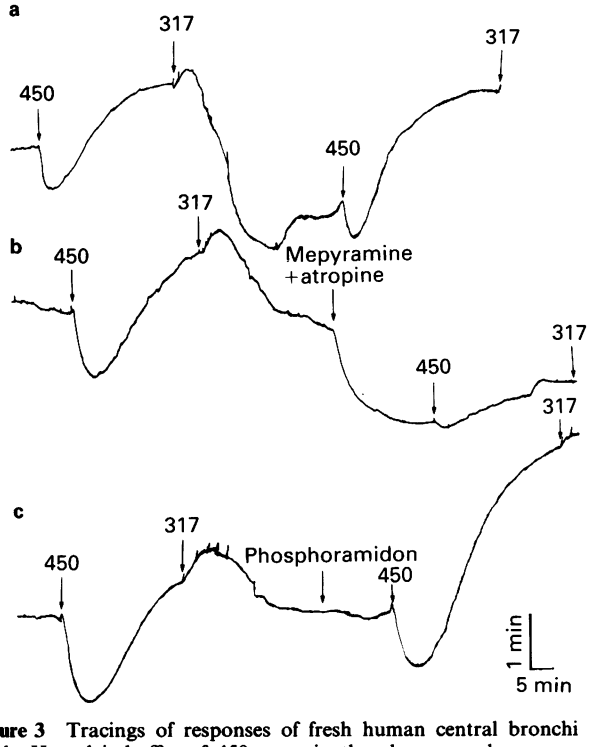

Figure 3 Tracings of responses of fresh human central bronchi to Krebs-Henseleit buffer of 450 mosM in the absence and presence of different drugs. The arrows indicate where the osmolarity was changed to 450 mosM (450) and back to 317 mosM (317) and where drugs were added. (a) Responses of a control preparation challenged with hyperosmolar buffer followed by washing with normal buffer and a second hyperosmolar challenge. (b) Responses to hyperosmolar buffer before and after addition of mepyramine (5×10^{-6} M) and atropine (1.2×10^{-6} M). The combination of these drugs reduces the baseline contractile state as well as the relaxation and the contractile phase of the response to hyperosmolarity. (c) Responses to hyperosmolarity in the absence and presence of phosphoramidon (5×10^{-6} M), which increases the contractile phase of the response to hyperosmolarity.

Hyperosmolarity and airway responses to electric field stimulation

Table 4 shows the responses to EFS before, during and after exposure to hyperosmolar buffer together with the responses of time parallel control preparations. The mean cholinergic contraction obtained during hyperosmolarity was 128.9% of the mean cholinergic contraction before hyperosmolarity whereas in controls this was only 89.4% ($P < 0.001$). A similar difference between controls and treated preparations was seen if the responses during and after hyperosmolarity were compared (121.8% vs. 91.1%), indicating that the increase of the cholinergic phase in hyperosmolar buffer was reversible. The relaxation phase tended to decrease in hyperosmolar buffer but this was not significant. The slow contractile phase did not change in hyperosmolar buffer. The concentration of methacholine prior to the third response to EFS was similar in controls and in preparations that had been treated with hyperosmolar buffer ($3.5 \pm 1.6 \times 10^{-7}$ M vs. $6.5 \pm 3.0 \times 10^{-7}$ M, NS).

Discussion

Our study shows that hyperosmolar Krebs-Henseleit buffer of 450 mosM elicits a biphasic relaxation-contraction response in human fresh isolated central airways. None of the drugs that we used specifically inhibited the initial rapid relaxation phase, and this phase was unchanged in the presence of α-chymotrypsin or after removal of the epithelium. The contraction phase was inhibited by a histamine (H_1) receptor antagonist, a muscarinic receptor antagonist and a local anaesthetic and it was potentiated in the presence of a NEP inhibitor, a combination of a NEP inhibitor with other peptidase inhibitors and after removal of the epithelium. In hypersomolar buffer the sensitivity and maximal response to methacholine as well as the cholinergic twitch to EFS were increased.

Our results only partly concur with those of Finney *et al.* (1987) who reported that in human isolated airways, hypertonic buffer gives an initial relaxation followed by a slow (30–40 min) return to baseline. We also found a relaxation phase followed by a slow contraction phase, but the contraction phase was about twice as big as the relaxation phase, resulting in a net contraction (Figure 1). This difference might be explained by different handling of the tissue (fresh airways versus airways that had been stored overnight) and different methods of measurement (isotonic versus isometric).

Table 2 Values of BCS_2, the relaxation and the contraction phase in the absence and presence of drugs which interfere with the degradation of neuropeptides, and of epithelium

		BCS_2 (% MACR)	*Relaxation phase* (% first)	*Contraction phase* (% first)	n
Phosphoramidon	−	29.5 ± 3.6	74.9 ± 12.8	136.0 ± 9.5	
	+	32.6 ± 4.1	101.0 ± 16.8	174.0 ± 12.6*	10
Phosphoramidon plus captopril plus bacitracin	−	29.2 ± 4.1	81.4 ± 15.3	126.9 ± 13.6	
	+	32.9 ± 5.3	105.9 ± 12.3	166.0 ± 19.3**	8
α-Chymotrypsin	−	28.0 ± 5.5	91.6 ± 13.8	113.2 ± 12.3	
	+	24.3 ± 5.1	76.2 ± 14.2	104.6 ± 12.0	5
Epithelium	+	30.0 ± 4.2	−8.8 ± 2.8†	37.4 ± 5.3†	6
	−	32.8 ± 5.4	−12.8 ± 4.0†	49.3 ± 4.6†*	

† expressed as % MACR, see data analysis.
Abbreviations: BCS_2: the baseline contractile state before the second response to hyperosmolar buffer expressed as a percentage of the maximum active contractile range (% MACR), which is the difference between the maximal contraction to methacholine (10^{-4} M) and the maximal relaxation to isoprenaline (10^{-4} M) in calcium-free buffer with EDTA (1.5×10^{-3} M).
The relaxation and contraction phase are expressed as a percentage of the first response on the same preparation.
Statistical significance: $*P < 0.05$, $**P < 0.01$ for comparison of responses in the presence (+) and absence (−) of a drug.

Table 3 Responses of segments of central human airways to methacholine at different osmolarities

Treated	317 mosM	450 mosM	317 mosM
$-\log EC_{50}$	5.89 ± 0.05	6.11 ± 0.8***	5.86 ± 0.07
E_{max}	100.5 ± 2.4	108.7 ± 2.1***	93.7 ± 2.3
Control	317 mosM	317 mosM	317 mosM
$-\log EC_{50}$	5.90 ± 0.07	5.91 ± 0.04	5.93 ± 0.07
E_{max}	95.0 ± 1.9	94.1 ± 1.4	90.0 ± 1.5

Abbreviations: $-\log EC_{50}$: the negative logarithm of the methacholine concentration that caused 50% of the maximal effect. E_{max}: maximal effect of methacholine expressed as a percentage of the maximum active contractile range (% MACR), which is the difference between the maximal contraction to methacholine (10^{-4} M) at the beginning of the experiment and the maximal relaxation to isoprenaline (10^{-4} M) in calcium-free buffer with EDTA (1.5×10^{-3} M). For each preparation a CCRC was obtained before, during and after a change in the osmolarity of the bathing fluid. In controls the osmolarity remained unchanged.
Mean results from 7 different lung tissue specimens.
Statistical significance: $***P < 0.001$ compared to control.

In earlier experiments on peripheral human airways we found that hyperosmolar buffer of 450 mosM evoked a monophasic contractile response, while we found no evidence for the release of histamine; anticholinoceptor drugs were not tested in this previous study (Jongejan *et al.*, 1990). These two differences between the present and the previous study may well be related to methodological factors. Firstly, in the present study we raised the osmolarity by adding NaCl directly to the organ bath while in the previous study (Jongejan *et al.*, 1990) we washed the iso-osmolar buffer in the organ bath away with hyperosmolar buffer. During this washing procedure we probably missed the initial phase of the response. Secondly, in the present study the contribution of differences between preparations to the total variability in the responses was minimized by stimulating each preparation twice so that it could serve as its own control. In the earlier study (Jongejan *et al.*, 1990) the responses of drug-treated preparations were compared to those of a time-parallel control. This increases the risk of a type II error (false negative result) since differences between preparations and differences due to drug effects will both contribute to the observed variability. Thus, it seems possible that in the previous study (Jongejan *et al.*, 1990) we did not detect the relaxation phase because of different ways of increasing the osmolarity of the buffer, and that we missed a histamine-effect because of a type II error. Indeed, in later experiments on fresh peripheral airways where we added extra NaCl directly to the organ bath we also observed a rapid relaxation followed by a contraction which was virtually abolished in the presence of mepyramine together with atropine (R.C. Jongejan, unpublished observations).

The effect of hyperosmolarity seems to differ between species. A study on the trachea of anaesthetized dogs showed that hyperosmolar buffer had no effect on the external tracheal diameter (Deffenbach *et al.*, 1989). In precontracted guinea-pig trachea, luminal application of hyperosmolar buffer resulted in an epithelium-dependent relaxation (Munakata *et*

Table 4 Responses of human airways to electric field stimulation at different osmolarities

Treated	317 mosM	450 mosM	317 mosM
Cholinergic twitch	20.4 ± 7.7	26.3 ± 7.2***	21.6 ± 7.5
Non-adrenergic relaxation	−9.5 ± 3.3	−5.4 ± 3.4	−3.2 ± 1.6
Slow contraction	7.7 ± 3.0	11.7 ± 2.8	12.0 ± 4.6
Control	317 mosM	317 mosM	317 mosM
Cholinergic twitch	22.8 ± 6.5	20.4 ± 5.7	22.6 ± 5.6
Non-adrenergic relaxation	−10.1 ± 5.4	−11.7 ± 5.4	−10.7 ± 4.7
Slow contraction	11.0 ± 4.3	12.6 ± 6.2	15.8 ± 5.1

The results are expressed as a percentage of the maximum active contractile range (% MACR), which is the difference between the maximal contraction to methacholine (10^{-4} M) and the maximal relaxation to isoprenaline (10^{-4} M) in calcium-free buffer with EDTA (1.5×10^{-3} M). For each preparation a response to electric field stimulation (EFS) was obtained before, during and after a change in the osmolarity of the bathing fluid. In controls the osmolarity remained unchanged.
Mean results from 7 different lung tissue specimens.
Statistical significance: $***P < 0.001$ compared to control.

al., 1988), while we observed a biphasic relaxation-contraction response. However, we did not selectively apply the osmotic stimulus to the mucosal surface of the airway *in vitro* and this could also account for the difference in response pattern.

What are the mechanisms of the relaxation and contraction phase? The reduction of the relaxation phase in the presence of mepyramine, mepyramine plus atropine and lignocaine was probably nonspecific since these drugs reduced BCS to below 25% MACR, a level associated with low values of R. The relaxation phase is probably not caused by relaxing peptides which contain aromatic amino acyl bonds such as VIP or PHI nor by an epithelium-derived relaxing factor, since it remained unchanged in the presence of α-chymotrypsin or after removal of the epithelium. We would therefore suggest, by exclusion, that the relaxation phase is caused by the release of an unknown relaxing substance or a direct effect of hyperosmolarity on the airway muscle. As suggested by Finney *et al.* (1987), it seems possible that an increased extracellular Na^+ concentration leads to the influx of Na^+ into the smooth muscle cell via the Na^+/Ca^{2+}-exchanger (Rodger, 1985). This exchanger couples the influx of Na^+ to the efflux of Ca^{2+}, which will cause relaxation. However, this possibility requires further investigation.

The reduction of the contraction phase with atropine or mepyramine indicates that histamine and acetylcholine contribute to this phase. That the reduction of contraction phase with mepyramine plus atropine was significantly greater than with atropine and mepyramine alone points to a separate and additive contribution of acetylcholine and histamine to smooth muscle contraction in hypertonic buffer. Several explanations are possible for the inhibitory effect of lignocaine on the contraction phase. Firstly, it has been suggested that lignocaine may block muscarinic receptors (Murlas *et al.*, 1982) but this seems unlikely since in the present study lignocaine did not change the response to methacholine. Secondly, lignocaine may inhibit nervous conductance. However, because TTX which inhibits nervous conductance too, had no effect on the contractile phase this seems an unlikely explanation for the effect of lignocaine. Thirdly, lignocaine may have inhibited the release of histamine via a direct effect on mast cells (Kazimierczak *et al.*, 1976) but this seems unlikely since this effect is only seen at concentrations above 10^{-3} M while we used 10^{-5} M. Finally, lignocaine may have blocked the release of neuropeptides from afferent nerves (Persson *et al.*, 1986), a process that is insensitive to TTX (Lundberg *et al.*, 1984). The potentiation of the contraction phase with phosphoramidon further supports the possibility that peptides that are sensitive to degradation by NEP are implicated in the contraction phase. A recent study by Umeno *et al.* (1990) on rat trachea also indicates that hyperosmolarity releases neuropeptides from afferent nerves. The potentiating effect of epithelial denudation on the contraction phase is also in keeping with a contribution of NEP-sensitive peptides to this phase, since in the airways NEP is predominantly located in the epithelium (Johnson *et al.*, 1985). Alternatively, removal of the epithelium increases the sensitivity of the airway muscle to acetylcholine and histamine (Aizawa *et al.*, 1988) and this could also have contributed to an increase of the contraction phase. Thus, the relaxation phase of the response to hyperosmolarity may involve the release of an unknown muscle relaxing substance, which is not of epithelial origin, or a direct relaxing effect of hyperosmolar buffer on the airway muscle caused by the influx of sodium into the smooth muscle cell coupled to the efflux of calcium via the Na^+/Ca^{2+} exchanger. The contractile phase may result from stimulation of afferent nerves and the release of neuropeptides, acetylcholine and histamine. These mechanisms may well be interrelated, as has been demonstrated in rat airways (Joos *et al.*, 1988) where neuropeptides may release histamine and acetylcholine, but this remains to be elucidated for human airways.

Our finding that the sensitivity to methacholine increases slightly in hyperosmolar buffer confirms our previous results on peripheral airways (Jongejan *et al.*, 1990). This increased sensitivity of the airway muscle could also explain the increased cholinergic response to EFS that we have found. Alternatively, hyperosmolarity may facilitate the release of acetylcholine during EFS. The mechanism of this hyperosmolarity-induced airway hypersensitivity remains to be investigated.

Can our findings contribute to the understanding of bronchoconstriction to inhalation of hypertonic saline in asthmatics? It has already been pointed out (see Methods: effect of hyperosmolarity and its mechanism) that the challenge with buffer of 450 mosM that we have used may well be relevant to the *in vivo* situation. However, several differences between the *in vivo* and *in vitro* situation make it difficult to know how the *in vitro* response relates to the *in vivo* one. First, we could not use asthmatic airways. Second, unlike *in vivo*, the osmotic stimulus *in vitro* was not selectively applied to the mucosa. It is possible that selective application of the hyperosmolar stimulus to the mucosa would result in an initial contraction, since the contraction phase seems of mucosal origin. In this case the response pattern *in vivo* and *in vitro* would be more alike. The relaxation phase, which does not seem to originate from the mucosa, may not be seen or even follow the initial contraction, in case hyperosmolarity penetrates to the level of the smooth muscle. Third, the time course of the responses *in vivo* and *in vitro* cannot be simply compared since contractions *in vitro* are often slower than bronchoconstriction *in vivo*. For instance, *in vivo* the response to inhalation of a dose of methacholine will reach a maximum after about 3 min whereas in our experiments the maximum of the response to a dose of methacholine will be reached after 12–15 min. Based on this comparison we would estimate that the contraction phase of the response to hypertonicity *in vivo* would take 5–6 min, which is comparable to the time course of hyperosmolarity-induced bronchoconstriction *in vivo*. Fourth, we do not know how the BCS *in vitro* relates to baseline airway tone *in vivo*. Finally, the mucosal swelling which probably occurs *in vivo* after the release of neuropeptides or histamine and which might contribute to airway narrowing does not occur *in vitro*. However, the fact that in asthmatics, H_1-receptor antagonists (Finnerty *et al.*, 1989; Finney *et al.*, 1990) and blockers of muscarinic cholinoceptors (Boulet *et al.*, 1989) provide partial protection against the bronchoconstriction to inhaled hyperosmolar saline suggests that the response *in vivo* and *in vitro* have similar mechanisms. In this respect two of our findings are potentially important. First, that hyperosmolarity may not only release histamine and acetylcholine in the airway wall but also neuropeptides. Secondly, that the absence of the epithelium potentiates the contractile effect of hyperosmolarity. Since in asthmatic airways the epithelium seems to be damaged (Laitinen *et al.*, 1985; Kirby *et al.*, 1987; Jeffery *et al.*, 1990), this might be a factor which contributes to the hyperresponsiveness to inhalation of hypertonic saline in asthma.

In conclusion, hyperosmolarity causes a biphasic relaxation-contraction response in central human airways. The relaxation phase is probably not due to relaxing peptides such as VIP or PHI or the release of an epithelium-derived relaxing factor, but may result from a direct effect of hyperosmolar buffer on the muscle. The release of acetylcholine, histamine and neuropeptides contributes to the contractile phase. This contractile phase is increased after removal of the epithelium. Prostaglandins or LTC_4/D_4 do not seem to contribute to the contractile phase.

This study was supported by the Netherlands Asthma Foundation. The authors wish to thank the departments of pathology, pulmonology, and thoracic surgery of the Ikazia Hospital (Rotterdam), the Zuiderziekenhuis (Rotterdam), the Leyenburg Hospital (The Hague) and the University Hospital of Erasmus University (Rotterdam) for their help in providing the lung tissue and clinical data.

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(Received June 20, 1990
Revised November 8, 1990
Accepted November 29, 1990)

Br. J. Pharmacol. (1991), **102**, 938–942

Characterization of the 5-HT receptor mediating endothelium-dependent relaxation in porcine vena cava

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1 5-Hydroxytryptamine (5-HT) relaxes rings of neonatal porcine isolated vena cava by both an endothelium-dependent and an endothelium-independent mechanism. The receptor mediating the latter response has been shown to be a 5-HT_1-like receptor (positively coupled to adenylyl cyclase) located on the vascular smooth muscle. The features of the endothelium-dependent response to 5-HT in this preparation are now described.

2 In ring preparations contracted with the stable thromboxane-A_2-mimetic, U-46619 (10 nM), and in the presence of the 5-HT_2 receptor antagonist ketanserin (1 μM), low concentrations of 5-HT (1–100 nM) evoked an endothelium-dependent, rapid, 'spike-like' relaxation. Higher concentrations of 5-HT (0.1–10 μM) elicited a more sustained, but endothelium-independent relaxation.

3 Relaxation induced by low concentrations (1–100 nM) of 5-HT was abolished by endothelium removal, and was markedly (but not totally) inhibited by the guanylate cyclase inhibitor, methylene blue (10 μM) or by the inhibitor of endothelium-derived nitric oxide (NO) synthesis, L-N^G-monomethylarginine (L-NMMA; 100–500 μM).

4 The endothelium-dependent response to 5-HT was mimicked by α-methyl-5-HT, 5-methoxytryptamine, tryptamine and 2-methyl-5-HT, but not by sumatriptan or 8-hydroxy-di-n-propylaminotetralin (8-OH-DPAT) at concentrations up to 10 μM. In contrast, relaxation evoked by 5-carboxamidotryptamine (5-CT) was endothelium-independent.

5 The endothelium-dependent relaxation induced by 5-HT or α-methyl-5-HT was antagonized by methysergide, methiothepin, cyproheptadine and metergoline, but not by ketanserin, spiperone, ondansetron, verapamil, cyanopindolol, mesulergine, ICS 205-930, or indomethacin.

6 These results suggest that the endothelium-dependent relaxation of porcine vena cava induced by 5-HT is largely mediated by the release of NO (although other endothelium-derived relaxing factors may also be involved) and that 5-HT is acting at a receptor which is not '5-HT_1-like', 5-HT_2, 5-HT_3 or 5-HT_4 and is not comparable to recognised 5-HT receptor ligand binding sites. The characteristics of this receptor are discussed in relation to the endothelial 5-HT receptor types in other blood vessels.

Keywords: Endothelium; EDRF; 5-HT receptor; α-methyl-5-HT; porcine vena cava

Introduction

Relaxation of vascular smooth muscle by 5-hydroxytryptamine (5-HT) can be brought about by at least two mechanisms. We have previously described an endothelium-independent relaxation of neonatal porcine isolated vena cava by 5-HT and have shown this to be mediated through a '5-HT_1-like' receptor located on the vascular smooth muscle, activation of which leads to increases in intra-cellular adenosine 3':5'-cyclic AMP (cyclic AMP) and smooth muscle relaxation (Sumner *et al.*, 1989).

Relaxation of vascular smooth muscle by 5-HT can also be elicited through an endothelium-dependent mechanism. The 5-HT receptors mediating such responses, which have been studied extensively in coronary artery preparations, have been designated '5-HT_1-like'. Such a classification has largely been based on the lack of effect of 5-HT_2 and 5-HT_3 receptor antagonists (Cocks & Angus, 1983; Leff *et al.*, 1987; Molderings *et al.*, 1989; reviewed in Angus & Cocks, 1989). An endothelium-dependent relaxation to 5-HT in the neonatal porcine isolated vena cava preparation has previously been described (Sumner & Humphrey, 1988). The characteristics of this response are now described in more detail.

Methods

Measurement of vascular relaxation

This was performed as previously described (Sumner *et al.*, 1989). Briefly, lengths of abdominal vena cava were removed from neonatal pigs (Large White variety, 6–10 days of age, killed by captive-bolt pistol and exsanguination) and were divided into 4 rings (each 2–3 mm in length). These were mounted for isometric recording of tension changes and were maintained at 37°C in a gassed Krebs solution under a resting tension of 0.2–0.5 g, and allowed to equilibrate for 60 min, with changes of Krebs solution every 15 min. After this period, preparations were 'primed' with 80 mM KCl which was then washed out with fresh Krebs solution. After a further 30 min, preparations were contracted with 10 nM U-46619, a concentration that had previously been shown to produce 70–90% of its maximal response. When a stable tone had been established, cumulative concentration-effect curves to 5-HT were produced in all preparations. Throughout this study, the thromboxane A_2 receptor agonist, U-46619 was used to contract porcine vena cava ring preparations in the presence of the 5-HT_2 receptor antagonist, ketanserin (1 μM) to prevent the contractile effects of 5-HT_2 receptor agonists (Cocks & Angus, 1983).

Agonist potency measurements

Cumulative concentration-effect curves (over the range 0.1 nM to 10 μM) to agonists were constructed in preparations contracted with U-46619 (10 nM), each successive concentration being applied when the response to the previous concentration had reached a plateau. Agonist potencies were assessed relative to 5-HT. Thus, on one ring preparation (control), cumulative concentration-effect curves were repeated to assess changes in tissue sensitivity to 5-HT (geometric mean agonist concentration-ratio, with 95% confidence intervals, was 1.23, 0.38–1.97, $n = 28$). On three other ring preparations from the same vessel, concentration-effect curves to 5-HT were followed

by curves for test agonists. Relaxant responses were expressed as a percentage of U-46619-induced tone, this being the difference between resting and sustained tensions (0.5–1 g). Relaxation was measured at the peak response and expressed as a percentage relative to the initial tone preceding agonist addition. The concentration of an agonist required to produce 50% of its own maximal relaxation represented the EC_{50} value; where quoted, the pEC_{50} is the negative logarithm$_{10}$ of the molar EC_{50} value.

Antagonist potency measurements

Potential antagonists of the relaxation induced by 5-HT or α-methyl-5-HT were assessed in preparations contracted with U-46619 in the presence of ketanserin (1 μM).

For these experiments, four ring preparations from the same blood vessel were used. Cumulative concentration-effect curves to 5-HT or α-methyl-5-HT were produced for each preparation. After washing, three rings were exposed to antagonist or vehicle, whilst the fourth ring remained untreated (control). After 30 min, the preparations were contracted to a constant tone and cumulative concentration-effect curves to 5-HT or α-methyl-5-HT were repeated.

After correcting for any sensitivity changes over successive agonist curves, concentration-ratios were calculated by dividing the EC_{50} for the agonist in the presence of antagonist (second curve) by the EC_{50} for the agonist in the absence of antagonist (first curve). In control preparations, the geometric mean concentration-ratios (with 95% confidence intervals) over two consecutive 5-HT or α-methyl-5-HT concentration-effect curves were 1.53 (0.51–2.13) and 1.71 (0.29–2.39) respectively ($n = 26$). Concentration-ratios were used to calculate pA_2 (slope) values by linear regression analyses of Schild plots (Arunlakshana & Schild, 1959).

Endothelium removal

In experiments to examine the endothelium dependency of 5-HT-induced relaxant responses, lengths of porcine vena cava were divided in half. One half was endothelium-denuded by scraping the lumen with an inflated balloon catheter (Swan-Ganz, 0.5 ml capacity), whilst the other was left intact (control). The vessel sections were then divided into rings and mounted in organ baths. Absence of the endothelium was confirmed by loss of relaxant response to acetylcholine or carbachol (1 μM).

Drugs

The following compounds were purchased or generously donated: 5-hydroxytryptamine creatinine sulphate, tryptamine hydrochloride, methylene blue, 5-methoxytryptamine hydrochloride and verapamil hydrochloride (Sigma), spiperone, ketanserin (Janssen), methiothepin maleate (Hoffman-La Roche), metergoline (Farmitalia), methysergide hydrogen maleate, mesulergine (a kind gift from Dr G. Engel, Sandoz), L-N^G-monomethylarginine citrate (Salford Ultrafine Chemicals, Manchester), and ICS 205–930 (Research Biochemicals).

The following compounds were synthesized by Chemistry Research, Glaxo Group Research, Ware, Herts: 5-carboxamidotryptamine, α-methyl-5-HT maleate, 2-methyl-5-HT sulphate, 8-hydroxy-2(di-n-propylamino)tetralin hydrobromide, (±)-cyanopindolol, U-46619 (11,9-epoxymethanoprostaglandin), sumatriptan and ondansetron.

Statistics

Values are expressed as arithmetic means (± s.e.mean) or geometric means (with 95% confidence limits) from n observations, which is also the number of animals used.

Results

In ring preparations of neonatal isolated vena cava of the pig contracted with U-46619 (10 nM), and in the presence of the 5-HT_2 receptor antagonist ketanserin (1 μM), low concentrations of 5-HT (1–100 nM) elicited a complex relaxant response (Figure 1). This response (exemplified by 10 nM 5-HT) was comprised of an initial, rapid 'spike-like' phase of relaxation, followed by a less marked but more sustained reduction in tone (Figure 1a). The full response required the continued presence of 5-HT, since washout with Krebs solution containing U-46619 (10 nM) restored the level of contraction to that prior to the addition of 5-HT (Figure 1b). Removal of the endothelium abolished all responses elicited by 5-HT at concentrations below 100 nM (Figure 1c).

Agonist potency measurements

The 'spike-like', endothelium-dependent relaxation induced by 5-HT ($pEC_{50} = 8.4$) was reproduced, both qualitatively and quantitatively by α-methyl-5-HT ($pEC_{50} = 8.8$). Similar responses were obtained with 5-methoxytryptamine, tryptamine and 2-methyl-5-HT (Table 1), but not with 8-hydroxy-di-n-propylaminotetralin (8-OH-DPAT) or sumatriptan at concentrations up to 10 μM. Concentration-effect curves for 5-HT receptor agonists were bell-shaped (Figure 2), with responses decreasing at higher concentrations.

At concentrations greater than 30–100 nM, 5-HT also elicited a relaxation which was endothelium-independent (Figure 3), a response that was mimicked by the 5-HT_1-like receptor agonist, 5-carboxamidotryptamine (5-CT; 1–100 nM). Thus, endothelium removal did not affect either the sensitivity or the pattern of relaxation to higher concentrations (30 nM–10 μM) of 5-HT or to 5-CT (no evidence for an endothelium-dependent relaxation to 5-CT could be obtained over the concentration range 1 pM–1 μM). It should be noted, however, that the endothelium-independent relaxation to 5-CT was maximal at 100 nM, and therefore any activity of 5-CT on the endothelial

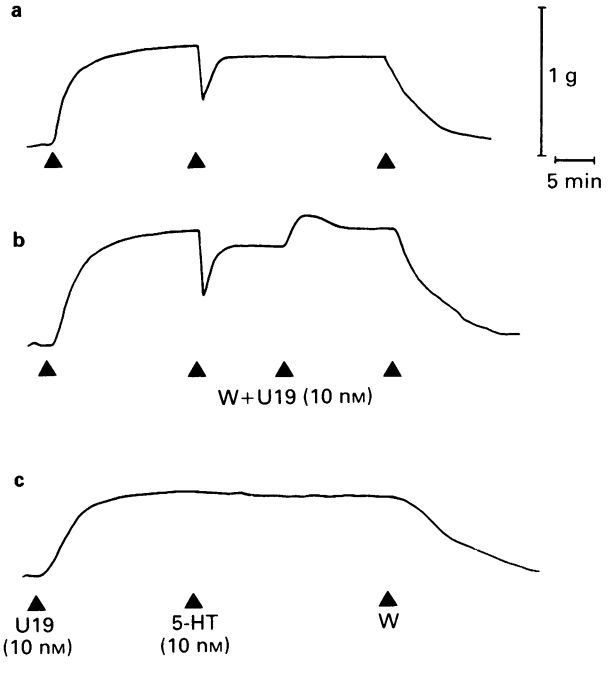

Figure 1 Relaxation to a single concentration of 5-hydroxytryptamine (5-HT, 10 nM) in endothelium-intact (a and b) or endothelium-denuded (c) ring preparations of neonatal porcine isolated vena cava contracted with U-46619 (U19; 10 nM) in the presence of ketanserin (1 μM). The sustained phase of relaxation induced by 5-HT (a) was reversed by washout (W) with Krebs solution containing U19 (10 nM, b). No response to 5-HT was evident in an endothelium-denuded preparation (c).

Table 1 Endothelium-dependent relaxant activity of 5-HT receptor agonists in neonatal porcine isolated vena cava ring preparations contracted with U-46619 (10 nM) in the presence of ketanserin (1 μM)

Agonist	pEC_{50}	% *maximal relaxation*	n
α-Methyl-5-HT	8.8 ± 0.1	70 ± 10	13
5-HT	8.4 ± 0.6	85 ± 3	20
5-Methoxytryptamine	8.4 ± 0.7	81 ± 33	4
Tryptamine	8.0 ± 0.2	91 ± 15	4
2-Methyl-5-HT	7.2 ± 0.1	62 ± 11	4
5-CT	*		
8-Hydroxy-DPAT	< 5.0	0	3
Sumatriptan	< 5.0	0	3

Results are means ± s.e.mean from *n* experiments.
* At a concentration of 100 nM, 5-CT produced a maximal relaxation through an endothelium-independent mechanism which precluded an assessment of any activity of this agonist at the endothelial cell 5-HT receptor.

5-HT receptor at higher concentrations could not be determined. In an attempt to unmask endothelium-dependent effects of 5-CT, responses were examined in the presence of spiperone (1 μM), an antagonist at the smooth muscle cell 5-HT receptor (Sumner *et al.*, 1989). This concentration produced a 10 fold rightward shift in the concentration-effect curve to 5-CT, but did not reveal any endothelium-dependent activity at 5-CT concentrations up to 1 μM. Attempts to use higher concentrations of spiperone were unsuccessful due to an action at the endothelial cell 5-HT receptor at concentrations of 3 μM or greater (results not shown). In contrast to 5-CT, α-methyl-5-HT evoked only an endothelium-dependent relaxation (Figure 3), producing no response (at concentrations up to 1 μM) in the absence of an intact endothelium.

Antagonist studies

Endothelium-dependent relaxations induced by 5-HT or α-methyl-5-HT were abolished in preparations treated with 1 μM methiothepin, methysergide, or cyproheptadine. Antagonism by lower concentrations of methysergide (1 and 10 nM) was

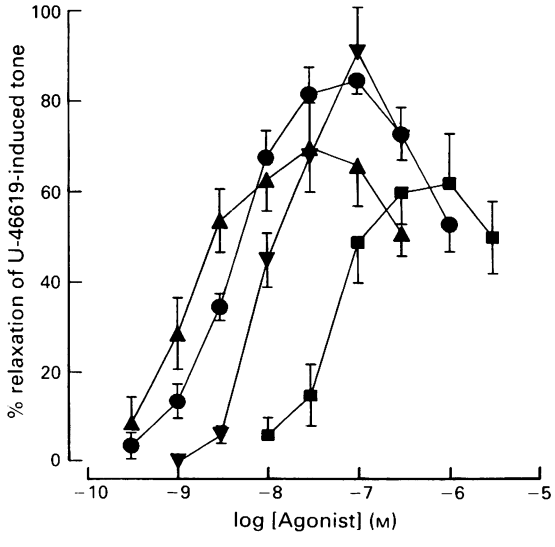

Figure 2 Concentration-effect curves for the endothelium-dependent relaxation of neonatal porcine isolated vena cava by 5-hydroxytryptamine (5-HT) (●), α-methyl-5-HT (▲), tryptamine (▼) and 2-methyl-5-HT (■). Ring preparations were contracted with U-46619 (10 nM), in the presence of ketanserin (1 μM), and cumulative curves established. Results are means from at least four separate determinations, with relaxation being measured at the peak response ('spike') in each case; vertical bars show s.e.mean.

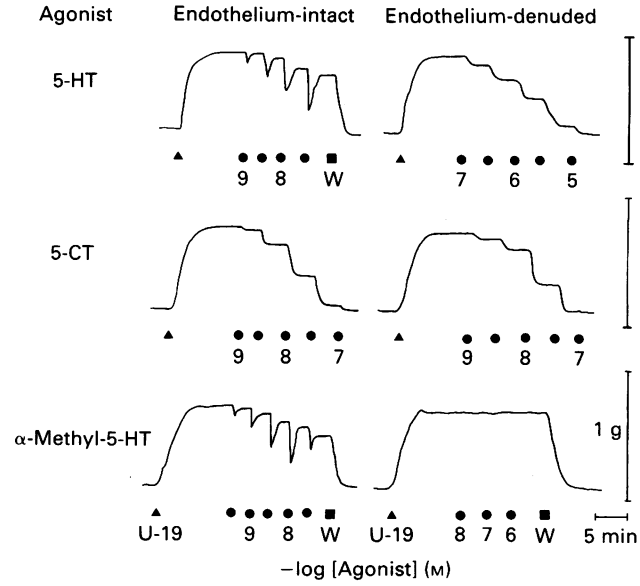

Figure 3 Effect of endothelium removal on relaxant responses to 5-HT, 5-carboxamidotryptamine (5-CT) and α-methyl-5-HT. Ring preparations of neonatal porcine isolated vena cava were contracted with U-46619 (U-19; 10 nM), in the presence of ketanserin (1 μM), and cumulative concentration-effect curves (3 fold increments) were constructed for each of the 5-HT receptor agonists. Note that although, unlike the other two agonists, 5-HT produced both endothelium-dependent and independent relaxations, the endothelium-independent relaxation occurred at considerably higher relative concentrations of 5-HT (0.1–10 μM).

accompanied by a depression in the maximum response to the relaxant agonist (Figure 4a). Only metergoline appeared to behave as a competitive antagonist (Figure 4b; pA_2 7.2 ± 0.3, slope 1.22 ± 0.18 versus α-methyl-5-HT). Specificity of antagonism was assessed at 1 μM versus carbachol. Carbachol produced a concentration-dependent relaxation (pEC_{50} 7.7 ± 0.2) of the porcine vena cava preparation, eliciting 100% relaxation at 1 μM. This response was totally dependent upon an intact endothelium, there being no relaxation in endothelium-denuded preparations. Methiothepin (geometric mean concentration-ratio (CR) with [95% confidence limits] of 10.2[2.1–17.3]) and methysergide (CR = 5.7, [2.3–9.3]), but not metergoline (CR < 2), produced some antagonism (at 1 μM) of the relaxant responses to carbachol (1 nM–1 μM). Antagonists which failed to modify endothelium-dependent relaxation to either 5-HT or α-methyl-5-HT included mesulergine, cyanopindolol, ketanserin, spiperone and ondansetron, all at 1 μM, and ICS 205-930 at 10 μM. In addition, sumatriptan and 8-OH-DPAT were not antagonists (at 10 μM).

Mechanistic studies

Relaxation of porcine vena cava induced either by 5-HT (1–100 nM) or α-methyl-5-HT (0.3–100 nM) was clearly abolished by endothelium removal (Figure 3). Responses evoked by these agonists were not mediated by the release of prostacyclin, since relaxation was unaffected by the cyclo-oxygenase inhibitor, indomethacin (2.8 μM); furthermore, prostacyclin produced only a contractile response in this preparation (Sumner, unpublished observation). The calcium channel blocker verapamil (1 μM) also failed to inhibit responses to α-methyl-5-HT (the effect on responses to 5-HT was not investigated). However, relaxation induced either by 5-HT or α-methyl-5-HT was markedly, but not totally, attenuated (Figure 5) by the inhibitor of soluble guanylate cyclase, methylene blue (10 μM; Ignarro & Kadowitz, 1985) and by the inhibitor of endothelium-derived nitric oxide (NO) synthesis, L-N^G-monomethylarginine (L-NMMA, 500 μM; Rees *et al.*, 1989) with mean CR values (calculated at the 20% level) of 250[98–288] and 56[10–228] respectively. Both of these inhibitors

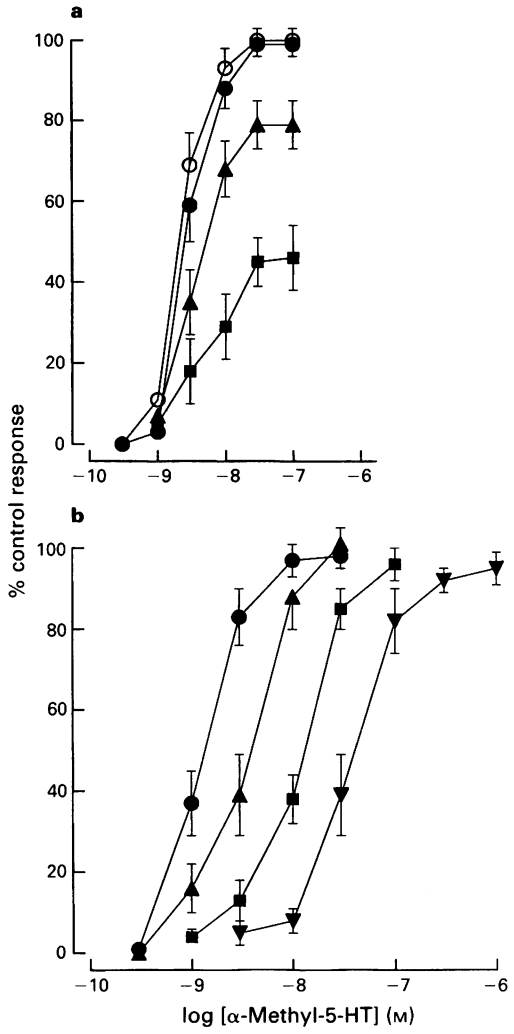

Figure 4 Antagonism of relaxant responses to α-methyl-5-hydroxytryptamine (α-methyl-5-HT) in ring preparations of neonatal porcine isolated vena cava by methysergide (a) and metergoline (b). Control responses to α-methyl-5-HT were repeated in preparations which had been pretreated (30 min) with either Krebs solution (○, ●) or antagonist solution. Methysergide at 1 nM (▲) or 10 nM (■); metergoline 0.1 μM (▲), 0.3 μM (■) or 1 μM (▼). Results are means of 3 determinations (with s.e.mean shown by vertical bars), and are expressed relative to the control response to α-methyl-5-HT in each preparation. Ketanserin (1 μM) was present throughout.

caused a small contraction (about 15% of the U-46619 response) of resting ring preparations.

Discussion

The neonatal porcine isolated vena cava preparation appears to contain two types of 5-HT receptor mediating smooth muscle relaxation. A 5-HT_1-like receptor is located on the vascular smooth muscle and is coupled to adenylyl cyclase (Sumner *et al.*, 1989). This receptor is stimulated by 5-HT and 5-CT, but does not recognise either α-methyl-5-HT or tryptamine. Furthermore, the smooth muscle receptor can be antagonized by methiothepin, methysergide, metergoline, mesulergine and spiperone (Sumner *et al.*, 1989).

The results of the present study suggest that relaxation of the porcine vena cava by 5-HT can also be elicited through an action at an endothelial cell 5-HT receptor. Activation of this receptor appears to evoke the release of an endothelium-derived relaxing factor (EDRF), principally NO, which in turn causes smooth muscle relaxation by elevating intracellular guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels (reviewed in Ignarro & Kadowitz, 1985). In keeping with this

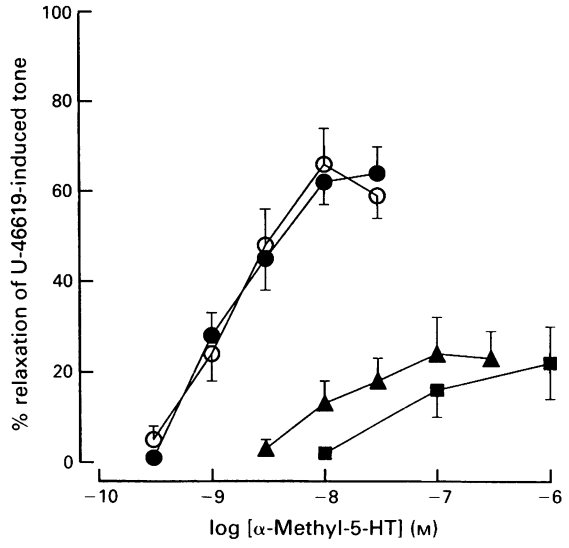

Figure 5 Effects of L-N^G-monomethylarginine and methylene blue on relaxant responses to α-methyl-5-hydroxytryptamine (α-methyl-5-HT). Ring preparations of neonatal porcine vena cava were exposed to Krebs solution (○, ●), monomethylarginine (▲, 500 μM) or methylene blue (■, 10 μM) for 15 min prior to contraction with U-46619 (10 nM) and, in the presence of ketanserin (1 μM), cumulative concentration-effect curves were constructed for α-methyl-5-HT (results are means from 3 determinations; s.e.mean shown by vertical bars).

mechanism, relaxation evoked by 5-HT or its analogue, α-methyl-5-HT, was attenuated by the guanylate cyclase inhibitor, methylene blue (Ignarro & Kadowitz, 1985) or by the inhibitor of EDNO synthesis, L-NMMA (Rees *et al.*, 1989). It should be noted, however, that these compounds did not totally abolish responses to α-methyl-5-HT, thus raising the possibility that more than one EDRF could be involved.

The two types of 'relaxant' 5-HT receptors in this preparation can readily be distinguished by removal of the endothelium. In addition, these receptors can also be differentiated on the basis of their pharmacology. Thus, the smooth muscle relaxant 5-HT_1-like receptor is selectively stimulated by 5-CT and antagonized by spiperone or mesulergine (Sumner *et al.*, 1989), whereas the endothelial cell 5-HT receptor is activated by α-methyl-5-HT and tryptamine, does not appear to recognise 5-CT, and is not antagonized by spiperone or mesulergine (at concentrations up to 1 μM). Higher concentrations of spiperone also blocked the endothelial cell 5-HT receptor, and hence could not be used to reveal any endothelium-dependent relaxation to 5-CT at concentrations above 1 μM.

An endothelium-dependent relaxation to 5-HT has been widely reported (Cocks & Angus, 1983; Molderings *et al.*, 1989; reviewed in Angus & Cocks, 1989). In the majority of cases, responses have been studied in porcine or canine coronary blood vessels and have demonstrated both equipotent agonist activity for 5-HT and 5-CT, and antagonism by methiothepin or methysergide, but not by ketanserin. This has prompted the classification of this type of receptor as 5-HT_1-like (see Bradley *et al.*, 1986). An extensive receptor characterization has also been undertaken for the endothelium-dependent response to 5-HT in rabbit jugular vein (Leff *et al.*, 1987), the pharmacology of which appears similar to that described here. Interestingly, the pattern of response ('spike-like') and the relative agonist potencies, particularly with respect to α-methyl-5-HT and 5-CT in the studies using venous tissue, are somewhat different from those seen with coronary artery preparations. Furthermore, recent publications have reported significant agonist activity with sumatriptan (Schoeffter & Hoyer, 1989; 1990) and antagonism by cyanopindolol (Molderings *et al.*, 1989) in the porcine coronary artery, observations that contrast with those made

in this study, in which sumatriptan and cyanopindolol were without effect either as agonists or antagonists. It is tempting, therefore, to speculate that these differences reflect endothelial cell 5-HT receptor heterogeneity, a possibility which is under investigation at present. Whether or not this proves to be the case, the apparent lack of response (this study) or the weaker activity, relative to 5-HT (Leff *et al.*, 1987), of the 5-HT_1-like receptor agonist, 5-CT, raises questions about including the venous endothelial cell receptor in the 5-HT_1-like class. By contrast, the coronary artery endothelial cell 5-HT receptor is clearly of the 5-HT_1-like class, and shows similarities to the 5-HT_{1D} receptor (Schoeffter & Hoyer, 1989; 1990).

In conclusion, the neonatal porcine vena cava contains an endothelial cell 5-HT receptor, activation of which leads to EDRF release and subsequent vascular relaxation. The potent agonist, α-methyl-5-HT may provide a useful tool for further investigating the significance of this receptor, which does not appear to correspond to reported 5-HT receptors or to radioligand binding sites. Thus the lack of effect (as agonists or antagonists) of 5-CT, 8-OH-DPAT, spiperone, cyanopindolol, mesulergine, sumatriptan, ondansetron, ketanserin and ICS 205–930 would rule out a role for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, 5-HT_{1D}, smooth muscle contractile and relaxant 5-HT_1-like, 5-HT_2, 5-HT_3 and 5-HT_4 receptors.

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(Received August 27, 1990
Revised November 23, 1990
Accepted November 26, 1990)

Br. J. Pharmacol. (1991), **102**, 943–949

a_2-Adrenoceptor blocking profile of SK&F 104078: further evidence for receptor subtypes

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1 The ability of the putative, selective post-junctional α_2-adrenoceptor antagonist, SK&F 104078 to antagonize the effects of structurally-diverse agonists at pre-junctional α_2-adrenoceptors in the guinea-pig ileum and rat vas deferens *in vitro* and in the rat heart *in vivo*, and at post-junctional α_2-adrenoceptors in the rabbit ear vein *in vitro*, was examined. Results obtained with SK&F 104078 were compared with those obtained with yohimbine.

2 Xylazine and B-HT933 each caused a concentration-dependent inhibition of the field-stimulation-evoked twitch responses of the guinea-pig ileum and rat vas deferens. SK&F 104078 did not antagonize either agonist in the guinea-pig ileum and exerted only minimal blocking activity against xylazine in the rat vas deferens. In contrast, SK&F 104078 competitively antagonized B-HT933 in the rat vas deferens ($pA_2 = 6.45$). Yohimbine competitively antagonized both agonists in each tissue (pA_2 values ranged between 7.46 and 7.88).

3 In the pithed rat xylazine and B-HT933, injected intravenously, caused a dose-dependent reduction in the tachycardia elicited by stimulation of the cardiac preganglionic sympathetic nerves. SK&F 104078 ($10\,mg\,kg^{-1}$, i.v.) caused a 20–30 fold rightward displacement of the dose-response curve to xylazine, but did not affect responses to B-HT933. Yohimbine ($1\,mg\,kg^{-1}$, i.v.) antagonized both agonists to a similar degree.

4 In the rabbit ear vein xylazine, B-HT933, noradrenaline and UK14304 elicted vasoconstrictor responses. Prazosin was without effect, but in contrast, SK&F 104078 was a competitive antagonist of each of the agonists (pA_2 values ranged between 6.63 and 6.72). Yohimbine also competitively antagonized each of the agonists in this preparation (pA_2 values ranged between 7.81 and 8.07).

5 SK&F 104078 was also a competitive antagonist ($pA_2 = 6.20$) against noradrenaline at post-junctional α_1-adrenoceptors in the rabbit aorta.

6 These data show that SK&F 104078 is a competitive antagonist at post-junctional α_1- and α_2-adrenoceptors. Its antagonist potency at pre-junctional α_2-adrenoceptors is agonist- and tissue-dependent. Yohimbine does not discriminate between pre- and post-junctional α_2-adrenoceptors. The findings are discussed in terms of the possible existence of subclasses of α_2-adrenoceptors.

Keywords: α_2-adrenoceptors; rat vas deferens; guinea-pig ileum; rabbit ear vein; rabbit aorta; SK&F 104078; yohimbine

Introduction

The subclassification of α-adrenoceptors into two broad groups, α_1- and α_2-adrenoceptors, is widely acknowledged. Furthermore, there is growing evidence that both of these classes of receptors may be further divisible (for review, see Docherty, 1989). Various authors (e.g. Bylund, 1985; Drew, 1985) have drawn attention to data that suggest that α_2-adrenoceptors may not be a homogeneous group, but no drug has yet clearly established that this is the case. Recently, however, it was proposed that SK&F 104078 is an antagonist at post-, but not pre-junctional α_2-adrenoceptors (Hieble *et al.*, 1986; Ruffolo *et al.*, 1987; Daly *et al.*, 1988). Although this view was supported by Kelly *et al.* (1989), others have found contradictory evidence (Bertie *et al.*, 1988; Connaughton & Docherty, 1988; Connaughton *et al.*, 1989). In order to investigate the basis for these conflicting findings, we have re-evaluated the antagonist potency of SK&F 104078 at pre- and post-junctional α_2-adrenoceptors, against a number of α-adrenoceptor agonists. A preliminary account of this work has already been presented to the British Pharmacological Society (Akers *et al.*, 1989).

Methods

In vitro

Guinea-pig isolated ileum Guinea-pig ileum preparations were set up according to the method of Drew (1978). Guinea-pigs (Dunkin Hartley, Interfauna, males 300–800 g) were killed by cervical dislocation and the ileum removed. The 10 cm portion nearest the ileo-caecal junction was discarded. Segments, 2–3 cm in length, were selected from the terminal portion of the ileum and suspended under a resting tension of 1 g in modified Krebs-Henseleit solution, containing propranolol (1×10^{-6} M) to block β-adrenoceptors. Four preparations were simultaneously subjected to field stimulation at 0.1 Hz (square wave pulses, 1 ms in duration at supra-maximal voltage) and contractions of the longitudinal muscle were measured isometrically with Dynamometer UF1 2 oz strain gauges and displayed on a Lectromed MX8 chart recorder. When constant twitch responses had been obtained, cumulative concentration-effect curves to either of the selective α_2-adrenoceptor agonists, B-HT933 or xylazine, were constructed using 0.5 log unit increments. The response to each concentration was allowed to develop fully before adding the next concentration. The agonist concentration-effect curve was repeated after a 60 min wash-out period until constant (i.e. less than 2 fold shift in the agonist concentration-effect curve) and then again after incubation with a single concentration of SK&F 104078 (3×10^{-7}–3×10^{-6} M), yohimbine (1×10^{-7}–1×10^{-6} M) or vehicle. Agonist concentration-ratios were determined from the concentrations causing 50% inhibition of the twitch response (IC_{50}) in the absence and presence of the antagonist and corrected for the spontaneous shift in the vehicle-treated preparation. Where appropriate, results were plotted according to the method of Arunlakshana & Schild (1959) and pA_2 values and slopes of regressions were calculated.

In these, and in all other experiments *in vitro*, antagonist/vehicle contact time was 45 min.

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In pilot experiments, it was found that SK&F 104078 caused concentration-dependent inhibition of the twitch response of the longitudinal muscle. To assess the effects of different twitch heights on the potency of B-HT933, in a separate series of experiments, atropine was used at various concentrations to reduce the twitch height to levels similar to those produced by the different concentrations of SK&F 104078. B-HT933 concentration-effect curves were constructed at these reduced twitch heights and the IC_{50} values compared with control values.

In order to determine whether SK&F 104078 or yohimbine exerted any non-specific pre-junctional actions on the ileum, concentration-effect curves to [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (DAMGO), a μ-opioid receptor agonist, were repeated until constant and then after incubation with the highest concentrations of the antagonists previously used against the α-adrenoceptor agonists.

Rat isolated vas deferens Vasa deferentia of the rat were set up according to a modification of the method of Drew (1977) and Anton *et al.* (1977). Male rats (AH/A albino 200–500 g) were killed by stunning and cervical dislocation. The vasa were removed, desheathed and divided into two. The prostatic portion of each vas deferens was suspended in modified Krebs-Henseleit solution, under an initial resting tension of 1 g. Preparations were subjected to field stimulation at 0.3 Hz (square wave pulses, 2 ms duration at supramaximal voltage) and contractions were measured and displayed as described above.

From pilot experiments, it was found that only a single concentration-effect curve to either of the selective α_2-adrenoceptor agonists, B-HT933 or xylazine, could be obtained in any individual preparation; neither agonist could be removed despite repeated washing. The following protocol was therefore adopted: experiments were performed on paired prostatic portions of vasa deferentia from a single rat. One portion of the pair was treated with a single concentration of antagonist, while the other was treated with the vehicle. After drug administration, the stimulator was switched off. Thirty-five minutes later, the stimulator was switched on and, after a further 10 min, a concentration-effect curve to one of the agonists was constructed in both portions. Agonist concentration-ratios were determined from the concentrations causing 50% inhibition of the twitch response in the absence and presence of the antagonists. Where appropriate, the results were pooled and plotted as described above and the pA_2 and slope of the regression were calculated.

A similar protocol was used to test for any non-specific, pre-junctional actions of SK&F 104078 and yohimbine in this tissue, by using DAMGO in place of the α-adrenoceptor agonists.

Rabbit isolated ear vein rings Rabbit isolated ear vein rings were set up following a modification of the method of Daly *et al.* (1988). Rabbits (NZW albino, male, Froxfield, 2.2–3.5 kg) were killed by captive bolt. The central ear vein was cannulated with a 1.5 mm diameter perspex rod, dissected free of connective tissue and removed. The vein was removed from the perspex rod and sectioned into 4 rings of 3–4 mm in length. Each segment was suspended in modified Krebs-Henseleit solution, under an initial resting tension of 0.5 g, between two parallel stainless steel wires (200 μm diameter), one of which was anchored, and the other connected to a Dynamometer UFI 2 oz strain gauge. The Krebs-Henseleit solution contained cocaine (3×10^{-5} M), corticosterone (4×10^{-5} M), propranolol (1×10^{-6} M), indomethacin (3×10^{-6} M) and ascorbic acid (1×10^{-4} M) to block neuronal and extraneuronal uptake, β-adrenoceptors, spontaneous prostanoid production and drug oxidation respectively. Contractions were measured and displayed as above. After an equilibration period of 30 min, the tissues were exposed to one, or more, priming doses of the agonist under investigation. This was followed by repeated washing of the tissue every 15 min. Sixty minutes after the initial washing, cumulative concentration-effect curves were constructed to one of the agonists. Three further agonist concentration-effect curves were obtained sequentially, each after incubation with either vehicle or increasing concentrations of SK&F 104078, yohimbine or prazosin.

Agonist concentration-ratios were determined from the EC_{50} values (concentration causing 50% maximal contraction) in the absence and presence of increasing concentrations of the antagonist, and subjected to Schild analysis as previously described. Any non-specific, post-junctional actions of SK&F 104078 or yohimbine were evaluated by obtaining agonist concentration-effect curves to prostaglandin $F_{2\alpha}$, before and after treatment with the highest concentrations of the antagonists used.

Rabbit isolated aorta strips Rabbits (NZW albino, males, Froxfield, 2.2–3.5 kg) were killed by captive bolt and the aorta removed immediately. The tissues were cut spirally into four strips; 2.0–2.5 cm lengths of tissue were suspended in modified Krebs-Henseleit solution, similar to that used with the rabbit isolated ear vein preparation (except that no indomethacin was present), under an initial tension of 1 g. Concentration-effect curves to noradrenaline were repeated until constant, and then after incubation with a single concentration of SK&F 104078. Three separate concentrations of the antagonist were studied in any one experiment. Agonist equipotent concentration-ratios were obtained as in the ear vein and corrected for spontaneous shift in the vehicle control. pA_2 values and slopes of regression were calculated as above.

Composition of the physiological salt solution All the isolated tissues were immersed in modified Krebs-Henseleit solution of the following composition (mM): Na^+ 143.4, K^+ 5.9, Mg^{2+} 0.6, Ca^{2+} 1.3, Cl^- 124.5, HPO_4^- 1.2, SO_4^{2-} 0.6, HCO_3^- 25.0 and glucose 11.1.

The bathing fluid was maintained at 37 °C and bubbled continuously with 95% O_2 and 5% CO_2.

In vivo

Male rats (AH/A albino, 240–410 g) were anaesthetized with a mixture of either enfluorane or isofluorane (4% in O_2) and nitrous oxide, in a ratio of 3:1. They were then pithed and prepared for stimulation of the preganglionic cardiac sympathetic nerves, broadly as described by Drew (1976). Atropine (1 mg kg^{-1}) and (+)-tubocurarine (1 mg kg^{-1}) were administered intravenously. Body temperature was maintained at 37 °C with a heating blanket. Following preparation, the preganglionic cardiac sympathetic nerves were stimulated (0.5 Hz, 1 ms square wave pulses, supramaximal voltage) continuously; this increased heart rate by 80–100 beats min^{-1}. When the tachycardia was stable, doses of B-HT933 (1.8–540 μg kg^{-1}) or xylazine (1–300 μg kg^{-1}) were injected in a cumulative manner, sufficient time being allowed to elapse between successive doses for the ensuing decrease in heart rate to stabilize. After the highest dose of drug had been administered, the stimulator was switched off. One hour later it was turned on again, and the tachycardia re-established. When the heart rate had stabilized, SK&F 104078 (10 mg kg^{-1}) or yohimbine (1 mg kg^{-1}) was injected intravenously; 15 min later the dose-response curve to B-HT933 or xylazine was constructed again. Preliminary (unpublished) experiments confirmed that in the absence of antagonist treatment, 1 h was sufficient for the effects of the initial series of doses of either agonist on heart rate to have worn off, and that repeated administration of either agonist yielded a reproducible dose-response curve.

Drugs used

The following drugs were used: ascorbic acid (Sigma), atropine sulphate (BDH), B-HT933 (2-amino-6-ethyl-5,6,7,8-tetrahydro-4H-oxazolo-[4,5-d]-azepine dihydrochloride,

Boehringer), cocaine hydrochloride (May and Baker Ltd), corticosterone, (Sigma), DAMGO ([D-Ala^2,Me Phe^4, Gly-ol] enkephalin, Bachem UK) indomethacin (Sigma), (−)-noradrenaline bitartrate (Sigma), prazosin hydrochloride (Pfizer), propranolol hydrochloride (Sigma), SK&F 104078 (6-chloro-9-[(3-methyl-2-butenyl)oxy]-3-methyl-2,3,4,5-tetrahydro-1H-3-benzazepine, Glaxo and Smith Kline and French), (+)-tubocurarine (Burroughs Wellcome), UK14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate, Pfizer), xylazine (Bayer A.G.) and yohimbine hydrochloride (Sigma).

All drugs were dissolved in distilled water or saline, except corticosterone which was dissolved in ethanol, and indomethacin which was dissolved in 10% bicarbonate (prior to adding to the Krebs-Henseleit solution). Solutions of noradrenaline contained ascorbic acid (1×10^{-4} M) to inhibit oxidation. All stock solutions were kept on ice throughout the experiments.

Statistics

Data are expressed as arithmetic mean (with 95% confidence intervals). The pA_2 value (with 95% confidence intervals) obtained in the rat isolated vas deferens was a single estimate, calculated from a Schild plot using pooled data from all the equipotent concentration-ratios obtained with a particular agonist. Tests for significance were carried out with either Student's unpaired or paired t test where appropriate. Data were considered significant when $P < 0.05$.

Results

In vitro

Guinea-pig isolated ileum Field stimulation of the guinea-pig ileum produced individual twitch responses which were inhibited by B-HT933 (1×10^{-7}–3×10^{-5} M) and xylazine (1×10^{-8}–1×10^{-5} M) in a reproducible and concentration-dependent manner. SK&F 104078, 3×10^{-7}, 1×10^{-6} and 3×10^{-6} M reduced the twitch responses by 15.5 ± 7.5, 41.3 ± 5.8 and 54.3 ± 18.6% respectively ($n = 4$). At these new basal twitch heights, concentration-effect curves to B-HT933 and xylazine, after correction for spontaneous shifts in control preparations, were displaced less than 2 fold to the right by SK&F 104078 (Figure 1). Higher concentrations of SK&F 104078 abolished the twitch response. To determine whether the change in twitch height had affected the potency of the α_2-adrenoceptor agonists, B-HT933 concentration-effect curves were obtained in the absence and presence of atropine. The concentrations of atropine required to reduce the twitch responses to levels similar to that produced by SK&F 104078 ranged between 3×10^{-10} and 3×10^{-9} M. At all concentrations of atropine, a <3 fold leftward displacement of B-HT933 concentration-response curves was observed.

In contrast to SK&F 104078, yohimbine (1×10^{-7}–1×10^{-6} M) did not depress the twitch response, but caused concentration-dependent, rightward displacements in the B-HT933 and xylazine concentration-effect curves. pA_2 values and slopes of regression are shown in Table 1. The μ-receptor agonist, DAMGO (1×10^{-10}–1×10^{-6} M) caused a concentration-dependent inhitition of the twitch response. Yohimbine (1×10^{-6} M) or SK&F 104078 (3×10^{-6} M) did not significantly displace DAMGO concentration-response curves.

Rat isolated vas deferens Field stimulation of the rat isolated vas deferens at 0.3 Hz produced individual twitch responses. These twitch responses were inhibited in a concentration-dependent manner by B-HT933 (1×10^{-8}–1×10^{-6} M) and by xylazine (1×10^{-9}–1×10^{-6} M). SK&F 104078 (1×10^{-6}–1×10^{-5} M) caused concentration-dependent, rightward displacements of the concentration-effect curves to B-HT933

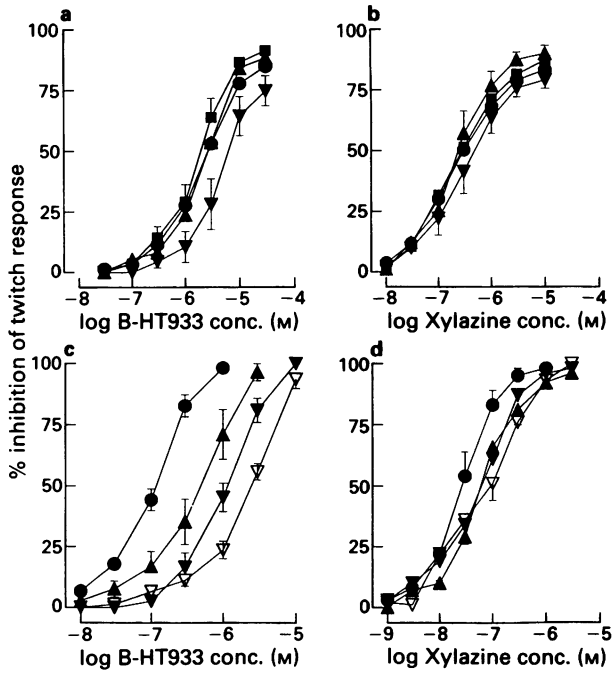

Figure 1 The twitch-inhibitory effects of B-HT933 and xylazine in guinea-pig ileum (a and b respectively) and rat vas deferens (c and d respectively) in vehicle-treated preparations (●) and in preparations treated with SK&F 104078, 3×10^{-7} M (■), 1×10^{-6} M (▲), 3×10^{-6} M (▼) or 1×10^{-5} M (▽). Results are expressed as % inhibition of the twitch response (mean with s.e.mean shown by vertical bars).

(Figure 1). A pA_2 value (95% CI) of 6.45 (6.22–6.84) and a slope of regression (95% CI) of 0.96 (0.69–1.24) was obtained. SK&F 104078 (1×10^{-6} M) displaced the xylazine concentration-response curve 3.2 fold to the right. At higher concentrations of SK&F 104078 (3×10^{-6} and 1×10^{-5} M), the mean displacements were only 3.2 and 3.5 fold, respectively (Figure 1). Yohimbine (1×10^{-7}–1×10^{-6} M) caused concentration-dependent, rightward displacements in both B-HT933 and xylazine concentration-effect curves; pA_2 values are shown in Table 1. The μ-receptor agonist, DAMGO (1×10^{-9}–3×10^{-6} M) also caused a concentration-dependent inhibition of the twitch responses. Neither SK&F 104078 (1×10^{-5} M) nor yohimbine (1×10^{-6} M) displaced the DAMGO concentration-effect curve (data not shown). At the concentrations used in these experiments, neither the antagonists nor their respective vehicles, had any consistent effects on the twitch response of the rat vas deferens preparation.

Rabbit isolated ear vein rings B-HT933 (1×10^{-8}–1×10^{-5} M), xylazine (1×10^{-8}–1×10^{-5} M), UK14304 (1×10^{-9}–1×10^{-6} M) and noradrenaline (1×10^{-9}–1×10^{-5} M) caused concentration-related constrictions of the rabbit isolated ear vein. Administration of acetylcholine (1×10^{-6} M), either caused a small contraction, or had no effect on the sustained contraction elicited by noradrenaline, suggesting that there was no functional endothelium present in these tissues. There was no significant difference in the maximum response, or the EC_{50} values for any given agonist, from repeated concentration-effect curves in vehicle-treated preparations. No attempt was made, therefore, to correct the agonist concentration-ratios in the presence of the antagonists for spontaneous changes in the control preparations.

Prazosin (1×10^{-7}–1×10^{-6} M) caused less than a 2 fold rightward displacement in the concentration-effect curves to any of the agonists.

SK&F 104078 (3×10^{-7}–3×10^{-6} M; Figure 2) and yohimbine (1×10^{-7}–1×10^{-6} M) caused concentration-dependent,

Table 1 Antagonist potencies of SK&F 104078 and yohimbine at pre- and post-junctional α_2-adrenoceptors and at post-junctional α_1-adrenoceptors *in vitro*

		SK&F 104078		*Yohimbine*	
Tissue	*Agonist*	pA_2	*Slope*	pA_2	*Slope*
Guinea-pig ileum	B-HT933	NC	—	7.84 (7.13–8.64)	0.85 (0.54–1.33)
	Xylazine	NC	—	7.88 (7.72–8.05)	0.68 (0.53–0.87)
Rat vas deferens	B-HT933	6.45 (6.22–6.84)	0.96 (0.69–1.24)	7.46 (7.23–7.84)	1.19 (0.86–1.52)
	Xylazine	NC	—	7.54 (7.19–8.42)	1.05 (0.58–1.52)
Rabbit ear vein	B-HT933	6.67 (6.56–6.77)	1.04 (0.94–1.15)	7.96 (7.76–8.16)	0.98 (0.82–1.16)
	Xylazine	6.70 (6.51–6.89)	1.09 (0.99–1.19)	8.00 (7.59–8.43)	1.04 (0.84–1.29)
	UK14304	6.63 (6.30–6.98)	1.22 (1.04–1.43)	7.81 (7.69–7.93)	1.22 (1.06–1.41)
	Noradrenaline	6.72 (6.26–7.22)	1.03 (0.68–1.56)	8.07 (7.91–8.24)	0.89 (0.70–1.13)
Rabbit aorta	Noradrenaline	6.20 (5.88–6.54)	0.91 (0.73–1.12)	NT	

Results are expressed as arithmetic mean (and 95% confidence intervals) of 4 or more experiments.
NC: Not calculable.
NT: Not tested.

parallel, rightward displacements in the concentration-effect curves to each agonist studied, but had no effect on the maximum responses obtained. The pA_2 values and the slopes of the Schild plots for these antagonists are shown in Table 1.

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1×10^{-9}–1×10^{-5} M) caused reproducible concentration-effect curves. Neither SK&F 104078 (3×10^{-6} M) nor yohimbine (1×10^{-6} M) had any effect on the $PGF_{2\alpha}$-induced responses.

Rabbit isolated aortic strips Noradrenaline (1×10^{-8}–3×10^{-6} M) caused reproducible concentration-dependent contractions in the rabbit isolated aortic strip preparation. SK&F 104078 (3×10^{-6}–3×10^{-5} M) produced concentration-dependent rightward displacements of the noradrenaline concentration-effect curves (Figure 2d). The pA_2 value and slope of regression are shown in Table 1.

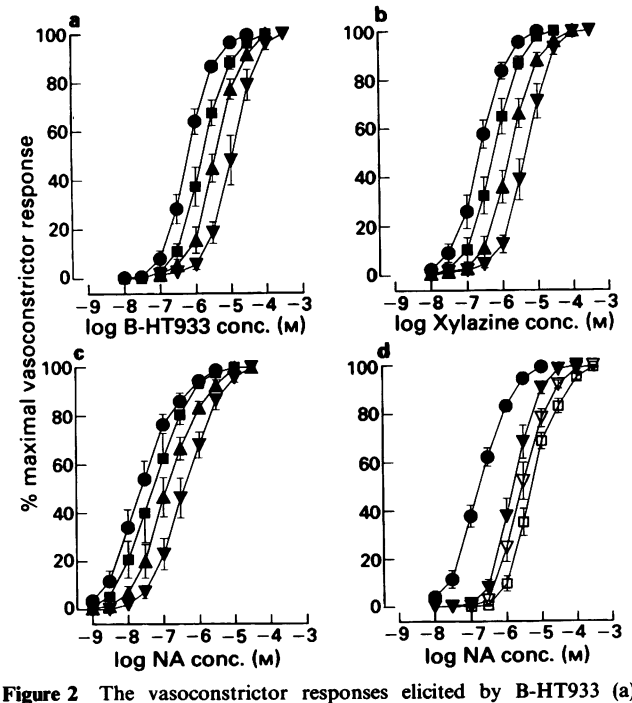

Figure 2 The vasoconstrictor responses elicited by B-HT933 (a), xylazine (b) and noradrenaline (NA) (c) in rabbit ear vein, and by noradrenaline in rabbit aorta (d). Preparations were treated with vehicle (●) or SK&F 104078, 3×10^{-7} M (■), 1×10^{-6} M (▲), 3×10^{-6} M (▼), 1×10^{-5} M (▽) or 3×10^{-5} M (□). Results are expressed as mean % of the maximum response obtained; s.e.mean shown by vertical bars.

In vivo

B-HT933 (1.8–540 μg kg^{-1} i.v.) and xylazine (1–300 μg kg^{-1} i.v.) produced dose-dependent reductions in the tachycardia elicted by nerve stimulation in pithed rats, which were well sustained at each dose level. Blood pressure was also increased, but this effect was less well sustained (see also Drew, 1976). For this reason, the effects of the agonists on blood pressure were not quantified.

SK&F 104078 (10 mg kg^{-1}, i.v. given over 5 min) and yohimbine (1 mg kg^{-1}, i.v., given similarly) each reduced the tachycardia transiently. However, the tachycardia completely recovered within 15 min and was not significantly different from pre-antagonist levels, when the second agonist dose-response curve was initiated.

SK&F 104078 displaced the dose-response curve to xylazine 19.9 (7.5–53.4) fold to the right (geometric mean and 95% confidence limits), but had no effect on that to B-HT933 (dose-ratio = 1.5 (0.48–4.8)). Results are shown in Figure 3. In contrast, yohimbine caused about a 20–30 fold rightward displacement in the dose-response curves to each agonist.

Discussion

The aim of this study was to re-examine the claims that SK&F 104078 selectively blocks post- rather than pre-junctional α_2-adrenoceptors (Hieble *et al.*, 1986; Ruffolo *et al.*, 1987). If true, the concept of heterogeneity of α_2-adrenoceptors would be substantiated. To this end, we have used two well-characterized, preferential α_2-adrenoceptor agonists, B-HT933 and xylazine, in a series of preparations in which their effects are mediated primarily, if not exclusively, via α_2-adrenoceptors. In addition, the antagonist potency of SK&F 104078 against the sympathetic neurotransmitter, noradrenaline was established at post-junctional α_2-adrenoceptors and compared with that determined at α_1-adrenoceptors. The results of our experiments suggest that

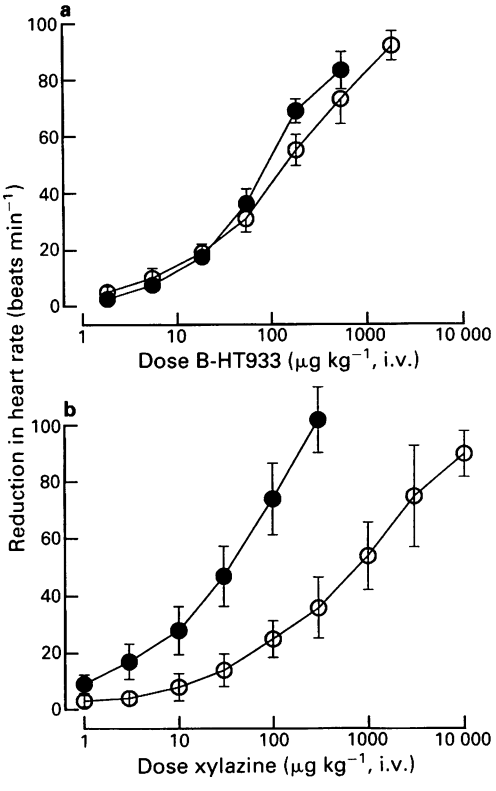

Figure 3 The inhibition by B-HT933 (a) or xylazine (b) of the stimulation-evoked tachycardia in pithed rats pretreated with vehicle (●) or SK&F 104078 (10 mg kg^{-1}, i.v.; ○). Results are expressed as mean decreases in heart rate with s.e.mean shown by vertical bars (n = 4–5).

the claims made for the selectivity of action of SK&F 104078, although true in part, do not fully describe its profile of action. Although the data described in this paper were obtained with a sample of SK&F 104078 synthesized in the Chemistry Research Division, Glaxo Group Research, chemical analysis and pharmacological comparison failed to show any detectable differences from an authentic sample of the compound, generously supplied by Smith, Kline & French.

Consistent with the observation reported by Ruffolo *et al.* (1987), that SK&F 104078 did not antagonize the α-methylnoradrenaline-induced inhibition of the twitch response in the guinea-pig ileum, we found that SK&F 104078 in concentrations up to 3 × 10^{-6} M, did not antagonize the twitch-inhibitory effects of B-HT933 or xylazine. Analysis of the results is complicated by virtue of the fact that at all concentrations used, SK&F 104078 itself reduced the twitch response, which could have led to a substantial, underlying, leftwards shift in the agonist dose-response curves, thus opposing the influence of the antagonist. This seems unlikely, however, since SK&F 104078 at the highest concentration used (3 × 10^{-6} M), did not influence the responsiveness of the ileum to the twitch-inhibitory effects of DAMGO. This view is further supported by the finding that atropine, which reduced twitch responses to a similar extent to SK&F 104078, had minimal effects on the sensitivity of the tissue to B-HT933. Thus, despite the complication of the direct effects of SK&F 104078 on the twitch response it seems that SK&F 104078 has little or no affinity for the pre-junctional α_2-adrenoceptors in the guinea-pig ileum. The possibility that the twitch-inhibitory effect of SK&F 104078 might reflect agonist activity at pre-junctional α_2-adrenoceptors can be discounted because it was unaffected by pretreatment with yohimbine (1 × 10^{-6} M: n = 4); its mechanism remains unknown (results not shown).

The same conclusion might be drawn concerning the affinity of SK&F 104078 for the pre-junctional α_2-adrenoceptors in the rat vas deferens, since the compound produced only small, concentration-independent, displacements of the xylazine concentration-effect curves in this tissue. However, contrary to this view is the finding that SK&F 104078 appeared to exert specific, competitive antagonism towards B-HT933 in the same preparation. Furthermore, the pA_2 of 6.45 obtained is comparable to affinity estimates made against *p*-aminoclonidine (pA_2 = 6.47; Bertie *et al.*, 1988) and UK-14,304 ($pK_B \simeq 5.85$; Hieble *et al.*, 1988), although antagonism towards the latter agonist did not appear to be truly competitive in nature. Our potency estimate for SK&F 104078 in this tissue is also similar to that initially reported against xylazine (pA_2 = 6.39; Connaughton *et al.*, 1989). Thus, there is no doubt that SK&F 104078 does show affinity for the pre-junctional α_2-adrenoceptors in the vas deferens. We cannot explain why SK&F 104078 exerted such weak antagonism against xylazine in our hands, compared with the findings of Connaughton *et al.* (1989). Although the original potency estimate of SK&F 104078 against xylazine was made with a single concentration of the antagonist, later work has confirmed its competitive nature (pA_2 = 6.45; slope = 0.98; Connaughton & Docherty, 1990a). In view of these findings, we re-investigated the effects of the highest concentration of SK&F 104078 (1 × 10^{-5} M) against xylazine. In these experiments, SK&F 104078 produced a mean 4.2 fold shift to the right in the xylazine concentration-effect curve (range 1.7–9.4). These findings were similar to those previously obtained (mean 3.5 fold shift; range 2.5–6.2). The reason for the difference between our results and those of Connaughton & Docherty (1990a) remain obscure, although we cannot discount the possibility that small differences in experimental protocol (single pulses compared to continuous stimulation at 0.3 Hz) might be responsible. Clearly, SK&F 104078 can antagonize xylazine just as effectively as B-HT933 at α_2-adrenoceptors, because it was equipotent against these agonists at post-junctional receptors in the rabbit ear vein. (Our studies with prazosin confirm that the vasoconstrictor effects of these agonists in this tissue are not mediated via post-junctional α_1-adrenoceptors; see also Daly *et al.*, 1988). Furthermore, SK&F 104078 was equipotent in antagonizing the selective α_2-adrenoceptor agonist, UK-14304 and the non-selective agonist, noradrenaline in this tissue. The antagonism of these agonists was specific and competitive. The range of pA_2 values (6.63–6.72) obtained for SK&F 104078 against all the agonists at the post-junctional α_2-adrenoceptors in the rabbit ear vein is very similar to that obtained against B-HT933 at pre-junctional receptors in the rat vas deferens (6.45). The values we obtained are also similar to those obtained against B-HT920 at post-junctional α_2-adrenoceptors in the dog saphenous vein in the presence (pA_2 = 6.8) and absence (pA_2 = 7.12) of nifedipine (derived from Hieble *et al.*, 1988). Using the same tissue, we have derived a pA_2 (slope) of 6.91 (0.83) against B-HT920, in the absence of nifedipine (unpublished observations).

In complete contrast to its effects in the rat vas deferens, SK&F 104078 antagonized xylazine, but not B-HT933, at pre-junctional α_2-adrenoceptors in the rat heart *in vivo*. The degree of the antagonism exerted by SK&F 104078 10 mg kg^{-1} against xylazine in our experiments was similar to that reported for a dose of 5 mg kg^{-1}, by Connaughton & Docherty (1988). The failure of SK&F 104078 to antagonize B-HT933 at pre-junctional α_2-adrenoceptors in the rat heart *in vitro* is in keeping with its lack of activity against B-HT920 in rat atria *in vitro* (Ruffolo *et al.*, 1987).

Finally, to complete the profile of action of SK&F 104078, we determined its antagonist potency against noradrenaline at α_1-adrenoceptors in the rabbit aorta (Docherty & Starke, 1981). The pA_2 of 6.21 in this preparation is slightly lower than that derived from the data reported by Hieble *et al.* (1986) against noradrenaline in the rabbit aorta (pA_2 = 6.81), or against phenylephrine in rat aorta (pA_2 = 6.76; Bertie *et al.*, 1988). Nevertheless, it confirms that SK&F 104078 does exert antagonism at α_1-adrenoceptors over a similar range of

concentrations to those that are effective at α_2-adrenoceptors (at least in some tissues and against some agonists).

In summary, therefore, it is clear that SK&F 104078 is an antagonist at post-junctional α_2-adrenoceptors, and is equally effective against all the agonists we have examined. However, its antagonist activity is not restricted to post-junctional α_2-adrenoceptors, but its ability to block pre-junctional α_2-adrenoceptors seems to be both tissue- and agonist-dependent. In contrast, and in broad agreement with other reports in the literature, yohimbine shows no such diversity of action.

The most obvious explanation for these findings is that α_2-adrenoceptors do not constitute an homogeneous group, and that the different subtypes are not conveniently located pre- or post-junctionally. If taken at face value, at least three subtypes of α_2-adrenoceptor are needed to explain our results. In order to distinguish between these subtypes (simply for convenience and for discussion within this paper, and without the intention of proposing the more widespread adoption of this nomenclature) we will arbitrarily designate them as α_{2a}, α_{2b}, and α_{2c} receptors. Accordingly, the α_2-adrenoceptor in the vas deferens at which SK&F 104078 antagonizes the B-HT933-induced inhibition of the twitch response may be designated α_{2a}. The receptor in the rat heart at which SK&F 104078 antagonized xylazine-induced inhibition of the stimulation-evoked tachycardia could then be termed the α_{2b} adrenoceptor, whereas the receptor at which SK&F 104078 fails to antagonize xylazine or B-HT933-induced twitch inhibition in the guinea-pig ileum would be designated the α_{2c}-adrenoceptor. Using this nomenclature, we have to propose that the twitch-inhibitory effect of xylazine in the rat vas deferens is mediated primarily through activation of α_{2c} receptors, rather than α_{2a} receptors through which B-HT933 acts. However, the small and variable degree of blockade exerted by SK&F 104078 against xylazine in this tissue may reflect some degree of interaction at α_{2a} receptors. By the same token, the sympatho-inhibitory effect of B-HT933 in the rat heart *in vivo*, would be mediated via α_{2c}-adrenoceptors, which are not susceptible to blockade by SK&F 104078. Presumably, both α_{2a}- and α_{2b}-adrenoceptors are present in the rabbit ear vein, and SK&F 104078 does not distinguish between them. At first sight, this analysis may seem unnecessarily complicated, but there is already evidence to support the view that (at least) 3-subtypes of α_2-adrenoceptors exist. Regan *et al.* (1988) described the characteristics of two subtypes, the 'α_2-C4' and 'α_2-C10' adrenoceptors. B-HT933 showed equal affinity for these receptor subtypes, as did yohimbine and SK&F 104078. Only prazosin showed any marked difference, having a 40 fold higher affinity for the α_2-C4 subtype. There is also evidence for a third α_2-adrenoceptor subtype ('α_2-C2'), but little is known of its pharmacological characteristics. Bylund (1988) has also described the presence of 3 subtypes of α_2-adrenoceptors, which he designated α_2A, α_2B and α_2C-adrenoceptors. According to his nomenclature, the α_2A-subtype is analogous to the α_2-C10 subtype of Regan *et al.* (1988); the α_2B subtype may also equate to the α_2-C4 subtype although this is not certain. Furthermore, the α_2C-receptor described by Bylund, and found in OK cells (an opossum kidney-derived cell line) may be the same as the α_2-C2 subtype described by Regan *et al.* (1988). Finally, Flordellis *et al.* (1989) have reported that there are at least 3 mRNA isoforms in the rat, which do not correspond to previously described α-adrenoceptor mRNA species.

In view of the paucity of information on the pharmacological characteristics of the subtypes of α_2-adrenoceptors, it is difficult to explain our findings in terms of actions at α_2A (α_2-C10), α_2B (α_2-C4?) and α_2C (α_2-C2?) receptors. However, it is clear that the receptor(s) which B-HT933 and xylazine (and α-methylnoradrenaline; Ruffolo *et al.*, 1987) act upon in the guinea-pig ileum cannot be of the α_2A or α_2B type, for which SK&F 104078 had demonstrable affinity. Therefore the prejunctional α_2-adrenoceptors in the guinea-pig ileum may be of the α_2C subtype. Furthermore, Connaughton & Doherty (1990b) have concluded on the basis of an extensive correlation coefficient analysis of the effects of a wide range of antagonists, that the pre-junctional α_2-adrenoceptors in the rat vas deferens and rat atrium may be of the α_2A and α_2B subtypes, respectively.

It is conceivable that the preparations that we have chosen in which to evaluate the α_2-adrenoceptor blocking activity of SK&F 104078 contain mixtures of the different α_2-adrenoceptor isoforms described by Bylund (1988) or Flordellis *et al.* (1989). If so, it might be anticipated that the relative potencies of the agonists, B-HT933 and xylazine would differ from preparation to preparation. In this context, the finding that xylazine is approximately 24 times more potent than B-HT933 in the guinea-pig ileum, but only about 11 and 2 times more potent in the rat vas deferens and rat heart respectively, may support this proposal. However, before any subdivision of α_2-adrenoceptors, based on data of the type described in this report can be accepted with confidence, further evaluation is necessary including, perhaps, analysis of data obtained from stably transfected cell lines expressing a single subtype of each of the α_2-adrenoceptors.

The authors wish to thank Dr D. Butina, Medicinal Chemistry, Glaxo Group Research, for synthesis of SK&F 104078, and Mr A. Green for excellent technical assistance.

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(Received August 30, 1990
Revised November 29, 1990
Accepted December 3, 1990)

Br. J. Pharmacol. (1991), **102**, 950–954

Interactions between the vascular peptide endothelin-1 and sensory neuropeptides in gastric mucosal injury

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1 The interactions between endogenous and exogenous sensory neuropeptides on gastric mucosal injury induced by endothelin-1 (ET-1) have been investigated in the anaesthetized rat.

2 Close intra-arterial infusion of ET-1 (4–20 pmol kg^{-1} min^{-1}) dose-dependently induced vasoconges-tion and haemorrhagic necrosis in the gastric mucosa.

3 Capsaicin-pretreatment, two weeks earlier to deplete sensory neuropeptides from primary afferent neu-rones, augmented the mucosal damage induced by ET-1, as assessed by both macroscopic and histological examination.

4 The damage induced by threshold doses of ET-1 alone or in capsaicin-pretreated rats was further enhanced by administration of indomethacin (5 mg kg^{-1}, i.v.), indicating a modulatory influence of endogenous prostanoids.

5 Morphine administration (3 mg kg^{-1}, i.v.), which can prevent neuropeptide release, augmented the damage induced by threshold doses of ET-1, this effect being reversed by naloxone (1 mg kg^{-1}, i.v.).

6 Concurrent local intra-arterial infusion of rat α-calcitonin gene-related peptide (10–50 pmol kg^{-1} min^{-1}) dose-dependently reduced the mucosal injury induced by ET-1.

7 These findings suggest interactions between ET-1 and sensory neuropeptides, which may reflect an important influence of these peptide mediators in the regulation of mucosal integrity.

Keywords: Vasoactive mediators; gastric mucosal damage; vascular endothelium; endothelin-1; calcitonin gene-related peptide; primary sensory neurones; prostanoids

Introduction

An interplay between local mediators, such as those that can exert vasoactive actions, may be involved in the maintenance of tissue integrity. In the stomach, studies with cyclo-oxygenase inhibitors have suggested that the local biosynthesis and release of the prostanoids, prostacyclin and prostaglandin E_2 are important for the mucosa to withstand noxious challenge (Whittle & Vane, 1987). The local release of sensory neuropeptides such as calcitonin gene-related peptide (CGRP) from primary afferent neurones has also been proposed as an endogenous protective mechanism within the gastric mucosa (Szolcsanyi & Bartho, 1981; Holzer & Sametz, 1986).

This concept of the role of endogenous neuropeptides has arisen from the findings that acute administration of capsaicin, which stimulates afferent sensory neurones, can protect against damage (Holzer *et al.*, 1990), while chronic pretreatment with capsaicin which can deplete such neurones of their neuropeptides, substantially augments mucosal injury induced by a number of ulcerogens (Szolcsanyi & Bartho, 1981; Holzer & Sametz, 1986; Esplugues *et al.*, 1989; Esplugues & Whittle, 1990). Furthermore, morphine administration, which can prevent neuropeptide release from sensory neurones (Lembeck & Donnerer, 1985) can likewise augment gastric mucosal damage induced by Paf (Esplugues *et al.*, 1989; Pique *et al.*, 1990) or topical application of ethanol (Esplugues & Whittle, 1990).

The biosynthesis of a novel 21-residue peptide by vascular endothelial cells, now known as endothelin-1 (ET-1) has been described, which exhibits vasoconstrictor actions both *in vitro* and *in vivo* (Yanagisawa *et al.*, 1988; Inoue *et al.*, 1989). Local intra-arterial infusion of picomole quantities of ET-1 induces substantial gastric mucosal injury in the rat (Whittle & Esplugues, 1988; Whittle *et al.*, 1989), while intravenous infusion of ET-1 can augment damage induced by intragastric instillation of ethanol or acid (Wallace *et al.*, 1989; MacNaughton *et al.*, 1989). In the current study, the influence of endogenous sensory neuropeptides on the gastric damage induced by locally administered ET-1 has been investigated in the anaesthetized rat, by use of capsaicin-pretreatment or morphine administration. Furthermore, the interaction of endogenous sensory neuropeptides with gastric prostanoids in the protection of the mucosa against ET-1-induced injury has been explored by the use of indomethacin. In addition, the actions of local infusion of the sensory neuropeptide, rat α-CGRP on mucosal damage induced by ET-1 have also been evaluated.

A preliminary account of this work has been presented to the British Pharmacological Society (Lopez-Belmonte & Whittle, 1990).

Methods

Gastric damage induced by the local intra-arterial infusion of endothelin-1

Male Wistar rats (230–260 g), deprived of food but not water for 18–20 h before the experiments, were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and the stomach exposed by a mid-line incision. The left gastric artery was cannulated with a short 23 g Teflon cannula (Esplugues *et al.*, 1989), the oesophagus and pylorus ligated and 2 ml of 0.1 M HCl in saline was instilled into the gastric lumen via a needle inserted through the forestomach. ET-1 (4–20 pmol kg^{-1} min^{-1}) or isotonic saline was infused locally for 10 min (10 μl min^{-1}), and the stomachs removed 20 min later for macroscopic inspection of the gastric mucosa.

Gastric damage following capsaicin-pretreatment or indomethacin

Adult rats (190–210 g) were treated with increasing doses of capsaicin for three consecutive days (20, 30 and 50 mg kg^{-1}, s.c.) to deplete neuropeptides in primary afferent neurones as described previously (Esplugues *et al.*, 1989). All capsaicin

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injections were made under halothane anaesthesia and, to counteract any respiratory impairment associated with the administration of capsaicin, the rats were pretreated with terbutaline (0.1 mg kg^{-1}, i.m.) and aminophylline (10 mg kg^{-1}, i.m.) prior to capsaicin injection. The animals were used 10–18 days after completion of the capsaicin treatment. Control animals received a similar regimen of treatment with the vehicle alone. In previous studies on gastric damage, this protocol for pretreatment of adult rats with capsaicin has been demonstrated to give comparable findings to that utilizing capsaicin treatment of neonatal rats (Pique *et al.*, 1990).

In further studies, indomethacin (5 mg kg^{-1}, i.v.) was administered 15 min prior to study in control or capsaicin-pretreated rats, in a dose previously shown to inhibit gastric mucosal prostanoid formation by over 90% (Whittle & Vane, 1987). ET-1 was then infused locally for 10 min, and the degree of mucosal injury subsequently assessed.

Effect of morphine administration

Rats were pretreated with morphine (3 mg kg^{-1}, i.v.), in a dose derived from previous studies on gastric mucosal damage (Esplugues *et al.*, 1989), 10 min prior to the local intra-arterial infusion of ET-1. In further experiments, the opioid antagonist naloxone (1 mg kg^{-1}, i.v.) was administered 10 min before the administration of morphine.

Effects of calcitonin gene-related peptide

In these studies, an intra-arterial 23 g teflon cannula attached to a bifurcated catheter was used, which allowed the concurrent close-arterial infusion of two substances, each at a rate of 10 μl min^{-1}. In control studies, local infusion of isotonic saline (20 μl min^{-1}) for 10 min did not induce any macroscopic or histologically-detected mucosal damage. Local infusion of rat α-CGRP (10–50 pmol kg^{-1} min^{-1}) or isotonic saline was started 2 min before the local infusion of ET-1 (20 pmol kg^{-1} min^{-1}) and maintained for the duration of the 10 min challenge period.

Mean systemic arterial blood pressure (BP) was measured from a cannula in a carotid artery connected to a pressure transducer (Elcomatic) and a chart recorder (Grass, model R-50), during close-arterial or intravenous infusion of CGRP.

Assessment of mucosal damage

Twenty minutes after terminating the local intra-arterial infusion of ET-1, the stomachs were removed and opened along the greater curvature. The stomachs were pinned out, mucosal side up, to a wax block and immersed in neutral buffered formalin and then photographed on colour transparency film. The extent of macroscopically-visible damage, involving regions of vasocongestion and haemorrhagic necrosis with associated surface cell exfoliation, was determined in a randomized manner from these projected transparencies via computerised planimetry. The area of mucosal damage was calculated as the % of the total gastric mucosa showing macroscopically visible damage.

For histological confirmation of the nature of the mucosal injury induced by ET-1 alone and in capsaicin-pretreated rats, samples of the fundus were excised from standardized regions and were processed by routine techniques before embedding in paraffin. Sections (4 μm) were stained with haematoxylin and eosin and examined under a light microscope. Each section was histologically assessed in a randomized manner, for glandular disruption and vasocongestion, and for deeper haemorrhagic necrosis. The length of each section exhibiting each type of damage was expressed as % of the total length of that section.

Materials

Rat α-calcitonin gene-related peptide (Cambridge Biochemicals, Cambridge) and endothelin-1 (human-porcine; Peninsula Labs; St. Helens, Merseyside) were dissolved in sterile distilled water and kept frozen (−20°C) in aliquots. Samples were freshly diluted in sterile isotonic saline when required.

Indomethacin (Sigma Chemical Co., Poole, Dorset) was dissolved in 5% sodium bicarbonate solution and diluted with distilled water prior to injection. Morphine hydrochloride (MacFarlane Smith, Edinburgh), naloxone hydrochloride (Endo Labs; New York, U.S.A.) and aminophylline (Sigma Chemical Co.), were dissolved in isotonic saline immediately before use. Capsaicin (Fluka Chemic AG, Buchs, Switzerland) was prepared in a 50 mg ml^{-1} solution containing absolute ethanol, tween 80 and isotonic saline (10:10:80 v/v/v). Terbutaline sulphate was obtained in injectable form from Astra Pharmaceuticals, Kings Langley.

Statistical analysis

All data are expressed as mean ± s.e.mean. Comparisons between groups were made by Student's t test for unpaired data where P values of less than 0.05 were taken as significant.

Results

Effects of capsaicin-pretreatment

Local intra-arterial infusion of submaximal doses of ET-1 (4–20 pmol kg^{-1} min^{-1} for 10 min) induced significant dose-related mucosal injury when assessed 20 min later (Figure 1). This damage was characterized macroscopically as regions of vasocongestion and localized areas of haemorrhage. In capsaicin-pretreated rats, the area of mucosal damage induced by each of these doses of ET-1 was substantially ($P < 0.05$) augmented, involving 79 ± 4% ($n = 4$) of the total mucosal area with the highest dose of ET-1 investigated (Figure 1). The increase in damage was apparent macroscopically as an increase in both the area and severity of the vasocongestion and the regions of haemorrhagic necrosis, while sloughing of the surface layers in these areas was widespread. In control experiments with vehicle- or capsaicin-pretreated rats, close-arterial infusion of isotonic saline (10 μl min^{-1}) did not induce any detectable damage (Figure 1).

On histological inspection of sections of the gastric mucosa, ET-1 (4–20 pmol kg^{-1} min^{-1}) induced dose-related vasocongestion and glandular disruption, as well as areas of deeper haemorrhage and necrosis. In control studies, neither intragastric instillation of acid-saline, nor capsaicin pretreatment induced any detectable vasocongestion or haemorrhage following close-arterial infusion of saline ($n = 3$ for each). However, capsaicin pretreatment significantly ($P < 0.05$) aug-

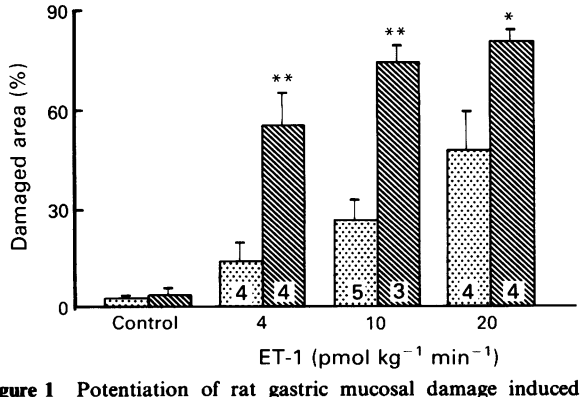

Figure 1 Potentiation of rat gastric mucosal damage induced by close-arterial infusion of endothelin-1 (ET-1; 4–20 pmol kg^{-1} min^{-1} for 10 min) by capsaicin-pretreatment, 2 weeks earlier. Results, shown as % of total mucosal area exhibiting macroscopic damage, for vehicle- (stippled columns) or capsaicin-treated (hatched columns) groups are the mean of (n) experiments, vertical bars show s.e.mean. Significant difference from ET-1 in vehicle-pretreated rats is given as * $P < 0.05$; ** $P < 0.01$.

mented the degree of vasocongestion and glandular disruption seen after challenge; thus following local infusion of ET-1 (10 pmol kg^{-1} min^{-1}), this damage was increased from 12 ± 6% (n = 3) to 67 ± 7% (n = 3; $P < 0.05$) of the section length. Likewise, deeper haemorrhagic necrosis was significantly ($P < 0.05$) increased by capsaicin-pretreatment from 1 ± 1% (n = 3) to 16 ± 4% (n = 3) of the total section length following ET-1 (10 pmol kg^{-1} min^{-1}) and from 6 ± 2% (n = 4) to 33 ± 7% (n = 4) of the total section length following ET-1 (20 pmol kg^{-1} min^{-1}).

Effect of indomethacin administration

Administration of indomethacin (5 mg kg^{-1}, i.v.) did not itself induce significant damage over this short experimental period in control rats (Figure 2). However, indomethacin pretreatment, 15 min prior to challenge, significantly ($P < 0.05$) potentiated the damage induced by the threshold dose of ET-1 (4 pmol kg^{-1} min^{-1}) as shown in Figure 2. The mucosal damage was again macroscopically apparent as areas of vasocongestion and haemorrhagic necrosis with epithelial sloughing.

The damage induced by the threshold dose of ET-1 and indomethacin administration was further augmented in capsaicin-pretreated rats, and involved 73 ± 9% (n = 4) of the total mucosal area, as shown in Figure 2. In capsaicin-pretreated rats, this dose of indomethacin alone induced damage to 8 ± 3% (n = 3) of the mucosal area over this time period.

Effect of morphine administration

Intravenous administration of morphine (3 mg kg^{-1}), 10 min prior to challenge, in a dose that itself did not induce mucosal injury, significantly ($P < 0.01$) potentiated the damaging actions of the threshold dose of ET-1 (4 pmol kg^{-1} min^{-1}) to involve 73 ± 12% (n = 4) of the total mucosal area, as shown in Figure 3. This mucosal injury was macroscopically comparable to that observed following local ET-1 administration in capsaicin-pretreated rats, with vasocongestion, haemorrhagic necrosis and accompanying surface cell exfoliation being clearly evident.

This effect of morphine was abolished by prior treatment with naloxone (1 mg kg^{-1}, i.v.) as shown in Figure 3. This dose

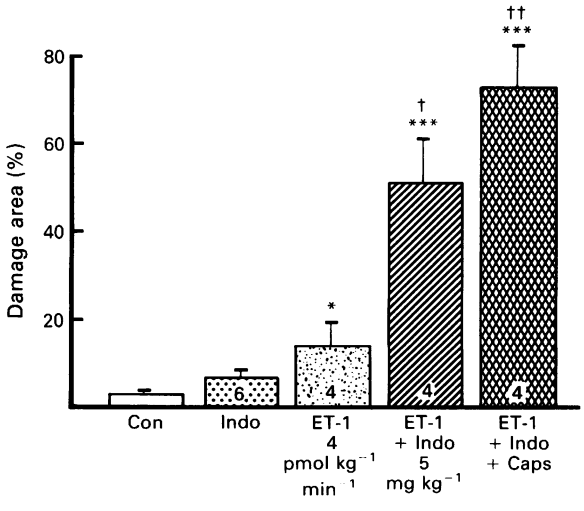

Figure 2 Effect of indomethacin administration (Indo; 5 mg kg^{-1}, i.v.), 15 min prior to challenge, on the gastric mucosal damage induced by close-arterial infusion of endothelin-1 (ET-1, 4 pmol kg^{-1} min^{-1}) in control or capsaicin (Caps)-pretreated (2 weeks earlier) rats. Results shown as % of total area exhibiting macroscopic damage, are the mean of (n) experiments, vertical bars show s.e.mean, where significant difference from control (Con) is shown as * $P < 0.05$; *** $P < 0.001$ and from ET-1 alone as † $P < 0.05$, †† $P < 0.001$.

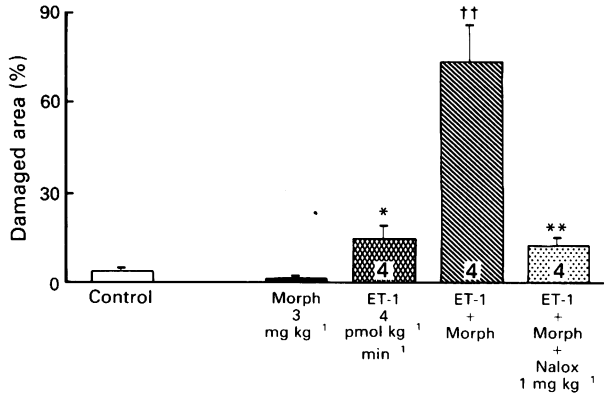

Figure 3 Potentiation of the gastric mucosal damage induced by close-arterial infusion of endothelin-1 (ET-1, 4 pmol kg^{-1} min^{-1} for 10 min) by administration of morphine (Morph, 3 mg kg^{-1} i.v.), 15 min prior to challenge and its inhibition by the opioid antagonist, naloxone (1 mg kg^{-1}, i.v.). Results, shown as % of total mucosal area exhibiting macroscopic damage, are the mean of (n) experiments, vertical bars show s.e.mean, where significant increase from control is given as * $P < 0.01$, from ET-1 alone is given as †† $P < 0.01$ and significant inhibition of ET-1 and morphine as ** $P < 0.001$.

of naloxone did not itself significantly affect the damage induced by ET-1 ($P > 0.05$; n = 3).

Inhibitory actions of calcitonin gene-related peptide

The actions of local intra-arterial infusion of rat α-CGRP on gastric mucosal damage induced by the submaximal dose of ET-1 (20 pmol kg^{-1} min^{-1}) were investigated. Concurrent close-arterial infusion of rat α-CGRP (10–50 pmol kg^{-1} min^{-1} for 10 min) caused a significant and dose-dependent reduction in the degree of damage induced by ET-1, as shown in Figure 4. Thus, rat α-CGRP (10, 25 and 50 pmol kg^{-1} min^{-1}) inhib-

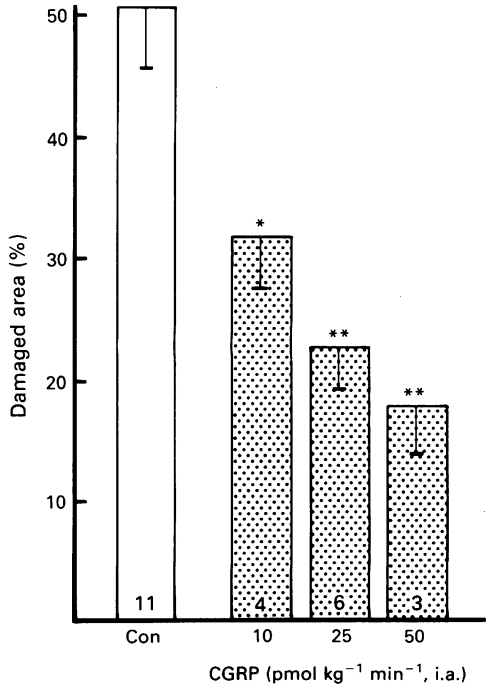

Figure 4 Inhibition of the gastric mucosal damage induced by close-arterial infusion of endothelin-1 (ET-1, 20 pmol kg^{-1} min^{-1} for 10 min) by concurrent close-arterial infusion of rat α-calcitonin gene-related peptide (α-CGRP, 10–50 pmol kg^{-1} min^{-1}). Results, shown as % of total mucosal area exhibiting macroscopic damage, are the mean of (n) experiments, vertical bars show s.e.mean, where significant inhibition is shown as * $P < 0.05$; ** $P < 0.01$.

ited mucosal damage by 36 ± 8% ($n = 4$, $P < 0.05$), 54 ± 7% ($n = 6$, $P < 0.01$) and 64 ± 8% ($n = 3$; $P < 0.01$) respectively.

These local intra-arterial doses of rat α-CGRP did not significantly alter systemic arterial BP, (change of 3 ± 3 mmHg and 4 ± 8 mmHg with 10 and 50 pmol kg^{-1} min^{-1} respectively, $n = 4$ for each). In contrast, significant dose-dependent hypotensive actions of rat α-CGRP were observed in these doses, when administered by the intravenous route, with a fall in systemic arterial BP of 8 ± 2 mmHg ($n = 4$; $P < 0.05$); 17 ± 9 mmHg ($n = 4$, $P < 0.05$) and 37 ± 1 mmHg ($n = 4$, $P < 0.01$) following intravenous infusion of rat α-CGRP (10, 25 and 50 pmol kg^{-1} min^{-1}, respectively).

Discussion

The present study demonstrates that the gastric mucosal injury induced by local intra-arterial infusion of ET-1 was augmented by capsaicin pretreatment or by the administration of morphine, as assessed by macroscopic or histological observation. Furthermore, local intra-arterial infusion of the sensory neuropeptide rat α-CGRP inhibited the mucosal damage induced by ET-1. Such findings thus indicate interactions between these peptides in the modulation of gastric mucosal integrity.

Previous studies with capsaicin-pretreatment, which disrupts primary afferent neurones in the gastric mucosa and depletes their neuropeptide content (Sternini *et al.*, 1987; Green & Dockray, 1988), have demonstrated enhanced mucosal damage following challenge with a number of proulcerogenic substances including indomethacin, ethanol and platelet activating factor (PAF) (Szolcsanyi & Bartho, 1981; Holzer & Sametz, 1986; Esplugues *et al.*, 1989; Esplugues & Whittle, 1990). Furthermore, capsaicin pretreatment augmented the deleterious reduction in gastric mucosal blood flow induced by PAF (Pique *et al.*, 1990). Since ET-1 has been demonstrated to exert vasoconstrictor actions on the rat isolated stomach (Wallace *et al.*, 1989), it is therefore possible that the increase in mucosal damage induced by ET-1 after capsaicin pretreatment, reflects enhanced vasoconstriction following depletion of vasodilator neuropeptides, although this awaits direct evaluation.

Other studies have demonstrated that the gastric damage induced by intravenous infusion of submaximal doses of ET-1 in the presence of topically applied 20% ethanol was augmented by pretreatment with indomethacin, as was the damage induced by intravenous ET-1 following intragastric instillation of 150 mM acid (Wallace *et al.*, 1989; MacNaughton *et al.*, 1989). Likewise, in the present study, pretreatment with indomethacin potentiated the mucosal damage induced by local infusion of threshold doses of ET-1, supporting a modulatory influence of endogenous prostanoids such as prostacyclin and prostaglandin E_2 (PGE_2). This damage following ET-1 and indomethacin administration was further increased in capsaicin-pretreated rats, suggesting that the concurrent reduction in both mucosal prostanoids and in endogenous sensory neuropeptides considerably increases the susceptibility of the gastric mucosa to challenge. Previous studies have also indicated an interaction between endogenous prostanoids and sensory neuropeptides in the maintenance of mucosal integrity (Holzer & Sametz, 1986; Whittle *et al.*, 1990).

Morphine administration has previously been demonstrated to enhance gastric damage induced by PAF or ethanol (Esplugues *et al.*, 1989; Esplugues & Whittle, 1990; Pique *et al.*, 1990). This action, which was exerted on peripheral opioid receptors, may reflect inhibitory actions on sensory neurones, since opioids have been shown to prevent neuropeptide release (Lembeck & Donnerer, 1985). As found with capsaicin-pretreatment, acute administration of morphine substantially enhanced the mucosal injury induced by threshold doses of local ET-1, which was of a similar nature to that observed in capsaicin-pretreated rats. This action was abolished by concurrent administration of the opioid μ-receptor antagonist, naloxone, indicating that this action of morphine was exerted on specific opioid receptors. These observations, along with previous studies with other ulcerogenic agents and with the neurotoxin, tetrodotoxin (Esplugues *et al.*, 1989) support the involvement of an opioid-sensitive neuronal pathway in the modulation of mucosal integrity.

The predominant neuropeptide localized by immunohistochemical techniques in capsaicin-sensitive neurones in the rat stomach is CGRP (Green & Dockray, 1988) and such neurones are found in close proximity to the submucosal microvasculature (Sternini *et al.*, 1987). This neuropeptide occurs principally in the form of α-CGRP in the sensory neurones innervating gastro-intestinal tissue (Mulderry *et al.*, 1988). In the present study, close-arterial infusion of rat α-CGRP dose-dependently reduced the macroscopic damage induced by ET-1. Previous studies have shown that intra-arterial administration of rat α-CGRP can also inhibit the gastric injury induced by intragastric instillation of aspirin or ethanol (Lippe *et al.*, 1989). Earlier studies had also demonstrated that subcutaneous administration of CGRP reduced gastric lesions induced by intragastric aspirin or indomethacin, but not the damage induced by ethanol, although this protective action was ascribed to anti-secretory actions (Maggi *et al.*, 1987). The mechanisms underlying the protective actions of rat α-CGRP in the present study are unlikely to be the consequence of anti-secretory actions since exogenous acid was instilled into the gastric lumen to ensure a consistent acidic environment throughout the experiment, while previous studies have demonstrated that ET-1-induced gastric damage is not inhibited by the antisecretory agent, cimetidine (Whittle & Esplugues, 1988).

CGRP is a potent endothelium-dependent vasodilator in many vascular beds and reduces systemic arterial BP following intravenous administration (Brain *et al.*, 1985; Marshall *et al.*, 1987). In the doses used in the present study, local intra-arterial infusion of rat α-CGRP had no effect on systemic BP suggesting that the protective actions observed were not the consequence of general systemic effects. Indeed, as these doses of rat α-CGRP did reduce BP following intravenous administration as observed previously (Lippe *et al.*, 1989), such findings suggest extensive metabolism of CGRP within the rat gastric or hepatic circulation, with minimal escape into the general circulation following local gastric infusion.

Previous studies have demonstrated that intravenous administration of CGRP in hypotensive doses did not protect the mucosa from ethanol or aspirin-induced injury (Lippe *et al.*, 1989). Whereas intravenous infusion of antisecretory doses of rat α-CGRP did not modify gastric mucosal blood flow in the rat (Leung *et al.*, 1987), intravenous injection of high doses of CGRP did increase blood flow to the rat stomach (Dipette *et al.*, 1987), while intravenous infusion of human CGRP elevated gastric blood flow in the rabbit (Bauerfeind *et al.*, 1989). Furthermore, intravenous infusion of human α-CGRP reversed the vasoconstrictor actions of ET-1 on the internal carotid vascular bed in the rat (Gardiner *et al.*, 1990), while local administration of CGRP has been demonstrated to attenuate the vasoconstrictor actions of local ET-1 in rabbit skin (Brain *et al.*, 1988). It will therefore be of interest in future studies to investigate the influence of locally infused CGRP on the vasoactive responses of the gastric mucosal microcirculation to the local administration of ET-1.

Whether ET-1 can induce direct damage to microvascular endothelium or other gastric cells, and the possibility that CGRP may directly enhance endothelial cell integrity and hence reduce mucosal damage is not yet known. Furthermore, it is of interest that ET-1 can be localized using *in situ* hybridization and immunostaining techniques in both sensory and motor neurones and dorsal root ganglia from human spinal tissue, and can co-exist with CGRP and substance P (Giaid *et al.*, 1989). In addition, ET-1 can induce depolarization of rat spinal neurones *in vitro*, which can be inhibited by a substance

P antagonist (Yoshizawa *et al.*, 1989). These findings may indicate an interaction between ET-1 and such neuropeptides in the modulation of sensory neuronal function. It is not yet possible to interpret the physiological significance of the present findings with exogenous administration of ET-1 or CGRP, since local levels of these mediators in the mucosal tissue cannot yet be appropriately determined. However, the opposing actions of CGRP and ET-1 on gastric damage observed in the current study could point to an important influence of such local peptide mediators in the control of mucosal injury and salubrity.

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(Received September 6, 1990
Revised November 22, 1990
Accepted November 26, 1990)

Br. J. Pharmacol. (1991), **102**, 955–961

A selective effect of protein kinase C activators on noradrenaline release compared with subsequent contraction in canine isolated saphenous veins

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1 Effects of protein kinase C (PKC) activators and inhibitors on both tritium overflow and subsequent contraction evoked by transmural nerve stimulation (TNS) were investigated in canine saphenous veins prelabelled with [^{3}H]-noradrenaline.

2 Activation of PKC by stepwise increasing concentrations (0.01 nM–1 μM) of 12-O-tetradecanoylphorbol 13-acetate (TPA), phorbol 12,13-dibutyrate (PDBu) or mezerein caused a significant and concentration-dependent enhancement of the tritium overflow evoked by TNS, while the activators failed to affect the corresponding contraction except with the highest concentration of PDBu when the contraction was significantly reduced. Phorbol, which is inactive on PKC, had no effects on the tritium overflow and contraction induced by TNS.

3 PKC inhibitors, polymyxin B (1 and 10 μM) and the isoquinolinesulphonamide, H-7 (1 μM), inhibited significantly the phorbol ester-potentiated tritium overflow evoked by TNS with no effects on the contraction. H-7 and the related inhibitor H-8 at 10 μM reduced significantly both responses to TNS in the presence of TPA, while they suppressed only the TNS-induced contraction in the absence of TPA.

4 None of the PKC activators or inhibitors affected the spontaneous tritium overflow.

5 PDBu (0.01 and 0.1 μM) elevated resting tension of the veins more effectively than TPA and mezerein.

6 These results suggest that PKC may modulate electrically stimulated noradrenaline release from adrenergic nerve endings of the canine saphenous veins and the PKC activators may act more selectively on presynaptic than postsynaptic sites, but have no apparent effect on postjunctional noradrenergic mechanisms.

Keywords: Protein kinase C activator; noradrenaline release; contraction; canine saphenous vein

Introduction

Depolarization of adrenergic nerve endings by electrical nerve stimulation leads to an elevation of intraneuronal Ca^{2+} levels, which triggers exocytotic release of noradrenaline (NA) (Blaustein, 1979). The intraneuronal responses between an increased Ca^{2+} concentration and NA release are not established. Recently, Ca^{2+}-phospholipid-dependent protein kinase (protein kinase C, PKC) has been shown to play a role in various biological responses including neurotransmitter release and neurotransmitter-induced contraction of vascular smooth muscle (Nishizuka, 1986). PKC is activated by 1,2-diacylglycerol, one of the products of phosphatidylinositol 4,5-bisphosphate hydrolysis in response to extracellular signals (Takai *et al.*, 1979), and is distributed in sympathetic nerves (Malhotra *et al.*, 1988) and various other tissues (Kuo *et al.*, 1980). Since tumour-promoting phorbol esters mimic the action of the endogenous PKC activator (Nishizuka, 1986), 1,2-diacylglycerol, the esters, 12-O-tetradecanoylphorbol 13-acetate (TPA) and phorbol 12,13-dibutyrate (PDBu) have been widely used as tools to study the involvement of PKC in the release of adrenergic neurotransmitters. Most of the investigations, however, have been performed with central neuronal tissues (Allgaier & Hertting, 1986; Allgaier *et al.*, 1987; Versteeg & Florijn, 1987; Versteeg & Ulenkate, 1987; Wang & Friedman, 1987; Hertting & Allgaier, 1988; Huang *et al.*, 1989), and few previous workers have examined simultaneously the implication of PKC in vascular adrenergic neurotransmission and subsequent neurotransmitter-induced contraction of smooth muscle. In the present study we investigated simultaneously the effect of PKC activators or inhibitors on both tritium overflow and following contraction evoked by transmural nerve stimulation (TNS) in canine isolated saphenous veins prelabelled with [^{3}H]-NA.

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Methods

Isotope experiments

Mongrel dogs of either sex, weighing 12–26 kg, were anaesthetized with sodium pentobarbitone (32 mg kg^{-1}, i.v.). Helical strips (3 × 30 mm) of the canine saphenous veins were incubated at 37°C for 2 h in Krebs-bicarbonate (Krebs) solution containing 134 nM (−)-[7-^{3}H]-NA (10 μCi in 5 ml of incubation medium) and 57 μM ascorbic acid, and mounted for superfusion according to our previous paper (Takata & Kato, 1984). The Krebs solution had the following composition (in mM): NaCl 118.2, KCl 4.6, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 24.8 and dextrose 10.0. The strips were then superfused at a rate of 3.6 ml min^{-1} with the Krebs solution maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. After a 60 min equilibration period, the veins were electrically stimulated (10 Hz, 0.3 ms, supramaximum voltage of 20 V, 150 pulses) at 16 min intervals through a pair of parallel platinum-wire electrodes with an electric stimulator (Nihon Kohden, SEN-7103). The TNS-induced contraction corresponds to a nearly half-maximal response, judging from the stimulus-contraction relationship. The TNS was repeated 8 times (S_1–S_8), if not indicated otherwise. Developed tension was recorded isometrically on an ink-writing oscillograph (Nihon Kohden, WI-640G) through a force-displacement transducer (Nihon Kohden, TB-611T). The superfusate samples were continuously collected at 2 min intervals from 4 min before S_2 throughout the experiment. One ml of each sample was mixed

with 10 ml of scintillation mixture (5.5 g DPO, 0.1 g POPOP, 667 ml toluene and 333 ml Triton X-100) and then counted in a liquid scintillation counter (Beckman, LS-9000 or Aloka, LSC-903). The TNS-evoked overflow of total tritium was estimated by subtracting the radioactivity obtained in the 2 min sample just before TNS from that in the 2 min sample during TNS. Thus, we have measured total evoked tritium overflow, and avoided lengthy chromatographic separation of the metabolites, because it more closely reflects neurotransmitter output than does the overflow of [^{3}H]-NA (Rorie *et al.*, 1980). Either PKC activators (0.01 nM–1 μM) or their vehicle, dimethyl sulphoxide (DMSO), at stepwise increasing concentrations was superfused 10 min before the onset of each stimulation of S_3 to S_8. The effects of PKC activators on the stimulation-evoked tritium overflow and contraction were expressed as the ratios of the responses to the stimulations (S_3–S_8) during the superfusion with PKC activators to the response to S_2, that is $S_3/S_2 - S_8/S_2$, and they were evaluated by comparing them with the corresponding ratios obtained from the DMSO-superfused veins. PKC inhibitors were superfused from 10 min before S_3 throughout the experiment. The effects of PKC inhibitors in combination with PKC activators were also expressed as the ratios, $S_3/S_2 - S_8/S_2$. These ratios were compared with the corresponding ratios obtained in the veins superfused with PKC activators alone. Radioactivity obtained from the 2 min sample just before stimulation of S_2–S_8 was considered as the spontaneous tritium overflow and designated as Sp_2–Sp_8, respectively. The effects of drugs on the spontaneous tritium overflow were estimated from the ratios, $Sp_3/Sp_2 - Sp_8/Sp_2$, and they were compared with the corresponding ratios in the absence of the drugs.

In some experiments the effect of 0.1 μM PDBu on the TNS-evoked tritium overflow was also investigated in the presence of tetraethylammonium (TEA), a K^+ channel blocker, because PKC activation causes blockade of K^+ currents (Baraban *et al.*, 1985) and because the blockade of K^+ currents results in augmentation of the stimulation-evoked NA release from canine saphenous veins (Kato & Takata, 1987). In this case the TNS was repeated 4 times (S_1–S_4). TEA (1 mM) was superfused from 10 min before S_3 throughout the experiment, and either PDBu (0.1 μM) or DMSO (0.001%) was further added to the superfusion medium from 10 min before S_4.

Non-isotope experiments

In these experiments, the TNS was applied twice every 16 min under the stimulation conditions described above. Thereafter, NA (10 nmol) was injected 3 times at 30 min intervals in a volume of 0.1 ml into the superfusion stream (NA_1–NA_3). The amount of NA used was sufficient to cause a contraction similar to that produced by the second TNS. Either PDBu (0.1 μM) or DMSO (0.001%) was superfused 10 min before NA_3. The effect of PDBu on the contraction induced by exogenous NA was expressed as the ratio NA_3/NA_2 and it was evaluated by comparing with the corresponding ratio in the DMSO-treated group.

Statistical analysis

All data are expressed as the mean ± s.e.mean. Statistical analyses were performed with an unpaired Student's two-tailed t test for two-sample comparison and one-way analysis of variance followed by Fisher's test of least significant difference or Dunnett test for multiple comparisons. In each case, P values less than 0.05 were considered significant.

Drugs

The following drugs were used: (−)-[7-^{3}H(N)]-NA (specific activity, 14.9 Ci mmol^{-1}, New England Nuclear), TPA (Sigma), PDBu (Sigma), mezerein (Sigma), phorbol (Sigma), polymyxin B sulphate (Sigma), 1-(5-isoquinolinesulphonyl)-2-methyl-piperazine dihydrochloride (H-7) (Seikagaku Kogyo), N-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride (H-8) (Seikagaku Kogyo), tetrodotoxin (Sigma), TEA chloride (Sigma), NA bitartrate (Sigma). PKC activators (10 mM) were dissolved in DMSO to prepare stock solution. Stock solutions (10 mM) of PKC inhibitors were prepared in distilled water. All drugs used were diluted with Krebs solution just before use.

Results

Effects of protein kinase C activators on TNS-evoked tritium overflow and contraction

In preliminary experiments we confirmed that tetrodotoxin (0.6 μM) abolished the TNS-evoked tritium overflow and the corresponding contraction during superfusion with PKC activators in the veins prelabelled with [^{3}H]-NA.

Figure 1 shows the effects of increasing concentrations of PKC activators on both tritium overflow and contraction induced by TNS. TPA and PDBu (0.01 nM–1 μM), PKC activating phorbol esters, caused a significant augmentation of the TNS-evoked tritium overflow in a concentration-dependent manner, compared with the DMSO-superfused veins. The

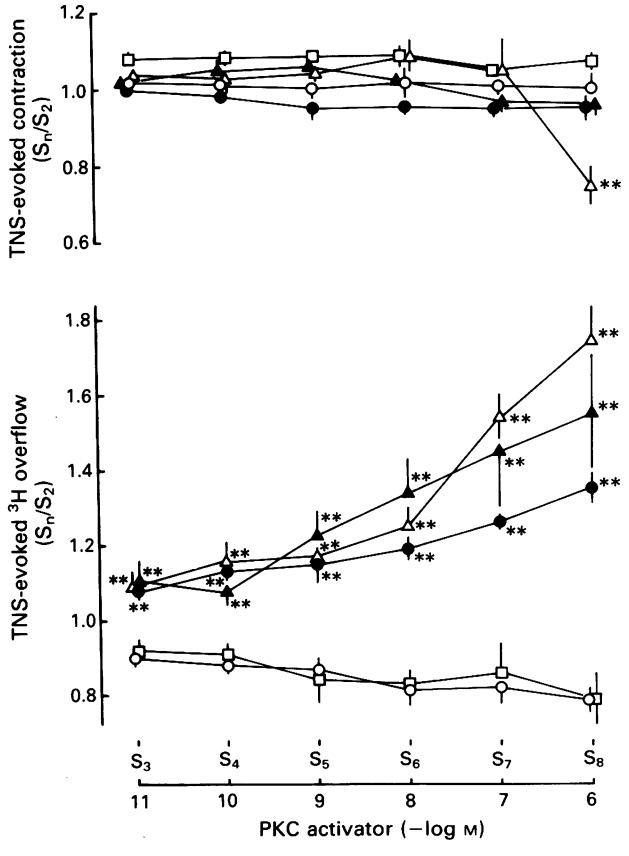

Figure 1 Effects of protein kinase C (PKC) activators on tritium overflow and contraction evoked by transmural nerve stimulation (TNS) in veins prelabelled with [^{3}H]-noradrenaline. Each PKC activator at increasing concentrations was superfused 10 min before each stimulation of S_3 to S_8. The radioactivities in the 2 min sample just before S_2 and the 2 min sample during S_2 were 4920 ± 265 and 13617 ± 1023 d.p.m. (n = 25), respectively. The corresponding contraction was 3.03 ± 0.20 g. Values are expressed as the mean from 5 experiments with s.e.mean shown by vertical bars. Symbols are as follows: DMSO (○); 12-O-tetradecanoylphorbol 13-acetate (●); phorbol 12,13-dibutyrate (△); mezerein (▲); phorbol (□). Asterisks indicate significant difference from the veins treated with dimethylsulphoxide: ** $P < 0.01$.

enhancement of the evoked tritium overflow caused by higher concentrations of PDBu (0.1 and 1 μM) was significantly ($P < 0.01$) greater than that by TPA. The TNS-induced contraction remained unchanged in the presence of TPA and PDBu except with the highest concentration of PDBu (1 μM) when the contraction produced by TNS was significantly ($P < 0.01$) inhibited. Mezerein (0.01 nM–1 μM), a non-phorbol PKC activator (Miyake *et al.*, 1984), also enhanced significantly the TNS-evoked tritium overflow with no effects on the associated contraction. The facilitatory effect of mezerein was not significantly different from that of PDBu. Phorbol (0.01 nM–1 μM), which is inactive on PKC (Castagna *et al.*, 1982), failed to affect either tritium overflow or the contraction caused by TNS.

The three PKC activators and phorbol had no significant effects on spontaneous tritium overflow (the ratio Sp_8/Sp_2 for DMSO was 0.68 ± 0.05; the ratios Sp_8/Sp_2 for each 1 μM of TPA, PDBu, mezerein and phorbol were 0.78 ± 0.04, 0.72 ± 0.03, 0.79 ± 0.05 and 0.79 ± 0.04, respectively). PDBu at 1 nM or more produced a concentration-dependent elevation of resting tension, the degree of the elevation being equivalent to 19.2 ± 3.4% for 1 nM, 22.2 ± 2.5% for 0.01 μM, 50.7 ± 8.7% for 0.1 μM and 89.6 ± 10.6% for 1 μM of the contraction (3.08 ± 0.22 g, $n = 5$) induced by S_2. There was a slight increase in the resting tension in the presence of the other PKC activators corresponding to 15.6 ± 4.2% ($n = 5$) for 1 μM TPA and 17.8 ± 4.7% ($n = 5$) for 1 μM mezerein over that induced by S_2. Phorbol failed to elevate resting tension.

Effects of protein kinase C inhibitors on the action of PKC activators on TNS-evoked tritium overflow and contraction

Polymyxin B (1 and 10 μM), a PKC inhibitor (Kuo *et al.*, 1983), produced a significant inhibition of the facilitatory effects of TPA on the TNS-evoked tritium overflow with no alteration of the contraction in comparison with the TPA-treated veins (Figure 2a). H-7 (1 and 10 μM), an isoquinolinesulphonamide PKC inhibitor (Hidaka *et al.*, 1984), also significantly inhibited the enhancement of the evoked tritium overflow by TPA, which was not significantly different from the inhibitory action of polymyxin B (Figure 2b). The corresponding contraction was inhibited only by 10 μM H-7 (Figure 2b). In the presence of TPA, H-8 (1 μM), another isoquinolinesulphonamide PKC inhibitor (Hidaka *et al.*, 1984), had no effects on the TNS-evoked tritium overflow and contraction, but at 10 μM it inhibited significantly both responses to TNS (Figure 2c).

The facilitation of the TNS-evoked tritium overflow by PDBu was significantly reduced by superfusion with 1 μM of each polymyxin B and H-7 but without effects on the contraction induced by TNS (Figure 3). There was no significant difference between the inhibitory actions of the two inhibitors.

The three PKC inhibitors used, by themselves, did not affect the TNS-evoked tritium overflow, whereas H-7 and H-8 (10 μM), but not polymyxin B, significantly inhibited the associated contraction (Figure 4). These inhibitors caused no change in spontaneous tritium overflow in the presence and absence of TPA and PDBu compared with the DMSO-treated veins.

Effects of phorbol 12,13-dibutyrate on TNS-evoked tritium overflow and contraction in the presence of tetraethylammonium

As shown in Table 1, TEA (1 mM) enhanced significantly both responses to TNS; the ratios S_4/S_2 were 2.16 ± 0.15 in overflow and 1.24 ± 0.03 in contraction. Since TEA (1 mM) does not increase the contraction induced by exogenous NA (Takata & Kato, 1988), the enhancing effect of TEA on the TNS-evoked contraction is due to an increased neurotransmitter release. PDBu (0.1 μM) in combination with TEA produced a further significant facilitation of the TNS-evoked tritium overflow, while it caused no additional increase in the contraction.

Effects of phorbol 12,13-dibutyrate on the contraction induced by exogenous noradrenaline

To confirm further a more selective action of PKC activators on presynaptic than postsynaptic sites, the effects of PDBu on

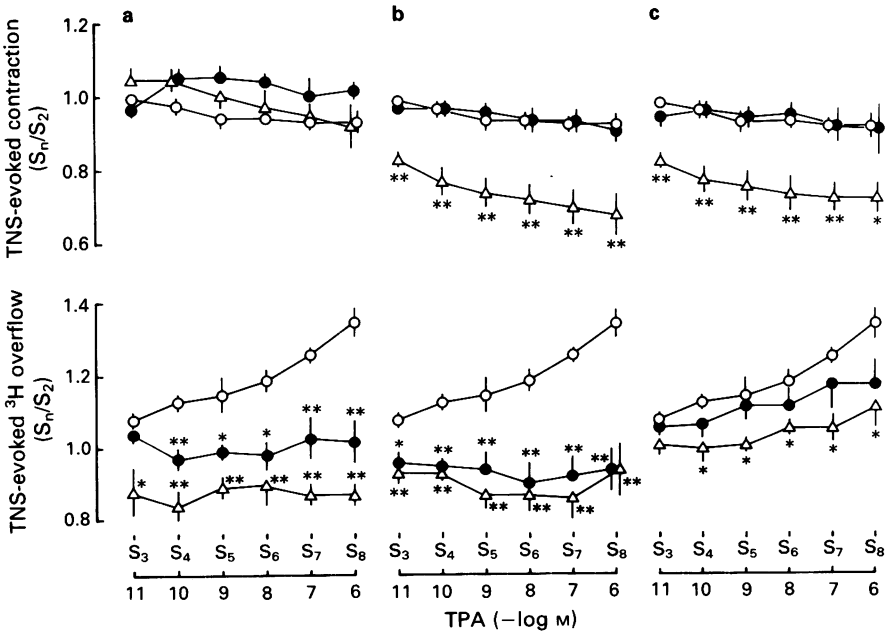

Figure 2 Effects of polymyxin B (a), H-7 (b) and H-8 (c) on tritium overflow and contraction evoked by transmural nerve stimulation (TNS) in the presence of 12-O-tetradecanoylphorbol 13-acetate (TPA) in veins prelabelled with [^{3}H]-noradrenaline. TPA at increasing concentrations was superfused 10 min before each stimulation of S_3 to S_8. Each protein kinase C inhibitor (1 μM (●), 10 μM (△), no inhibitor (○)) was superfused from 10 min before S_3 throughout the experiment. Values are expressed as the mean from 5 to 6 experiments with s.e.mean shown by vertical bars. Asterisks indicate significant difference from the veins treated with TPA alone (no inhibitor): * $P < 0.05$; ** $P < 0.01$.

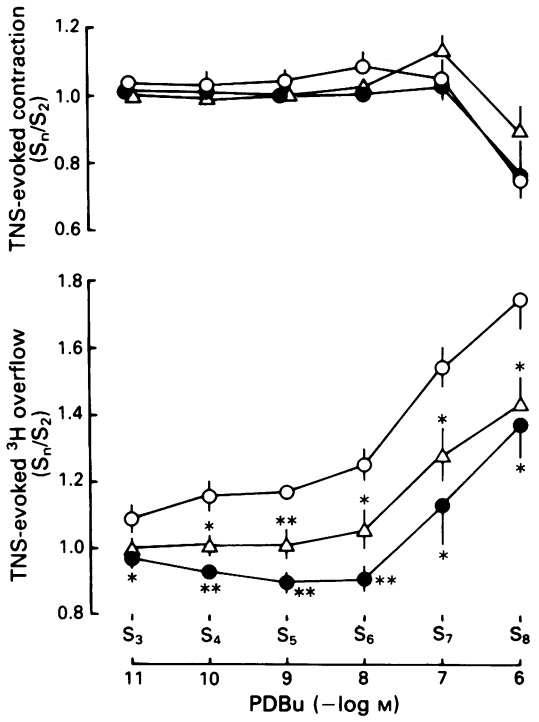

Figure 3 Effects of polymyxin B and H-7 on tritium overflow and contraction evoked by transmural nerve stimulation (TNS) in the presence of phorbol 12,13-dibutyrate (PDBu) in veins prelabelled with [^{3}H]-noradrenaline. PDBu at increasing concentrations was superfused 10 min before each stimulation of S_3 to S_8. Each protein kinase C inhibitor (1 μM polymyxin B (●), 1 μM H-7 (△), no inhibitor (○)) was superfused from 10 min before S_3 throughout the experiment. Values are expressed as the mean from 5 experiments with s.e.mean shown by vertical bars. Asterisks indicate significant difference from the veins treated with PDBu alone (no inhibitor): * $P < 0.05$; ** $P < 0.01$.

the contraction induced by exogenous NA (10 nmol) were investigated. In the vehicle group, the increases in tension to the second TNS and the second application of NA were 3.12 ± 0.43 and 3.40 ± 0.49 g ($n = 5$), respectively. The corresponding values in another group of veins before superfusion with 0.1 μM PDBu were 2.95 ± 0.49 and 3.22 ± 0.61 g ($n = 5$), respectively. No significant difference between the contractions caused by the TNS and NA was observed in either group. PDBu (0.1 μM) produced no significant potentiation of the contraction induced by 10 nmol NA (the ratios NA_3/NA_2 for the vehicle and PDBu groups were 1.07 ± 0.04 ($n = 5$) and 1.10 ± 0.02 ($n = 5$), respectively).

Table 1 Effects of phorbol 12,13-dibutyrate (PDBu, 0.1 μM) on transmural nerve stimulation (TNS)-evoked tritium overflow and contraction in the presence of tetraethylammonium (TEA, 1 mM) in veins prelabelled with [^{3}H]-noradrenaline

	TNS-evoked	
	^{3}H overflow	*Contraction*
Drugs	(S_4/S_2)	(S_4/S_2)
Control	0.99 ± 0.02	1.03 ± 0.03
TEA + DMSO	2.16 ± 0.15**	1.24 ± 0.03**
TEA + PDBu	3.21 ± 0.25**††	1.33 ± 0.06**

TEA was superfused from 10 min before S_3 throughout the experiment. PDBu was added to the superfusion medium for 16 min from 10 min before S_4. Values are expressed as the mean ± s.e.mean from 5 experiments. Significantly different from the control veins (** $P < 0.01$) or from the veins treated with TEA plus 0.001% dimethylsulphoxide (DMSO) (†† $P < 0.01$).

Discussion

In the present study TPA and PDBu caused a significant and concentration-dependent enhancement of tritium overflow induced by TNS in canine saphenous veins prelabelled with [^{3}H]-NA, while phorbol, which is insensitive to PKC (Castagna *et al.*, 1982), failed to enhance it. Mezerein, a non-phorbol PKC activator (Miyake *et al.*, 1984), also facilitated the TNS-evoked tritium overflow in a concentration-dependent manner. Together with the evidence that PKC is distributed in sympathetic nerves (Malhotra *et al.*, 1988), the results of the present study suggest that PKC may be involved in exocytotic NA release from the canine saphenous veins. Our present findings are qualitatively similar to previous

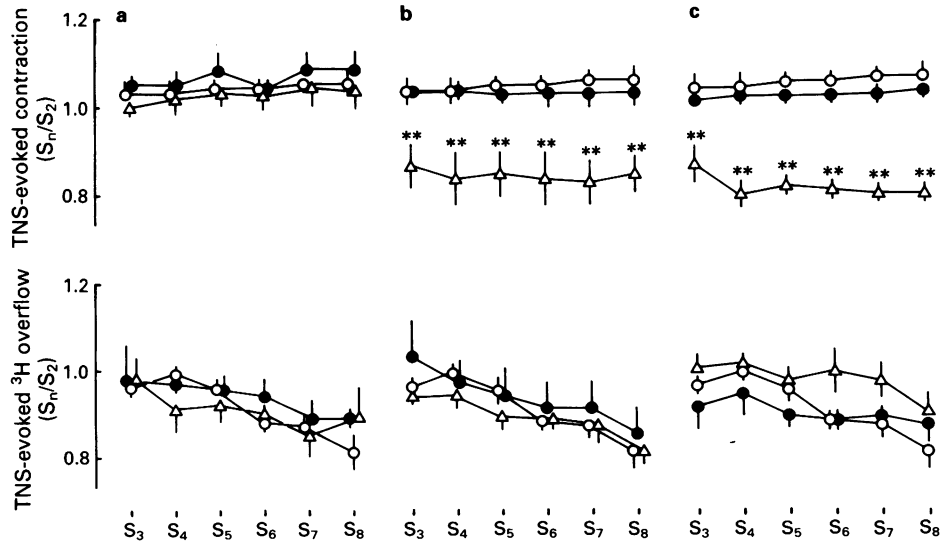

Figure 4 Effects of polymyxin B (a), H-7 (b) and H-8 (c) on tritium overflow and contraction evoked by transmural nerve stimulation (TNS) in veins prelabelled with [^{3}H]-noradrenaline. Each protein kinase C inhibitor (1 μM (●), 10 μM (△), no inhibitor (○)) was superfused from 10 min before S_3 throughout the experiment. Values are expressed as the mean from 5 experiments with s.e.mean shown by vertical bars. Asterisks indicate significant difference from the control veins (no inhibitor): ** $P < 0.01$.

reports on neurotransmitter release from both central (see Introduction) and peripheral adrenergic nerve endings (Wakade *et al.*, 1985; Shuntoh & Tanaka, 1986; Ishac & De Luca, 1988; Musgrave & Majewski, 1989). Polymyxin B and H-7, PKC inhibitors, counteracted significantly the PKC activator-induced facilitation of the tritium overflow evoked by TNS. H-7, but not polymyxin B (Mazzei *et al.*, 1982), also inhibits adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase at about the same concentration range (Hidaka *et al.*, 1984). Since the agents, which elevate cyclic AMP content, enhance NA release evoked by depolarization from the adrenergic nerve endings of canine saphenous veins (Takata & Kato, 1984; 1985), we investigated whether H-7 reduced the enhancing effects of PKC activators on the evoked tritium overflow by inhibiting cyclic AMP-dependent protein kinase, but not PKC. For this purpose, we used H-8, which is a more potent inhibitor of cyclic AMP-dependent protein kinase than PKC and is less potent than H-7 in inhibiting PKC (Hidaka *et al.*, 1984). Because H-8 did not inhibit the TPA-induced enhancement of the evoked tritium overflow more strongly than did H-7, these results suggest that the antagonistic action of H-7 may be due to inhibition of PKC and that the enhancing effect of PKC activators on the TNS-evoked NA release from the canine saphenous veins is attributable to activation of PKC. In spite of these suggestions, neither polymyxin B nor H-7, by themselves, inhibited the tritium overflow evoked by TNS. Since the two PKC inhibitors at 10 μM or more were previously shown not to inhibit stimulation evoked neurotransmitter release (Allgaier & Hertting, 1986; Wang & Friedman, 1987; Shuntoh *et al.*, 1988) although higher doses (> 30 μM) were effective, it is possible that the concentration of PKC inhibitors used to reduce the evoked tritium overflow in the present study may be too low.

PDBu at higher concentrations enhanced the tritium overflow evoked by TNS more markedly than did TPA, although TPA is more potent or equivalent to PDBu in activating PKC *in vitro* (Castagna *et al.*, 1982; Malhotra *et al.*, 1988). The reason for such potency differences is unknown but a greater potency of PDBu was also reported in NA releases from the rabbit hippocampus (Allgaier *et al.*, 1987) and from the mouse atria (Musgrave *et al.*, 1989). The former authors believe that a major reason for the reduced potency of TPA is that this highly lipophilic drug requires more time to equilibrate in the extracellular space compared with more hydrophilic analogues (Dunwiddie & Fredholm, 1985). On the other hand, Musgrave *et al.* (1989) showed that a smaller effect of TPA is not simply due to TPA requiring a longer time to produce its effect, because the potentiation caused by TPA was identical when the pretreatment period was prolonged from 15 to 75 min, and because PDBu produced a further enhancement of NA release even in the presence of the maximally effective concentration of TPA. From these results, they suggested that sympathetic nerves of the mouse atria possess multiple forms of PKC which are differentially activated by TPA and PDBu.

The precise mechanism by which PKC activators potentiate NA release remains unclear. The studies with rat salivary gland (Wakade *et al.*, 1985) and mouse atria (Musgrave & Majewski, 1989) indicate that the facilitatory action of PKC activators on electrically stimulated NA release is not due to blockade of presynaptic α_2-adrenoceptors. Furthermore, it has been reported that inhibition of neural NA reuptake is not responsible for the phorbol ester-induced potentiation of the evoked NA release (Shuntoh *et al.*, 1988). PKC activation causes blockade of K^+ currents (Baraban *et al.*, 1985) or facilitation of Na^+ (Dicker & Rozengurt, 1981) and Ca^{2+} currents (Wakade *et al.*, 1986), each of which leads to facilitation of the evoked NA release. PDBu caused a further significant enhancement of the TNS-evoked tritium overflow even in the presence of TEA. If PKC activators potentiate the depolarization-evoked NA release via facilitation of Na^+ currents following the enzyme activation, then it would be anticipated that the activators enhance the tritium overflow evoked by TNS, but not by elevated potassium ions, because the NA release induced by 40 mM KCl is not modified by drugs affecting Na^+ channel function in canine saphenous veins (Takata & Kato, 1985). However, the 40 mM KCl-evoked tritium overflow from canine saphenous veins preloaded with [^{3}H]-NA was also enhanced by PKC activators (unpublished data). Therefore, it appears that neither blockade of K^+ currents nor facilitation of Na^+ currents accounts for the PKC activator-potentiated tritium overflow. There is evidence for a synergistic action of PKC and Ca^{2+} in the stimulation-secretion coupling process (Nishizuka, 1984). The synergism occurs as a result of the increased sensitivity to Ca^{2+} of NA release processes triggered by Ca^{2+} following PKC activation (Shuntoh & Tanaka, 1986). Phorbol ester also causes a marked enhancement of electrically stimulated $^{45}Ca^{2+}$ accumulation in cultured sympathetic neurones of the chick embryo facilitating NA release (Malhotra *et al.*, 1988). This, together with the result that phorbol esters enhance only the Ca^{2+}-dependent NA release (Wakade *et al.*, 1985; Allgaier *et al.*, 1987), make it most likely that PKC activation and the resultant phosphorylation of neuronal proteins responsible for release processes lead to either enhanced influx of Ca^{2+} into the nerve endings or sensitization to Ca^{2+} of exocytotic processes facilitating NA release. The neuronal proteins which are substrates for PKC during NA release processes in the canine saphenous veins are still unknown.

Neither PKC activators nor inhibitors caused significant effects on spontaneous tritium overflow, in agreement with the findings in other tissues (Wakade *et al.*, 1985; Shuntoh & Tanaka, 1986; Ishac & De Luca, 1988; Shuntoh *et al.*, 1988; Musgrave & Majewski, 1989), suggesting that PKC activation may be responsible for modulating rather than for mediating neurotransmitter release.

PKC activators facilitated the TNS-evoked tritium overflow without producing a significant increase in subsequent contraction. Furthermore, PDBu failed to enhance the contraction produced by exogenous NA. Thus, it seems that PKC activators are more selective in their presynaptic than postsynaptic effects in the canine saphenous veins in contrast to the results with the perfused rat kidney (Sehic & Malik, 1989). Since the contraction produced by TNS was significantly increased in the presence of 1 mM TEA, the finding that PKC activators did not enhance the TNS-induced contraction in spite of an increase in the evoked tritium overflow cannot be explained by the view that no further increase in contraction occurred after PKC activators because the contraction was already maximal. The present study indicates that 1 mM TEA augmented electrically evoked tritium overflow 2.16 fold, whereas it enhanced the contraction only 1.24 fold. Therefore, the enhancement of the TNS-evoked overflow by PKC activators appears to be insufficient for significant potentiation of the contraction. Moreover, it is unlikely that under the conditions employed PKC plays a major role in the contractile response of the canine saphenous veins to NA released. A significant inhibition of the TNS-evoked contraction by the highest concentration (1 μM) of PDBu is likely to be due to a marked elevation of resting tension.

H-7 or H-8 (10 μM) inhibited significantly the TNS-evoked contraction regardless of the presence of PKC activators. The inhibitory effects of the two inhibitors are not due to a decrease in the evoked tritium overflow, since polymyxin B, which inhibited the evoked tritium overflow to a similar extent, did not reduce the corresponding contraction. Both H-7 and H-8 inhibit not only PKC but also Ca^{2+}-calmodulin-dependent protein kinase, the K_i values of H-7 and H-8 for Ca^{2+}-calmodulin-dependent kinase being 97 and 68 μM, respectively (Hidaka *et al.*, 1984). On the other hand, polymyxin B is over 100 fold more potent as an inhibitor of PKC than of Ca^{2+}-calmodulin-dependent kinase (K_i value; 6.5 μM vs 950 μM) (Wrenn & Wooten, 1984). It appears, therefore, that the inhibitory effects of H-7 and H-8 on the TNS-induced contraction may be due to the inhibition of Ca^{2+}-calmodulin-

dependent kinase rather than PKC. This view is also supported by the result that the PKC activators did not increase the contraction produced by TNS.

Higher concentrations of PDBu elevated resting tension much more effectively than TPA and mezerein. Phorbol did not raise resting tension. These findings suggest that the elevation of resting tension by the activators is probably due to PKC activation. Chiu *et al.* (1988) have reported that PDBu may activate the voltage-dependent Ca^{2+} channels through PKC activation to contract the canine saphenous veins. The lesser potency of TPA in raising resting tone might be in part explained by its highly lipophilic property as mentioned above. The elevation of resting tension induced by TPA and PDBu was not significantly suppressed by polymyxin B and H-7, in agreement with the result that H-7 at 10 μM caused no inhibition of the TPA-induced contraction in the aorta of normotensive Wistar Kyoto rats (Shibata *et al.*, 1990). On the other hand, Khalil & van Breemen (1988) found that H-7 at 20 μM reversibly inhibited the TPA-induced contraction in the rabbit aorta. Differences in animal species and experimental conditions might account for this discrepancy.

We thank Mr J. Sasaki, Miss S. Fujimori, Miss Y. Furuhashi and Miss Y. Nuijima for their excellent technical assistance.

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(Received May 10, 1990
Revised October 4, 1990
Accepted December 6, 1990)

Br. J. Pharmacol. (1991), **102**, 962–966

Bradykinin stimulates production of inositol (1,4,5) trisphosphate in cultured mesangial cells of the rat via a BK_2-kinin receptor

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1 Using [^{125}I-Tyr^0]-BK, as radiolabelled ligand, and various agonists and antagonists of bradykinin (BK) we identified a single class of specific BK_2-binding sites in mesangial cell membranes (B_{max} = 73 fmol mg^{-1} protein and K_d = 3.7 nM).

2 Following the addition of 0.1 μM BK, inositol (1,4,5) trisphosphate (IP3) formation increased within 20 s from a basal level of 64 to a maximal value of 175 pmol mg^{-1} protein.

3 Incubation in a Ca^{2+}-free medium did not change IP3 production but a 5 min preincubation with 1 mM EGTA completely prevented the BK-induced IP3 formation, suggesting that IP3 formation is partly dependent on extracellular calcium.

4 The BK_2 antagonist D-Arg-Hyp^3-D-Phe^7-BK (10 μM) but not the BK_1 antagonist (des-Arg^9-Leu^8-BK) abolished IP3 production in response to 0.1 μM BK. Pretreatment of mesangial cells with pertussis toxin was without effect on BK-induced IP3 formation, whereas phorbol 12-myristate 13-acetate significantly enhanced (by 25%) BK-induced IP3 formation.

5 The present data demonstrate that inositol phosphate breakdown in rat mesangial cells can be mediated via activation of a BK_2-kinin receptor and is under negative control of protein-kinase C.

Keywords: Bradykinin; rat cultured renal mesangial cells; inositol (1,4,5) trisphosphate; BK_2-receptor

Introduction

Mesangial cells, which represent about one third of the glomerular cell population, are contractile cells of the renal glomerulus (Kreisberg *et al.*, 1985; Schlondorff, 1987) that respond to vasoactive stimuli. It has been hypothesized that both contraction and relaxation of mesangial cells are involved in the regulation of the glomerular filtration rate. Contraction and relaxation of mesangial cells have been observed in response to a large number of agents (Pfeilschifter, 1989; Mene *et al.*, 1989). Agents such as angiotensin II, vasopressin, noradrenaline, platelet activating factor and thrombin, induce cell contraction; atriopeptide, guanosine 3':5'-cyclic monophosphate (cyclic GMP), cyclic AMP, prostaglandin E_2 (PGE_2) and dopamine are relaxing factors. Among the vasoactive agents, the effect of the nonapeptide bradykinin (BK) on mesangial cells has received less attention. However, micropuncture studies pointed out that intraglomerularly infused BK decreased the glomerular ultra-filtration coefficient (Kf) in rat (Baylis *et al.*, 1976), but at that time no mechanism was proposed. Kf is the product of the transcapillary hydraulic permeability and the total filtration area which is directly related to the contractile state of the cells. More recently it was demonstrated that BK stimulates a rise in cytosolic calcium in glomerular mesangial cells (Kremer *et al.*, 1987). Whether this rise in intracellular free calcium concentration is due to influx from extracellular calcium space or to mobilization of the intracellular store has not yet been fully investigated. On the basis of these preliminary observations, we recently characterized the presence of BK_2 receptor-like binding in glomerular membrane (Bascands *et al.*, 1989). The density of this binding site appeared to be down-regulated by the level of activity of the kinin-forming enzyme during changes in sodium intake (Emond *et al.*, 1989).

To confirm further two recent reports from our laboratory we investigate here the early response of cultured mesangial cells to BK and quantify this response by measuring inositol (1,4,5) trisphosphate (IP3) formation.

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Methods

Cell culture

Mesangial cells were obtained following culture of glomeruli isolated by graded sieving as described by Foidart *et al.* (1979). Under sterile conditions the kidneys were excised from anaesthetized normal Sprague Dawley rats (body weight 140–200 g), the cortex was removed, minced and washed several times in Hank's balanced salt solution and treated with collagenase (0.35 u l^{-1}). The tissue suspensions were then passed through three consecutive sterilized filters with decreasing pore sizes: 180, 125 and 75 μm. The isolated glomeruli were harvested from the surface of the 75 μm filter and plated into 25 cm^2 flask (NUNC) in a humidified atmosphere of 95% air 5% CO_2 and grown in complete medium consisting of RPMI 1640 supplemented with 15% foetal calf serum, 50 u ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 2 mM glutamine. Mesangial cells can be distinguished from fibroblasts by their ability to grow in a D-valine-containing medium (Gilbert & Migeon, 1975). In these conditions, it is well established that the first cell type to grow is the epithelial cells which persist for about one week and then start to senesce while with the high concentration of foetal calf serum used, the mesangial cells appear rapidly and proceed to overgrow the epithelial cells after 3 weeks of culture (Foidart *et al.*, 1979). The mesangial cells were identified by morphological and functional criteria: under phase-contrast microscopy, cells appeared large and stellate. The cells are sensitive to mitomycin (5 μg ml^{-1}) and do not stain with factor VIII antigen. Mesangial cells showed histochemical evidence of actin fibres revealed by NBD fluorescent phallacidin and myosin filaments revealed by specific antibodies. Angiotensin II (10^{-8} M)

and vasopressin (10^{-9} M) induced contraction of the cells and PGE_2 (10^{-8} M) relaxed the cells. As epithelial cells do not have angiotensin II receptors, the cell population obtained after 3 weeks of culture is a homogeneous mesangial cell culture, not contaminated with epithelial cells.

Membrane preparation and binding studies

The cultured cells were scraped off and suspended in the following medium: 50 mM Tris-HCl pH 7.4 containing 5 mM EDTA, 100 mM benzamidine, 10 mM leupeptin, 1 mM $NaHCO_3$, 2.5 mM $MgCl_2$, and manually homogenized in a tissue grinder (KONTES). The homogenate was centrifuged at 40,000 *g* for 30 min and the pellet was used as membrane preparation. Proteins were measured by the method of Lowry *et al.* (1951) after solubilization for 15 min at 100°C with 1 M NaOH; repeated cell counts revealed that 200 ± 50 μg of cellular protein was equivalent to 10^6 cells. The radiolabelled bradykinin used was [^{125}I-Tyr^0]-BK iodinated with Na^{125}I (Amersham) in the laboratory by the chloramin T method and immediately purified by high performance liquid chromatography (h.p.l.c.); the specific radioactivity was 280 Ci mmol^{-1}. Binding experiments were conducted as recently described (Bascands *et al.*, 1989). Briefly, saturation studies were performed with amounts of [^{125}I-Tyr^0]-BK increasing from 1 to 10 nM. For inhibition studies, a fixed concentration of [^{125}I-Tyr^0]-BK (1.7 nM) was used in the presence of increasing concentrations of agonists or antagonists (from 10^{-11} to 10^{-4} M) but also with other peptides for which receptors have been identified in mesangial cells such as angiotensin II, vasopressin and atrial natriuretic factor; the residual binding was expressed as the percentage of total specific binding. In both types of study, the final volume was 0.4 ml, the binding buffer consisted of 5 mM potassium phosphate, pH 7.2, containing 0.32 M sucrose and 0.1% lysozyme. After a 45 min incubation time at 37°C, 4 ml of washing buffer (binding buffer without lysozyme) was added and the total volume filtered on a GF/C Whatman filter previously soaked for at least 2 h in polyethylenimine (1%). The filters were washed four additional times with 4 ml of washing buffer. The filter-bound radioactivity was determined in a Cristal Multi RIA Packard gamma-counter. Specific binding was calculated by subtracting binding in the presence of excess unlabelled BK (10 μM) from the total binding in the absence of unlabelled peptide. The results of the saturation studies, the binding parameters (B_{max}, K_d), were analysed by use of the Kinetic EBDA Ligand computerized programme (Munson & Rodbard, 1980) which gives the linear transformation of the saturation data (Scatchard analysis, Hill plot (nH)). B_{max} is expressed as fmol of bradykinin bound per mg of protein (fmol mg^{-1} prot.) and K_d in nM. For competition studies the relative affinities of the different competitors were determined with one- or two-site models by the computerized programme mentioned above. Values are means ± s.e. of triplicate measurements of three independent experiments.

D-myo-Inositol (1,4,5) phosphate production and assay

After 21 days of culture, mesangial cells were seeded in 6-well culture trays (NUNC) at a density of 10^5 cells/well and cultured for 72 h with the complete medium. The cells were rendered quiescent by incubation in serum-free buffered medium for 24 h. After the 24 h period, some cultures were pretreated for 12 h with phorbol 12-myristate 13-acetate (PMA) 50 ng ml^{-1} or for 4 h with pertussis toxin (PT) 100 ng ml^{-1}; then the medium was drawn off and the cells were stimulated with bradykinin. The Ca^{2+}-free medium with or without EGTA (2 mM) added before stimulation with BK was not changed. The BK stimulation was stopped at the indicated times by adding 10% perchloric acid and then cells were kept on ice for 15 min. After neutralization with ice cold 1.5 M KOH, the samples were centrifuged at 2000 *g* for 15 min at 4°C. The supernatants were kept cold in ice and aliquots of 100 μl were used to measure IP3 with the [^{3}H]-D-myo-inositol 1,4,5-trisphosphate (IP3) RIA kit system (Amersham). The sensitivity range was from 0.5 to 75 pmol ml^{-1}, with 8 and 12% intra and inter-assay variation coefficients respectively.

Statistical analysis was performed with Student's *t* test and in all comparisons differences were considered significant at $P < 0.05$.

Drugs

Peptides were dissolved in water and 10^{-3} M stock solutions were stored frozen at −70°C. Peptides and drugs for these studies came from the following sources: collagenase (Boehringer Mannheim), penicillin, streptomycin, mytomycin, RPMI, foetal calf serum, D-valine medium, bradykinin (BK), lysyl-bradykinin (LBK), angiotensin II, vasopressin, PGE_2, EDTA, EGTA, benzamidine, leupeptin, pertussis toxin, polyethylenimine (Sigma, St. Louis, MO, U.S.A.), myosin antibody (Bio-Yeda), factor VIII antigen (Behring), NBD-phallicidin (N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin) was purchased from Molecular Probe; all the bradykinin agonists and antagonists were synthesized by the department of Pharmacology of Sherbrooke University.

Results

Binding studies

As shown in Figure 1, binding with increasing amounts of [^{125}I-Tyr^0]-BK on the mesangial cell membrane gave a maximum number of binding sites (B_{max}) of 73 ± 8 fmol mg^{-1} protein with a dissociation constant (K_d) of 3.7 ± 0.8 nM. The Scatchard plot (insert Figure 1) was linear and the Hill coefficient was not different from unity (0.96 ± 0.04), indicating that the [^{125}I-Tyr^0]-BK bound to only one class of binding site in the range of concentrations tested up to 10 nM. The specificity of [^{125}I-Tyr^0]-BK binding was tested by incubation with various agonist and antagonists (Figure 2). From the inhibition studies we deduced the following order of potency: BK > D-Arg-Hyp^3-D-Phe^7-BK > LBK and the respective IC_{50} values are 0.18 ± 0.03; 0.45 ± 0.04; 37 ± 5 nM. The BK_1 agonist, des-Arg^9-BK, was without effect on [^{125}I-Tyr^0]-BK binding. The binding of [^{125}I-Tyr^0]-BK was not inhibited by angiotensin II (10^{-4} M), vasopressin (10^{-4} M) or atrial natriuretic factor (10^{-4} M).

D-myo-Inositol (1,4,5) phosphate production

In Figure 3, the time course of IP3 formation following stimulation with 0.1 μM BK demonstrated a rapid increase from a basal level of 64 ± 14 pmol mg^{-1} protein to a maximal value

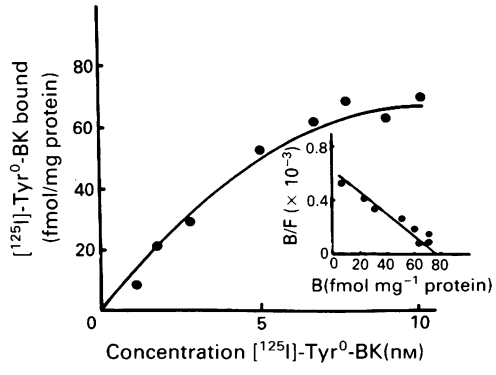

Figure 1 Binding curve and Scatchard analysis (inset) of [^{125}I]-Tyr^0-bradykinin to membrane from mesangial cells. Each point is the mean of triplicate determinations. Data are representative of those obtained in three separate experiments.

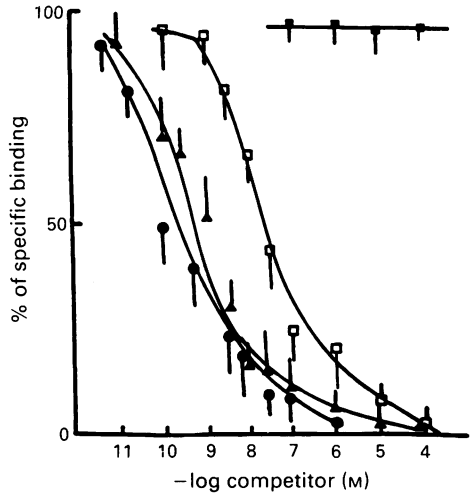

Figure 2 Inhibition of [^{125}I]-Tyr^0-bradykinin binding to membrane from mesangial cells by bradykinin (●), D-Arg-Hyp^3-D-Phe^7-bradykinin (▲), lysyl-bradykinin (□), and Des-Arg^9-bradykinin (■). Each point is the mean of triplicate determinations. Data are representative of those obtained in three separate experiments.

of 175 ± 25 pmol mg^{-1} protein which was reached after only 20 s. Return to basal value was observed within 2 min. Bradykinin induced a dose-dependent stimulation in IP3 formation (Figure 4). The maximal stimulation was achieved with 1 μM BK and the EC_{50} was between 10^{-8} and 10^{-9} M BK.

Pretreatment with pertussis toxin (100 ng ml^{-1}) for 4 h was without effect on BK-induced IP3 formation. In contrast, pretreatment with PMA for 12 h potentiated the BK stimulating effect by about 25% (Figure 3).

When the cells were incubated in a Ca^{2+}-free medium (Figure 5), no change in IP3 formation in response to 0.1 μM BK was observed when compared to the results obtained with a calcium containing medium (Figure 3). However, addition of EGTA to the Ca^{2+}-free medium completely abolished the BK-induced IP3 stimulation (Figure 5).

Incubation of the cells in the presence of the BK_2 antagonist, D-Arg-Hyp^3-D-Phe^7-BK (10 μM) inhibited the stimulating effect of 0.1 μM BK. The BK_2 antagonist alone did not demonstrate any stimulating or inhibiting effect on IP3 formation (Figure 6). The BK_1 antagonist des-Arg^9-Leu^8-BK had no inhibitory effect on the stimulating effect on IP3 formation.

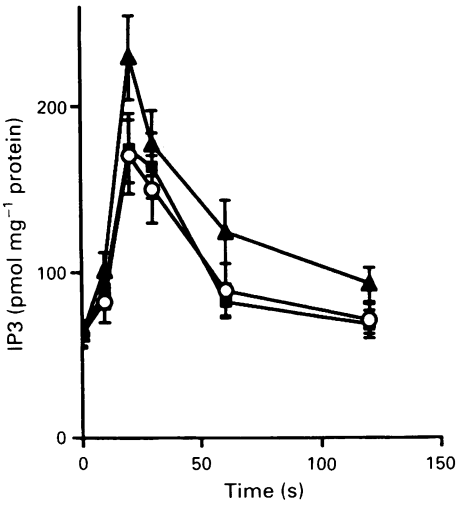

Figure 3 Kinetics of the production of inositol (1,4,5) trisphosphate (■) and effect of pretreatment with pertussis toxin (○) and phorbol 12-myristate 13-acetate (PMA, ▲) on bradykinin (0.1 μM)-stimulated inositol (1,4,5) trisphosphate formation in cultured rat mesangial cells. Bradykinin was added at time zero and the reaction was stopped at the indicated time. Each point is the mean of triplicate determinations. Data are representative of those obtained in three separate experiments.

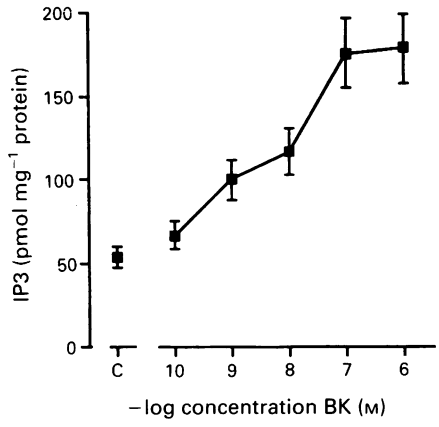

Figure 4 Dose-response curve for bradykinin (BK)-stimulated inositol (1,4,5) trisphosphate formation in cultured rat mesangial cells. C (control value). Cells were exposed to bradykinin for 20 s. Each point is the mean of triplicate determinations. Data are representative of those obtained in three separate experiments.

Discussion

The present results show that rat mesangial cells specifically bind bradykinin; this specific binding is coupled to phosphoinositide turnover. Although some of these results could have been expected from previous studies (Baylis *et al.*, 1976; Kremer *et al.*, 1987) the presence of bradykinin binding and the functional role of such binding were not demonstrated until now.

The pharmacological profile of the binding, as well as the inhibition of IP3 formation by D-Arg-Hyp^3-D-Phe^7-BK, strongly suggest the involvement of a BK_2-kinin receptor. The kinin receptors have been classified, according to the affinity of agonists and antagonists, into two classes named BK_1 and BK_2 (Régoli & Barabé, 1980). Specific binding with des-Arg^9-BK reveals the presence of a BK_1-kinin receptor, while bradykinin is the most specific agonist of the BK_2 receptor. Recently, on the basis of agonists and antagonists activities on uterine and ileal contractions, Braas *et al.* (1988), have suggested the presence of multiple bradykinin-BK_2-receptor subtypes. The present data are consistent with our previous

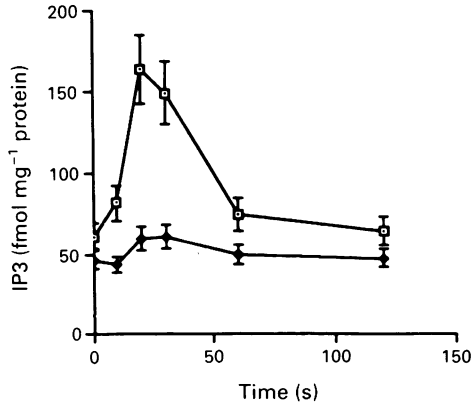

Figure 5 Effect of calcium on inositol (1,4,5) trisphosphate formation in bradykinin-stimulated cultured rat mesangial cells. Cells were preincubated in Ca^{2+}-free medium alone (□), or with EGTA (●) for 5 min before stimulation with 0.1 μM bradykinin which was added at time zero and the reaction was stopped at the indicated time. Each point is the mean of triplicate determinations. Data are representative of those obtained in three separate experiments.

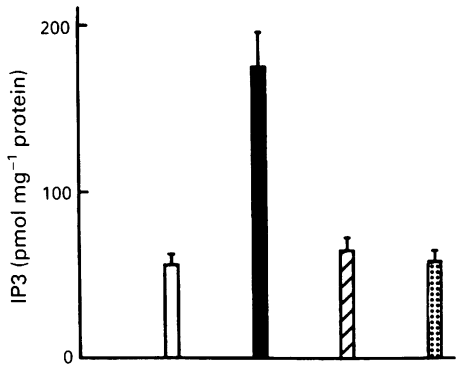

Figure 6 Inhibition of bradykinin-stimulated inositol (1,4,5) trisphosphate (IP3) formation by incubation with D-Arg-Hyp^3-D-Phe^7-bradykinin. Open column: control; solid column: bradykinin 0.1 μM; hatched column: bradykinin 0.1 μM + D-Arg-Hyp^3-D-Phe^7-bradykinin 10 μM; (stippled column) D-Arg-Hyp^3-D-Phe^7-bradykinin 10 μM. These data are representative of those obtained in three independent experiments.

findings on the glomerular membrane (Bascands *et al.*, 1989), but also provide a possible molecular support to explain the reduction of the ultra-filtration coefficient following intrarenal administration of BK (Baylis *et al.*, 1976). Very recently, it has been demonstrated that bradykinin stimulates phosphoinositide breakdown in intact rat glomeruli (Sekar *et al.*, 1990). However, the experiments were conducted with crude intact glomeruli which include mesangial, endothelial and epithelial cells. In other regions of the kidney or in other organs, specific BK-binding sites have been identified in rat intestinal membranes (Cox *et al.*, 1986), in rat duodenum (Boschcov *et al.*, 1984; Paiva *et al.*, 1989), in epithelial (Portilla & Morrisson, 1986) and endothelial cells (Lambert *et al.*, 1986). Therefore in intact glomeruli, mesangial cells may not be the sole target for BK. In addition, we found that BK potentiates IP3 formation by 173 $\pm$ 15% within 20 s, whereas on intact glomeruli (Sekar *et al.*, 1990) only a 47 $\pm$ 10% increase in total inositides was observed within 5 min in response to the same concentration of BK (0.1 μM). Although different methodologies are used to assess inositide breakdown, it should be noted that intact glomeruli contain kininase II (Chancel *et al.*, 1987) which could be responsible for a bradykinin degradation that was not assessed in the present investigation (Sekar *et al.*, 1990). All these aspects may explain the difference in the intensity of the BK-induced IP3 formation between our results and those of a recent study performed on intact glomeruli (Sekar *et al.*, 1990).

In the present study it was also shown that incubation of the cells in virtually Ca^{2+}-free medium did not inhibit IP3 formation. In most cell systems IP3 formation is considered to be extracellular Ca^{2+}-independent. However, addition of EGTA to the medium caused a 90% reduction in IP3 formation suggesting that slight trans-membrane Ca^{2+} efflux from the cells, sufficient to allow IP3 formation, is counteracted by the presence of EGTA. Such a Ca^{2+}-dependent IP3 formation has previously been reported in neuroblastoma-glioma hybrid cells (Fu *et al.*, 1988).

Furthermore, the results obtained in this study show that long term pretreatment of the mesangial cells with PMA potentiates, by 25%, the BK-stimulated effect on IP3 formation, suggesting that this effect is partly inhibited by protein kinase C (PKC). Long term pretreatment with PMA for 12 h results in a complete desensitization of PKC (Rodriguez-Pena & Rozengurt, 1984) and in this case the response to BK is increased. In contrast, short term exposure to PMA activates PKC transiently and may decrease the BK-stimulating effect as described recently in intact glomeruli (Sekar *et al.*, 1990). Such a negative feed-back of PKC has also been demonstrated for IP3 production in response to angiotensin II (Pfeilschifter, 1986) and vasopressin (Troyer *et al.*, 1988). On the other hand, we found that the release of IP3 is mediated by a pertussis-toxin-insensitive pathway confirming a previous report (Kremer *et al.*, 1987). Although the present report contains the first demonstration of BK-induced IP3 formation in mesangial cells, a similar effect has already been reported in other regions of the kidney: in medullary slices (Speziale *et al.*, 1985) in epithelial MDCK cells (Portilla & Morrisson, 1986) and in papillary collecting tubules (Shayman & Morrisson, 1985). In non-renal cell lines, there is also evidence that bradykinin stimulates phosphoinositides as in Swiss 3T3 fibroblasts (Burch & Axelrod, 1987), in bovine endothelials cells (Martin *et al.*, 1989) and in neuroblastoma cells (Fu *et al.*, 1988).

Therefore, as in other cells, BK induces IP3 formation which appears to be under negative control by PKC. In conclusion, IP3 formation in mesangial cells is induced by a wide variety of effectors which are all vasoactive peptides such as angiotensin II, vasopressin and now bradykinin. Furthermore the presence of such a large number of receptors linked to IP3 formation and smooth muscle-like activity, suggests the coexistence of different pathways for cell contractility.

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(Received June 5, 1990
Revised September 24, 1990
Accepted November 27, 1990)

Br. J. Pharmacol. (1991), **102**, 967–973

The involvement of endothelium-derived relaxing factor in the regulation of renal cortical blood flow in the rat

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1 In the present study the role of endogenous nitric oxide (NO) was investigated, in the regulation of renal cortical blood flow (RCBF) *in vivo* in anaesthetized rats under conditions in which prostacyclin involvement had been eliminated.

2 Infusions of the NO synthesis inhibitor N^G-monomethyl-L-arginine (MeArg) at 1 or 3 mg kg^{-1} min^{-1}, i.v., produced significant decreases in RCBF of 29 ± 7% and 35 ± 5%, respectively. These effects were reversed by co-infusion of a 3 fold excess of L-arginine (L-Arg).

3 Similarly, intravenous infusion of N_ω-nitro-L-arginine methyl ester (NO_2Arg) at 30 or 300 μg kg^{-1} min^{-1} attenuated RCBF by 21 ± 4% or 53 ± 4%, respectively, and these effects were reversed by L-Arg (3 or 10 mg kg^{-1} min^{-1}, i.v.). Most importantly, a low dose of NO_2Arg (30 μg kg^{-1} min^{-1}, i.v.), while having no pressor effect, considerably reduced RCBF, indicating that basal release of NO is important for the maintenance of renal cortical blood flow.

4 MeArg (3 mg kg^{-1} min^{-1}, i.v.) or NO_2Arg (300 μg kg^{-1} min^{-1}, i.v.) inhibited endothelium-dependent acetylcholine (ACh, 10 μg kg^{-1} min^{-1}, i.v. for 3 min) increases in RCBF in an L-Arg reversible manner, but did not affect endothelium-independent (dopamine 10 μg kg^{-1} min^{-1}, i.v., for 3 min) increases. Endothelin-1 (1 nmol kg^{-1}, i.v.), when given as a control for the vasoconstrictor effects of MeArg and NO_2Arg, produced a slight inhibition of the ACh-induced increase in RCBF, but this effect was significantly weaker than that produced by MeArg or NO_2Arg.

5 Our findings suggest that MeArg and NO_2Arg inhibit basal and ACh-stimulated release of NO in the renal cortical vasculature. Thus, endogenous NO formation may play an important role in the local regulation of renal cortical blood flow.

Keywords: Laser Doppler flowmetry; N^G-monomethyl-L-arginine; N_ω-nitro-L-arginine; L-arginine

Introduction

The endothelium releases several vasoactive agents which interact to regulate the underlying vascular smooth muscle tone, thus contributing to the control of blood pressure. One such agent is the labile humoral factor, endothelium-derived relaxing factor (EDRF), first described by Furchgott & Zawadzki (1980). In many vascular beds *in vitro*, including the rabbit isolated ear (Griffith *et al.*, 1988), rat lung (Cherry & Gillis, 1987), rat mesentery (Byfield *et al.*, 1986) and rat kidney (Bhardwaj & Moore 1988; Burton *et al.*, 1988), a role for EDRF in the control of arteriolar resistance has been proposed. However, relatively little is known about the role of EDRF in the control of renal vascular resistance *in vivo*.

EDRF, which was recently identified as nitric oxide (NO) (Palmer *et al.*, 1987) is formed from the terminal guanidino nitrogen atom(s) of L-arginine (L-Arg) (Palmer *et al.*, 1988a,b). N^G-monomethyl-L-arginine (MeArg), an inhibitor of arginine metabolism to nitrogen oxides (Hibbs *et al.*, 1987) prevents the release of NO from vascular endothelial cells (Palmer *et al.*, 1988a). In the anaesthetized rabbit (Rees *et al.*, 1989), rat (Whittle *et al.*, 1989) and guinea-pig (Aisaka *et al.*, 1989a), and in the conscious rat (Gardiner *et al.*, 1990a), MeArg causes a sustained rise in blood pressure suggesting that resting blood pressure is modulated by a continuous basal release of NO. Moreover, MeArg infusion into the forearm of human volunteers reduces blood flow by 40% and attenuates the dilator responses to acetylcholine but not to glyceryl trinitrate (Vallance *et al.*, 1989). In the rabbit aorta and rat mesentery *in vitro*, N_ω-nitro-L-arginine methyl ester (NO_2Arg) is a more potent inhibitor of endothelium-dependent vasodilatation than MeArg (Moore *et al.*, 1990). Although there is still debate as to whether EDRF is NO or a closely related substance such as a nitrosothiol (Myers *et al.*, 1990), the inhibition of EDRF formation by L-arginine analogues, such as MeArg or NO_2Arg, lends support to the identity of EDRF as NO. We shall refer to EDRF as NO in this paper.

NO causes vasodilatation by stimulating the formation of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) and a role of NO in the regulation of renal haemodynamics *in vivo* has recently been demonstrated, using excretion of urinary cyclic GMP as a biological marker of NO activity (Tolins *et al.*, 1990).

In the present study we have employed MeArg and NO_2Arg to elucidate the role of basal and stimulated release of NO in the haemodynamics of the renal cortex, using laser Doppler flowmetry for the continuous measurement of renal cortical blood flow. All animals were treated with indomethacin to eliminate any possible involvement of prostacyclin or other prostaglandins.

Methods

Surgical procedure

Male Wistar rats (195–462 g; Glaxo Laboratories, Greenford, Middx.), were anaesthetized with Trapanal (120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration and body temperature was maintained by 37°C by means of a rectal probe connected to a homeothermic blanket (BioScience, Sheerness, Kent). The right carotid artery was cannulated and connected to a Transamerica type 4-422-0001 pressure transducer for the measurement of systemic blood pressure and heart rate on a Grass 7D polygraph (Grass Instruments, Quincy, Mass., U.S.A.). The left jugular vein and right femoral vein were cannulated for the administration of drugs and the left femoral vein for the administration of saline (3 ml h^{-1}) to compensate for any fluid loss. All drugs were infused with a syringe pump (Perfuser VI, Braun, Melsunge, F.R.G.). The left

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kidney was exposed via a mid-line laparotomy to facilitate renal cortical blood flow measurements.

Laser Doppler flowmetry

Renal cortical blood flow was measured with a standard laser Doppler flow probe (Periflux PF3, Perimed, Stockholm, Sweden), the principle of which has been previously described (Walder *et al.*, 1990). The probe, which was gently placed on the ventral surface of the capsule of the left kidney, was fixed to a mobile arm pivoted about a fixed axis so that it moved as the kidney moved, thus reducing respiratory artefacts. Blood flow was continuously recorded on a microcomputer and expressed in arbitrary perfusion units. Drug-induced changes in blood flow were measured as percent change in perfusion units (PU) or as the area under the curve (AUC) (calculated from recorded data with the computer software package Perisoft, Perimed, Sweden) and expressed as units. The use of the laser Doppler technique for measurement of blood flow was first described by Riva *et al.* (1972) and has been validated as a method for study of renal blood flow (Stern *et al.*, 1977; Smits *et al.*, 1986). In particular relative changes in RCBF measured by laser Doppler flowmetry are closely correlated to absolute changes in total renal blood flow (as measured by electromagnetic flowprobes) or absolute changes in RCBF measured with radioactive microspheres (Smits *et al.*, 1986). In addition, removal of the capsule of the kidney is not a prerequisite to obtain reliable measurements of RCBF by laser Doppler flowmetry (Stern *et al.*, 1977; Smits *et al.*, 1986).

Experimental design

Effects of NO synthesis inhibition After surgery, all animals were allowed to stabilize for 10 min before receiving indomethacin ($5\,mg\,kg^{-1}$, i.v.). Twenty min later infusions were started of either N^G-monomethyl-L-arginine acetate (MeArg; $1\,mg\,kg^{-1}\,min^{-1}$, i.v., $n = 6$; or $3\,mg\,kg^{-1}\,min^{-1}$, i.v., $n = 5$) or N_ω-nitro-L-arginine methyl ester hydrochloride (NO_2Arg; $30\,\mu g\,kg^{-1}\,min^{-1}$, i.v.; $n = 4$; or $300\,\mu g\,kg^{-1}\,min^{-1}$, i.v., $n = 5$) at $0.04\,ml\,min^{-1}$ and continued throughout the experiment. These doses significantly elevate blood pressure in the anaesthetized rat *in vivo*, presumably via inhibition of NO formation (Walder *et al.*, 1990; Whittle *et al.*, 1990; Hecker *et al.*, 1990). After 20 min, the infusion of the NO synthesis inhibitors was continued but with a co-infusion of an excess of L-Arg (3 fold excess of L-Arg at either 3 or $10\,mg\,kg^{-1}\,min^{-1}$, i.v. for MeArg and a 100 fold excess of L-Arg at 3 or $30\,mg\,kg^{-1}\,min^{-1}$, i.v. for NO_2Arg). In some control experiments, L-Arg or D-Arg (3, 10 or $30\,mg\,kg^{-1}\,min^{-1}$, i.v.; $n = 3$–9) were infused for 10 min.

Effects of NO synthesis inhibition on stimulated release of NO Twenty min after indomethacin treatment, a submaximal dose of acetylcholine (ACh; $10\,\mu g\,kg^{-1}\,min^{-1}$, i.v.) was infused at a rate of $0.1\,ml\,min^{-1}$ for 3 min. This dose of ACh was repeated at 30 min and 60 min after the first dose. Ten min prior to the second ACh infusion, animals received either MeArg ($3\,mg\,kg^{-1}\,min^{-1}$, i.v.; $n = 5$) or NO_2Arg ($300\,\mu g\,kg^{-1}\,min^{-1}$, i.v.; $n = 5$), with the infusion continued throughout the experiment at a rate of $0.04\,ml\,min^{-1}$. Twenty min before the last ACh infusion, L-Arg was co-infused with either MeArg or NO_2Arg at a dose of $10\,mg\,kg^{-1}\,min^{-1}$ or $30\,mg\,kg^{-1}\,min^{-1}$, respectively. In some experiments, the endothelium-independent renal vasodilator, dopamine ($10\,\mu g\,kg^{-1}\,min^{-1}$, i.v.; $n = 3$), was infused at a rate of $0.1\,ml\,min^{-1}$, instead of ACh. In these experiments, the effect of only one of the inhibitors, namely NO_2Arg, was studied. In order to control for the general vasoconstriction and rise in blood pressure induced by MeArg or NO_2Arg, one group of animals received a bolus dose of endothelin-1 (ET-1; $1\,nmol\,kg^{-1}$, i.v.; $n = 4$) which preceded the second dose of ACh by 10 min. In this group, the animals received neither L-Arg nor a third infusion of ACh. In one group of experiments, the second infusion of ACh or dopamine was preceded by a 10 min infusion of L-Arg ($30\,mg\,kg^{-1}\,min^{-1}$, i.v.; $n = 4$–6).

Materials

Indomethacin was prepared as a $5\,mg\,kg^{-1}$ solution in 5% w/v sodium bicarbonate. Sodium-thiopentone (Trapanal) was obtained from Byk Gulden (Konstanz, F.R.G.). ACh, L-Arg, NO_2Arg, dopamine and indomethacin were obtained from Sigma (Poole, Dorset), MeArg was from Calbiochem (La Jolla, C.A., U.S.A.) and endothelin was from the Peptide Research Institute (Osaka, Japan).

Statistical comparisons

All values in the figures and text are expressed as mean ± s.e.mean of n observations. Statistical comparisons of differences within the same animal (i.e. before and after treatment) were made by Student's t test for paired determinations; comparisons of differences between groups of animals were made by Student's t test for unpaired determinations. A P value of <0.05 was considered significant.

Results

Mean resting values for mean arterial blood pressure (MAP) were 117 ± 3 mmHg ($n = 27$), for heart rate were 401 ± 6 beats min^{-1} ($n = 27$) and for renal cortical blood flow (RCBF) were 212 ± 15 PU ($n = 27$). These values were unaffected by pretreatment with indomethacin (122 ± 3 mmHg, $n = 27$, $P > 0.05$; 391 ± 7 beats min^{-1}, $n = 27$, $P > 0.05$; and 199 ± 16 PU, $n = 27$, $P > 0.05$).

Effects of NO synthesis inhibition

Infusion of MeArg ($1\,mg\,kg^{-1}\,min^{-1}$, i.v., $n = 6$; or $3\,mg\,kg^{-1}\,min^{-1}$, i.v., $n = 5$) produced dose-dependent increases in MAP of 33 ± 8 mmHg and 60 ± 4 mmHg, respectively. These effects were reversed by a 3 fold excess of L-Arg (3 or $10\,mg\,kg^{-1}\,min^{-1}$, i.v.) (Figure 1a). The increases in MAP brought about by 1 or $3\,mg\,kg^{-1}$ of MeArg were accompanied by concomitant decreases in RCBF of 29 ± 7% (45 ± 11 PU) and 35 ± 5% (56 ± 8 PU) which were not significantly different from each other. When calculated as change in conductance, MeArg (1 or $3\,mg\,kg^{-1}\,min^{-1}$) decreased renal vascular conductance by 43 ± 7% and 56 ± 4%, respectively. These values were also not significantly different from each other, suggesting that the low dose of MeArg ($1\,mg\,kg^{-1}\,min^{-1}$) was sufficient to achieve a maximal effect on RCBF. These reductions in RCBF were reversed by a 3 fold excess of L-Arg. An infusion of $10\,mg\,kg^{-1}\,min^{-1}$ of L-Arg not only reversed the effects of MeArg, but resulted in a 49 ± 10% increase in RCBF (Figure 2a). However, L-Arg had some vasodilator role of its own. Infusions of L-Arg (3, 10 and $30\,mg\,kg^{-1}\,min^{-1}$; $n = 3$–5) for 10 min significantly increased RCBF by 28 ± 3 PU, 47 ± 7 PU, 59 ± 3 PU, respectively ($P < 0.05$). The same doses of D-Arg did not significantly increase RCBF (18 ± 1 PU, 14 ± 2 PU, 29 ± 8 PU, respectively) when compared to saline (9 ± 5 PU) ($n = 3$–9).

When compared to MeArg ($1\,mg\,kg^{-1}\,min^{-1}$, i.v.), NO_2Arg ($300\,\mu g\,kg^{-1}\,min^{-1}$, i.v.; $n = 5$) produced a similar increase in MAP of 34 ± 7 mmHg indicating that NO_2Arg is approximately 3 fold more potent than MeArg (Figure 1b). An infusion of a 100 fold excess of L-Arg ($30\,mg\,kg^{-1}\,min^{-1}$), however, failed to reverse the pressor effect of NO_2Arg. This dose of NO_2Arg had a much greater effect on RCBF than MeArg ($1\,mg\,kg^{-1}\,min^{-1}$), significantly reducing it by 53 ± 4% (95 ± 16 PU) (Figure 2b). This higher potency of NO_2Arg on RCBF was also reflected by a significantly greater fall in renal vascular conductance (64 ± 2%). More importantly, a lower dose of NO_2Arg ($30\,\mu g\,kg^{-1}\,min^{-1}$; $n = 4$), without having any effect on MAP, significantly reduced RCBF by 21 ± 4% (56 ± 9 PU), (Figure 1b, 2b and Figure 3) and renal vascular

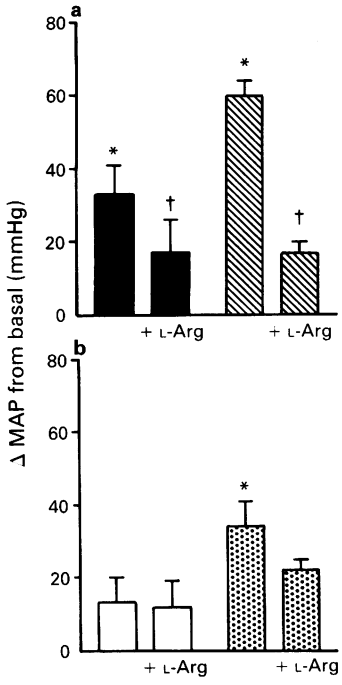

Figure 1 N^G-monomethyl-L-arginine (MeArg) or N_ω-nitro- L-arginine methyl ester (NO_2Arg) increase mean arterial blood pressure (MAP). (a) MeArg ($1\,mg\,kg^{-1}\,min^{-1}$; solid column; or $3\,mg\,kg^{-1}\,min^{-1}$; hatched column) increased MAP, whilst a 3 fold excess of L-arginine (L-Arg, $3\,mg\,kg^{-1}\,min^{-1}$; solid column or $10\,mg\,kg^{-1}\,min^{-1}$; hatched column, respectively) reversed this effect. (b) NO_2Arg ($30\,\mu g\,kg^{-1}\,min^{-1}$; open column) had no significant effect on MAP, whilst NO_2Arg ($300\,\mu g\,kg^{-1}\,min^{-1}$; stippled column) significantly increased MAP. A 100 fold excess of L-Arg ($3\,mg\,kg^{-1}\,min^{-1}$; open column or $30\,mg\,kg^{-1}\,min^{-1}$; stippled column, respectively) produced no significant reversal. Columns are means with s.e.mean shown by vertical bars. * $P < 0.05$ when compared to basal; † $P < 0.05$ when compared to MeArg.

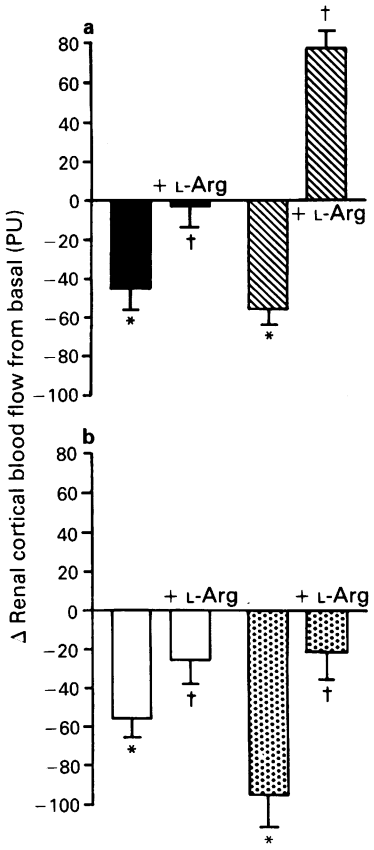

Figure 2 N^G-monomethyl-L-arginine (MeArg) or N_ω-nitro- L-arginine methyl ester (NO_2Arg) decrease renal cortical blood flow (RCBF) in an L-arginine (L-Arg) reversible manner. (a) MeArg ($1\,mg\,kg^{-1}\,min^{-1}$; solid column; or $3\,mg\,kg^{-1}\,min^{-1}$; hatched column) decreased RCBF, whilst a co-infusion of a 3 fold excess of L-Arg ($3\,mg\,kg^{-1}\,min^{-1}$; solid column) abolished this effect or ($10\,mg\,kg^{-1}\,min^{-1}$; hatched column) resulted in an increase in RCBF. (b) NO_2Arg ($30\,\mu g\,kg^{-1}\,min^{-1}$; open column or $300\,\mu g\,kg^{-1}\,min^{-1}$; stippled column) produced a more striking decrease in RCBF which was reversed by a 100 fold excess of L-Arg ($3\,mg\,kg^{-1}\,min^{-1}$; open column or $30\,mg\,kg^{-1}\,min^{-1}$; stippled, respectively). Columns are means with s.e.mean shown by vertical bars. * $P < 0.05$ when compared to basal; † $P < 0.05$ when compared to MeArg or NO_2Arg.

conductance by 28 ± 8%. The decreases in RCBF achieved with NO_2Arg were significantly reversed by a 100 fold excess of L-Arg (Figure 2b).

Effect of NO synthesis inhibition on stimulated release of NO

Figure 4 illustrates the fall in blood pressure in response to 3 min infusions of ACh in the presence and absence of MeArg. Infusion of ACh ($10\,\mu g\,kg^{-1}\,min^{-1}$, i.v.; $n = 14$) for 3 min resulted in a fall of MAP, which at maximum was 46 ± 2 mmHg and was 35 ± 3 mmHg after 3 min of infusion. This fall in MAP was not associated with any significant change in heart rate ($P > 0.05$; data not shown). Although MeArg ($3\,mg\,kg^{-1}\,min^{-1}$, i.v.) had no effect on the maximum fall in MAP induced by ACh, it did, however, reduce the duration of the response so that a significant inhibition was seen after 3 min of infusion (Figures 4 and 5a). This inhibition was partially, but significantly, reversed by a 20 min infusion of L-Arg. Similarly, NO_2Arg ($300\,\mu g\,kg^{-1}\,min^{-1}$, i.v.) did not affect the maximum fall in MAP produced by ACh, but significantly reduced the duration of the response. This effect was significantly reversed by a co-infusion of a 100 fold excess of L-Arg (Figure 5b).

The depressor response to ACh was associated with a hyperaemia of the renal cortex of 44 ± 3% amounting to an AUC of 19616 ± 2280 units ($n = 14$). Infusion of MeArg ($3\,mg\,kg^{-1}\,min^{-1}$, i.v.) significantly attenuated the ACh-induced hyperaemia by 86% (25711 ± 4300 to 4559 ± 3000 units; $n = 5$), whilst co-infusion of L-Arg ($10\,mg\,kg^{-1}\,min^{-1}$, i.v.) significantly reversed this inhibitory effect (25147 ± 2489 units) (Figure 6a). A representative trace of the effects of MeArg ($3\,mg\,kg^{-1}\,min^{-1}$, i.v.) on basal and ACh-stimulated release of NO is shown in Figure 7. A 3 fold lower concentration of NO_2Arg ($300\,\mu g\,kg^{-1}\,min^{-1}$, i.v.; $n = 5$) produced a similar 96% inhibition of the ACh-induced increase in renal blood flow (15667 ± 3064 to 837 ± 513 units), whilst a 100 fold excess of L-Arg ($30\,mg\,kg^{-1}\,min^{-1}$, i.v.) resulted in a partial, but significant reversal (8591 ± 2515 units) (Figure 6b).

Ten min after injection of ET-1 ($1\,nmol\,kg^{-1}$, i.v.) the MAP was increased by 32 ± 5 mmHg. The associated fall in RCBF (43 ± 7 PU) was not significantly different from that produced

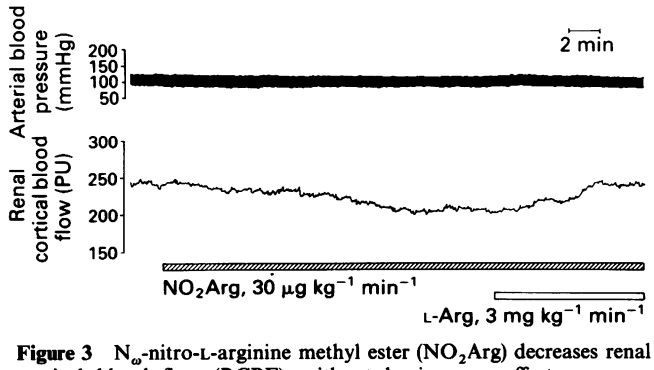

Figure 3 N_ω-nitro-L-arginine methyl ester (NO_2Arg) decreases renal cortical blood flow (RCBF), without having any effect on mean arterial blood pressure (MAP). NO_2Arg ($30\,\mu g\,kg^{-1}\,min^{-1}$; hatched bar) reduced RCBF, by approximately 20%, without any significant effect on MAP. A co-infusion of L-arginine (L-Arg, $3\,mg\,kg^{-1}\,min^{-1}$; open bar) reversed this effect.

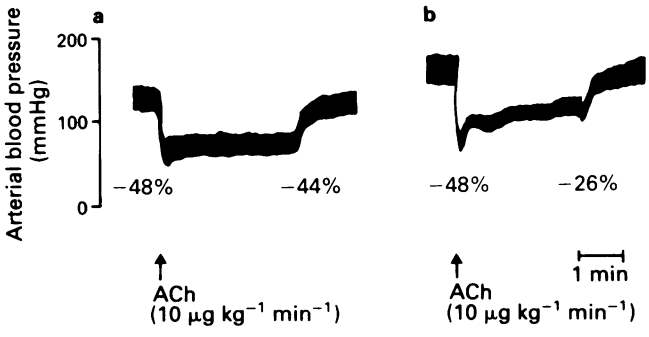

Figure 4 The figure shows two original traces illustrating the reductions in phasic arterial blood pressure in the anaesthetized rat in response to 3 min infusions of acetylcholine (ACh) (a) in the absence and (b) in the presence of N^G-monomethyl-L-arginine (MeArg). Infusion of ACh ($10\,\mu g\,kg^{-1}\,min^{-1}$, i.v. for 3 min) produced a maximum fall in mean arterial blood pressure (MAP) of 48%, which after 3 min remained at −44% (a). Although MeArg ($3\,mg\,kg^{-1}\,min^{-1}$, i.v.) did not affect the maximum fall in MAP induced by ACh (−48%), MeArg caused a partial inhibition of the ACh-induced reduction in blood pressure at 3 min (−26%; b).

by MeArg. Likewise, the increase in MAP brought about by ET-1 was not significantly different from that induced by NO_2Arg, even though the fall in RCBF was greater. Thus, the effects of ET-1 on either MAP or RCBF can be considered as a reasonable control for the changes brought about by both MeArg and NO_2Arg. This injection of ET-1 ($1\,nmol\,kg^{-1}$) did not significantly affect the depressor response to ACh at 3 min (Figure 5c). The renal cortical hyperaemia induced by ACh was inhibited to some extent by a bolus dose of ET-1 ($1\,nmol\,kg^{-1}$; $n = 4$) (16933 ± 2828 to 12019 ± 2560 units). However, this was significantly different from the inhibition produced by MeArg and NO_2Arg ($P < 0.05$; Figure 6c). A 3 min infusion of dopamine ($10\,\mu g\,kg^{-1}\,min^{-1}$) had no signifi-

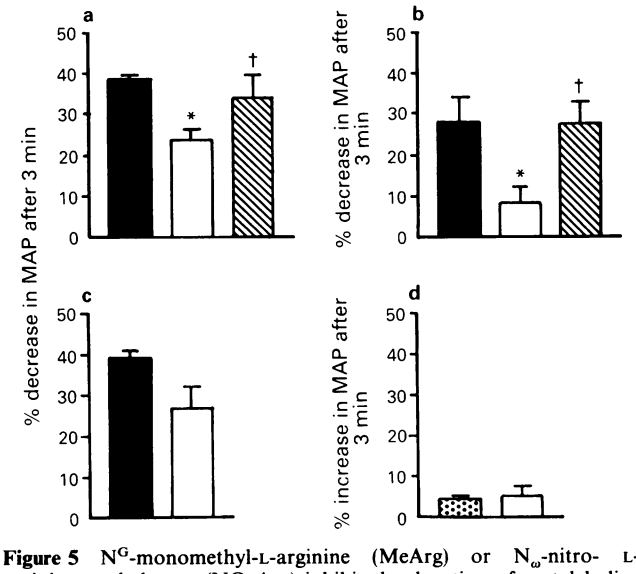

Figure 5 N^G-monomethyl-L-arginine (MeArg) or N_ω-nitro- L-arginine methyl ester (NO_2Arg) inhibit the duration of acetylcholine (ACh)-induced decreases in mean arterial blood pressure (MAP). (a) At the 3 min time point after commencing infusion, ACh ($10\,\mu g\,kg^{-1}\,min^{-1}$) produced a decrease in MAP which was attenuated by MeArg ($3\,mg\,kg^{-1}\,min^{-1}$; open column) and reversed by L-arginine (L-Arg, $10\,mg\,kg^{-1}\,min^{-1}$; hatched column). (b) The ACh-induced decrease in MAP is similarly attenuated by NO_2Arg ($300\,\mu g\,kg^{-1}\,min^{-1}$; open column) and reversed by L-Arg ($30\,mg\,kg^{-1}\,min^{-1}$; hatched column). (c) The fall in MAP produced by ACh was not significantly affected by endothelin-1 (ET-1; $1\,nmol\,kg^{-1}$). (d) Dopamine ($10\,\mu g\,kg^{-1}\,min^{-1}$; for 3 min) had no significant effect on MAP either in the presence (open column) or absence (stippled column) of NO_2Arg ($300\,\mu g\,kg^{-1}\,min^{-1}$). Data are expressed as means ± s.e.mean. * $P < 0.05$ when compared to control; † $P < 0.05$ when compared to MeArg or NO_2Arg.

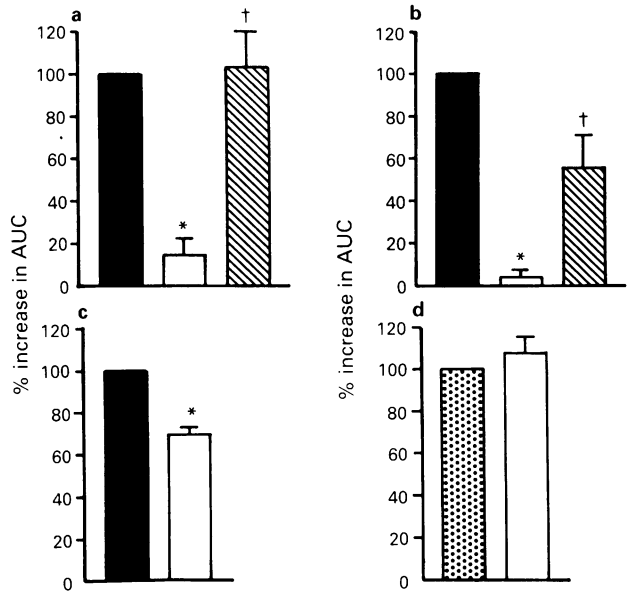

Figure 6 N^G-monomethyl-L-arginine (MeArg) or N_ω-nitro-L- arginine methyl ester (NO_2Arg) inhibit endothelium-dependent (acetylcholine, ACh) but not endothelium-independent increases in cortical blood flow (RCBF). RCBF responses were measured as the area under the curve (AUC). (a) ACh ($10\,\mu g\,kg^{-1}\,min^{-1}$; for 3 min) results in an increase in RCBF which is attenuated by MeArg ($3\,mg\,kg^{-1}\,min^{-1}$; open column) and reversed by L-arginine (L-Arg, $10\,mg\,kg^{-1}\,min^{-1}$; hatched column). (b) The ACh-induced increase in RCBF is similarly attenuated by NO_2Arg ($300\,\mu g\,kg^{-1}\,min^{-1}$; open column) and reversed by L-Arg ($30\,mg\,kg^{-1}\,min^{-1}$; hatched column). (c) The increase in RCBF produced by ACh was inhibited by endothelin-1 ($1\,nmol\,kg^{-1}$). However, this inhibition was significantly weaker than the inhibition seen with MeArg or NO_2Arg. (d) The increase in RCBF produced by dopamine ($10\,\mu g\,kg^{-1}\,min^{-1}$; for 3 min) was not significantly affected by NO_2Arg ($300\,\mu g\,kg^{-1}\,min^{-1}$). Data are expressed as means ± s.e.mean. * $P < 0.05$ when compared to control; † $P < 0.05$ when compared to MeArg or NO_2Arg.

cant effect on MAP, either alone or in the presence of NO_2Arg ($300\,\mu g\,kg^{-1}\,min^{-1}$) (Figure 5). However, an infusion of this dose of dopamine for 3 min resulted in an increase in renal cortical blood flow of 21 ± 1% amounting to an AUC of 10468 ± 880 units ($n = 3$), which was unaffected by NO_2Arg ($300\,\mu g\,kg^{-1}\,min^{-1}$) (10785 ± 815 units) (Figure 6d).

Infusions of high doses of L-Arg ($30\,mg\,kg^{-1}\,min^{-1}$, i.v.) did not affect the increase in RCBF induced by dopamine (7465 ± 1115 to 6442 ± 277 units, $n = 4$; $P > 0.05$). Although L-Arg potentiated the increase in RCBF induced by ACh in 3 out of 6 experiments, this effect failed to attain statistical significance (21951 ± 1851 to 38509 ± 7583 units, $n = 6$; $P > 0.05$).

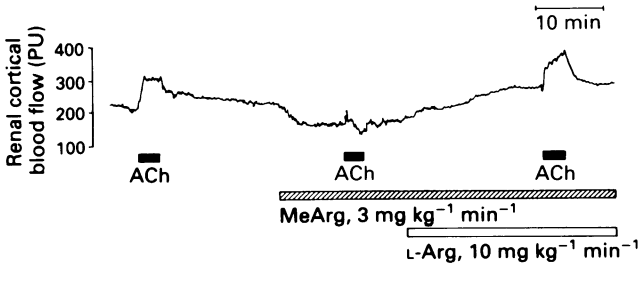

Figure 7 N^G-monomethyl-L-arginine (MeArg) inhibits the increase in renal cortical blood flow (RCBF) produced by acetylcholine (ACh), in an L-arginine (L-Arg) reversible manner. MeArg ($3\,mg\,kg^{-1}\,min^{-1}$; hatched bar) reduces the basal, and subsequently inhibits the ACh-stimulated ($10\,\mu g\,kg^{-1}\,min^{-1}$; for 3 min) increase in, RCBF whilst a co-infusion of L-Arg ($10\,mg\,kg^{-1}\,min^{-1}$; open bar) reverses these inhibitory effects of MeArg.

Discussion

After the discovery that aspirin-like drugs inhibit prostaglandin biosynthesis (Vane, 1971), many workers studied their effects on renal blood flow, in order to define any prostaglandin contribution (see Patrono & Dunn, 1987). There is now general agreement that prostaglandin formation does not contribute to normal renal blood flow but comes into play when the kidney is compromised, such as in circulatory shock or renal failure (Clive & Stoff, 1984; Carmichael & Shankel, 1985; Morrison, 1986; Patrono & Dunn, 1987; Oates *et al.*, 1988). Our results, in which indomethacin had no effect on renal cortical blood flow, fit into this general concept.

From our results, the arginine-NO system clearly participates in the physiological maintenance of renal cortical blood flow. The present study demonstrates that the NO synthesis inhibitor, MeArg, reduced renal cortical blood flow (RCBF) in an L-Arg reversible manner. Moreover, NO_2Arg produced a dose-dependent reduction in RCBF, which was partly reversed by L-Arg. In addition, both compounds inhibited endothelium-dependent (ACh), but not endothelium-independent (dopamine) increases in RCBF in an L-Arg reversible manner. Most importantly, a low dose of NO_2Arg, while having no pressor effect, considerably reduced RCBF.

Stimulated release of NO

ACh causes renal vasodilatation in isolated perfused kidneys (Burton *et al.*, 1987; Bhardwaj & Moore, 1988; Burton *et al.*, 1989) and *in vivo* (Baer *et al.*, 1970; Baylis *et al.*, 1976; Thomas *et al.*, 1988; Walder *et al.*, 1989). In the isolated perfused kidney of the rat, the vasodilator response to ACh and the concomitant increase in urinary cyclic GMP are inhibited by haemoglobin or gossypol (Burton *et al.*, 1990). More importantly, in the anaesthetized rat *in vivo*, ACh induces a dose-dependent increase in urinary cyclic GMP levels, the rates of increase of cyclic GMP being correlated with the fall in systemic blood pressure (Tolins *et al.*, 1990). The increase in urinary cyclic GMP levels, the hypotensive effect and renal haemodynamic effects of ACh are all inhibited by MeArg, suggesting that these effects of ACh are due to stimulation of NO formation, which then activates guanylate cyclase (Tolins *et al.*, 1990). This observation is supported by the work of Lahera *et al.* (1990) who also demonstrated that the ACh-induced increase in renal blood flow and diuresis in the dog are inhibited by MeArg.

In the current study, the ACh-induced systemic hypotension and increase in RCBF were inhibited by MeArg or NO_2Arg in an L-Arg reversible manner. Although ET-1, given to produce a similar pressor effect and fall in RCBF to that of MeArg or NO_2Arg, produced a slight inhibition of the ACh-induced increase in RCBF, this inhibition of the endothelium-dependent vasodilatation was significantly weaker than the one produced by MeArg or NO_2Arg. This suggests that a vasoconstrictor agent, such as ET-1, can exert a weak functional antagonism of endothelium-dependent vasodilator responses. However, the main mechanism of action of the inhibition of endothelium-dependent vasodilator responses produced by MeArg or NO_2Arg is most likely via specific inhibition of NO synthesis, for it was largely attenuated by L-Arg. Our finding here that the increase in RCBF induced by the endothelium-independent renal vasodilator dopamine (which acts via direct activation of cyclic AMP) was unaffected by NO_2Arg supports this conclusion. In addition, infusion of the highest dose of L-Arg did not significantly affect the ACh- or dopamine-induced increases in RCBF. This suggests that the L-Arg reversal of the inhibitory effects of MeArg and NO_2Arg on ACh-stimulated increases in RCBF are not due to direct effect of L-Arg.

Interestingly, the maximum systemic hypotensive effect produced by a 3 min infusion of ACh was unaffected by MeArg or NO_2Arg, but the duration of the response was shortened. Similarly, MeArg does not affect the maximum hypotensive effect produced by bolus injections of ACh in the anaesthetized guinea-pig, but significantly shortens the response to the endothelium-dependent vasodilator (Aisaka *et al.*, 1989b). In contrast, Rees *et al.* (1989) clearly demonstrated that the maximum hypotensive effects of ACh were inhibited by MeArg in the anaesthetized rabbit. In addition, Whittle *et al.* (1989) have also shown that MeArg inhibits the maximum hypotensive effects of ACh, ET-1, substance P and bradykinin in the anaesthetized rat *in vivo*. Although in our study, MeArg or NO_2Arg had only a weak inhibitor effect on the maximum systemic vasodilator responses brought about by ACh, it is clear that the ACh-induced increase in RCBF was largely attenuated by both inhibitors of NO formation. This finding indicates that the systemic hypotensive effect of ACh *in vivo* is only partly mediated by an ACh-stimulated release of NO. In support of this concept, NO_2Arg ($10\,mg\,kg^{-1}$, i.v.) did not affect the fall in blood pressure produced by bolus injections of ACh in conscious rats (Gardiner *et al.*, 1990c).

When compared to MeArg, at a tenth of the dose, NO_2Arg produced a similar inhibition of the duration of the hypotensive effect and inhibition of the increase in RCBF brought about by ACh. In addition, the effects of MeArg were reversed by a 3 fold excess of L-Arg, while the effects of NO_2Arg were only partially reversed by a 100 fold excess of L-Arg. These results support the current hypothesis that NO_2Arg is a more potent inhibitor of NO synthesis (Moore *et al.*, 1990; Ishii *et al.*, 1990; Hecker *et al.*, 1990; Gardiner *et al.*, 1990b). The recent finding that bovine endothelial cells in culture metabolize MeArg, but not NO_2Arg, to L-citrulline and subsequently to L-Arg may explain the differences in potency between MeArg and NO_2Arg observed *in vitro* and *in vivo* (Hecker *et al.*, 1990).

Basal NO release

The substantial renal cortical vasoconstriction induced by the inhibition of arginine metabolism clearly indicates that the 'basal' release of NO is important for the regulation and maintenance of cortical blood flow in anaesthetized rats. Our findings here that MeArg or NO_2Arg increase blood pressure, in an L-Arg reversible manner, are consistent with the current understanding that 'basal' NO release plays a major role in the modulation of systemic blood pressure *in vivo* (Rees *et al.*, 1989; Whittle *et al.*, 1989; Aisaka *et al.*, 1989; Vallance *et al.*, 1989; Hecker *et al.*, 1990; Gardiner *et al.*, 1990a).

Using laser Doppler flowmetry, we clearly demonstrate that the increases in MAP produced by MeArg or NO_2Arg were accompanied by L-Arg reversible falls in RCBF (ranging from 21 to 53%) demonstrating a local role for NO in the control of RCBF. Similarly, MeArg (Gardiner *et al.*, 1990a) and NO_2Arg (Gardiner *et al.*, 1990b) increase blood pressure and reduce total renal blood flow in the conscious rat chronically instrumented with pulsed Doppler flow probes. In contrast, Lahera *et al.* (1990), using an electromagnetic flow probe to measure renal arterial blood flow showed that intrarenal infusion of low concentrations of MeArg ($97\,\mu g\,kg^{-1}\,min^{-1}$) had no effect on basal renal blood flow. Similarly, when renal plasma flow is measured by *p*-aminohippurate (PAH) clearance, an intravenous bolus injection of MeArg ($15\,mg\,kg^{-1}$) failed to affect basal renal plasma flow (Tolins *et al.*, 1990). However, these studies (Lahera *et al.*, 1990; Tolins *et al.*, 1990) did not separate cortical from medullary flow which may account for the differences in our data on the effects of MeArg on basal RCBF.

In the present study we demonstrated that infusions of L-Arg, but not D-Arg, produced an increase in RCBF. This finding is not entirely surprising, for it is well documented that the oral intake of protein or the infusion of amino acids, in particular L-Arg, induces an increase in renal plasma flow and glomerular filtration rate in various species including man, dogs and rats (Bosch *et al.*, 1983; Palmore, 1983; Meyer & Brenner, 1983; Hostetter, 1986). Interestingly, Bhardwaj & Moore

(1989) showed that the vasodilator activity of L-Arg in the isolated perfused kidney of the rat can be abolished by removal of the endothelium. More recently, Tolins & Raij (1990) demonstrated that the increases in glomerular filtration rate and renal plasma flow, induced by amino acid infusion, were completely inhibited by intrarenal MeArg. Thus, on the one hand, NO may mediate some of the renal haemodynamic responses to L-Arg infusions. On the other hand, a direct vasodilator effect of L-Arg may contribute to some of its renal effects. At present it is not possible to differentiate between these two possibilities.

In addition, we have demonstrated that RCBF is much more sensitive than MAP to the effects of MeArg and NO_2Arg and to reversal by L-Arg. More importantly, a low dose of NO_2Arg, while having no systemic pressor effect, caused a 21% reduction in RCBF. This finding clearly indicates that the renal cortical vasculature has a relatively high basal release of NO, which substantially contributes to the regulation and maintenance of RCBF. Furthermore, our data here and our previous finding that MeArg and NO_2Arg did not influence basal blood flow in the rat gastric vasculature (Walder *et al.*, 1990), indicate that the pressor responses to MeArg and NO_2Arg in the anaesthetized rat *in vivo* are unlikely to be due to a uniform vasoconstriction in all vascular beds. Gardiner *et al.* (1990a,b), using pulsed Doppler probes, also showed differential haemodynamic effects of MeArg and NO_2Arg respectively, on renal, mesenteric and hindquarter vascular beds in the conscious rat. However, in those studies, the dose-dependent and long-lasting vasoconstrictions of both NO-inhibitors were more pronounced in the mesenteric than in the renal or hindquarter vascular beds.

It is still not clear whether the pressor responses to inhibitors of NO formation, such as MeArg, are entirely due to inhibition of 'basal' NO formation. Here, we demonstrate that MeArg and NO_2Arg substantially reduce RCBF at very low doses. Such a reduction in RCBF may be associated with an activation of the renin/angiotensin system. Thus, another potential underlying mechanism of the pressor effects obtained with MeArg or NO_2Arg *in vivo* may be related to an enhancement of renin/angiotensin II plasma levels secondary to a reduction in renal blood flow. However, recent work suggests that the renin/angiotensin II system is not responsible for the pressor effects of MeArg since the angiotensin II receptor antagonist, Sar-Gly-angiotensin II, did not prevent the rise in blood pressure induced by MeArg (Tolins & Raij, 1990).

In conclusion, our findings suggest that MeArg and NO_2Arg inhibit basal and ACh-stimulated release of NO in the renal cortical vasculature. The discovery that NO_2Arg can substantially reduce RCBF without having an effect on systemic blood pressure indicates that the renal cortical vasculature has a relatively high basal release of NO, which substantially contributes to the regulation and maintenance of RCBF.

This work was supported by a grant from Glaxo Group Research Limited.

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(Received September 10, 1990
Revised December 10, 1990
Accepted December 12, 1990)

Br. J. Pharmacol. (1991), **102**, 974–978

Flosequinan, a vasodilator with a novel mechanism of action

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1 The mechanism of action of flosequinan was investigated in ferret aortic smooth muscle by the simultaneous measurement of aequorin luminescence and isometric force.

2 The control calcium-force curve was obtained by plotting the calibrated aequorin luminescence against the force from potassium-depolarized muscles. Flosequinan relaxed potassium-depolarized muscles by causing parallel changes in $[Ca^{2+}]_i$ and force with no shift in the control $[Ca^{2+}]_i$-force relationship.

3 The $[Ca^{2+}]_i$-force relationship in the presence of a maximally effective concentration of phenylephrine was significantly shifted to the left of that for the control, potassium-depolarized muscle. Flosequinan relaxed the phenylephrine-contracted muscle by causing a large decrease in force with only a minimal decrease in $[Ca^{2+}]_i$, resulting in an apparent rightward shift of the $[Ca^{2+}]_i$-force relationship, toward the control curve.

4 In comparison, sodium nitroprusside caused relaxation of either the potassium- or phenylephrine-induced contraction solely by a decrease in $[Ca^{2+}]_i$ with no shift in either calcium-force relationship.

5 Milrinone caused no significant rightward shift of the calcium-force relationship during phenylephrine- or potassium-induced contractions, but when milrinone was added in the absence of vasoconstrictors, relaxation was obtained with no significant decrease in $[Ca^{2+}]_i$.

6 Flosequinan appears to differ in mechanism of action from both nitroprusside and milrinone. It relaxes depolarization-mediated contractions solely by decreasing $[Ca^{2+}]_i$ but also appears to be capable of reversing the apparent calcium sensitizing action of phenylephrine.

Keywords: Vasodilator; calcium; aequorin; flosequinan; vascular smooth muscle; milrinone; nitroprusside

Introduction

The mechanisms of action of vasodilators are varied. Vasodilators which are capable of increasing guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels, such as sodium nitroprusside, have been shown to decrease cytoplasmic ionized calcium levels ($[Ca^{2+}]_i$) (Morgan & Morgan, 1984b; Karaki *et al.*, 1988). Other vasodilators, typically those which are capable of increasing adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels, such as forskolin, isoprenaline and papaverine, are also capable of significantly uncoupling $[Ca^{2+}]_i$ from force production (Morgan & Morgan, 1984b; Takuwa *et al.*, 1988; Nishimura & van Breemen, 1989). The purpose of the present study was to investigate the mechanism of action of flosequinan, a novel arteriovenous dilator agent which is currently under clinical trial for use in both hypertension and heart failure (Cowley *et al.*, 1987; Kessler & Packer, 1987; Sim *et al.*, 1988; Dupont *et al.*, 1989). Its mechanism of action is currently unknown and what little is known suggests that it may have a novel mechanism of action. It has been reported to increase cyclic GMP levels; however, the magnitude of the increase in cyclic GMP has been stated to be inadequate to explain the magnitude of the relaxation observed (Allcock *et al.*, 1988). Recent work also suggests that it may have non-specific phosphodiesterase activity which could result in elevations of cyclic AMP as well; however, this effect has an IC_{10} of 1.0×10^{-4} M (Frodsham *et al.*, 1989).

The purpose of the present study was to investigate the mechanism of action of flosequinan in an *in vitro* preparation by determining its effects on $[Ca^{2+}]_i$ levels and on $[Ca^{2+}]_i$-force relationships.

Methods

Male ferrets (10 to 12 week old) were killed with an overdose of chloroform. All procedures were approved by the Institutional Committee on Animal Research. The descending thoracic aorta was quickly removed to a physiological saline solution oxygenated with 95% O_2, 5% CO_2. The aorta was cut into strips measuring 5 × 1 mm with the long axis parallel to the circular fibres. The endothelium was removed by gentle rubbing of the intimal surface of all preparations. Experiments were performed at 37°C in a physiological saline solution containing (mM): NaCl 120, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, NaH_2PO_4 1.2 and dextrose 11.5. Solutions containing elevated potassium concentrations were obtained by equimolar replacement of sodium chloride with potassium chloride.

All experiments were performed at 1.4 times the slack length, which we have previously found to be the optimal length for force production in the ferret aorta. In the organ bath experiments 1 h was allowed for equilibration after stretching and warming the muscle to 37°C. Force was recorded with a Gould UC2 transducer and stored on a magnetic FM tape deck.

Aequorin was loaded into ferret aorta strips by a method previously described (Morgan & Morgan, 1982). The method consisted of incubating the muscle in a series of ethylene*bis*-oxyethylenetrilotetraacetic acid- (EGTA) and adenosine triphosphate- (ATP) containing solutions at 2°C for 30–90 min. The first solution (containing 10 mM EGTA) was designed to make the cell membrane hyperpermeable; the second solution contained 0.5 mg ml^{-1} aequorin; and the third solution contained 0.1 mM EGTA and 10 mM $MgCl_2$ and is thought to re-seal the membrane. The muscles were allowed to equilibrate overnight, until basal light was absolutely stable. The light emitted by the aequorin contained in the muscle was detected with an EMI 9635QA photomultiplier tube specially selected for low dark current and is reported in nanoamperes of anode current. Calibration of aequorin light signals was performed in the manner of Allen & Blinks (1979). The muscle cells were lysed with 0.5% Triton X-100 in 50 mM $CaCl_2$ and maximal luminescence (L_{max}) was determined. Light levels (L) during the experiments were expressed as fractional luminescence (L/L_{max}) which was then converted to

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absolute Ca^{2+} concentrations by the use of an *in vitro* calibration curve (Jiang & Morgan, 1987). A value of $[Mg^{2+}]_i$ = 0.5 mM was assumed in these studies. This assumption was based on NMR measurements of $[Mg^{2+}]_i$ in smooth muscle cells (Kushmerick *et al.*, 1986).

Aequorin was obtained from the laboratory of Dr J.R. Blinks, Mayo Clinic (Rochester, MN, U.S.A.). The following drugs were used: flosequinan (Boots Pharmaceuticals, U.K.), sodium nitroprusside (Sigma, U.S.A.), milrinone (Sterling Winthrop, U.S.A.), (−)-phenylephrine HCl (Sigma, U.S.A.) and Triton X-100 (Fisher, U.S.A.).

Results are given as means ± s.e.mean. Statistical significance was accepted where the P value was less than 0.05 by Student's t test. Unless indicated otherwise, the comparisons were unpaired. All quantitative data points were taken from steady state measurements.

Results

Concentration-response relationships of flosequinan in ferret aorta

The effect of a range of concentrations of flosequinan, added cumulatively, in relaxing either 24 mM potassium (K)-induced contractions or phenylephrine (10^{-5} M)-induced contractions is shown in Figure 1. In both cases, unreasonably high concentrations of flosequinan would have been necessary to demonstrate a plateau in the concentration-response curve. It was noticed, however, that flosequinan, at 3×10^{-3} M, was capable of decreasing the phenylephrine-induced force from 22 mN to 6.4 mN, whereas the same concentration of flosequinan could only reduce the K-induced force from 18 mN to 11 mN in this tissue. In both cases, the vasodilator actions began between 10^{-4} and 10^{-6} M. All of the calcium-force studies were performed with 10^{-3} M flosequinan so as to produce a response large enough to be reliably measured. Therapeutic blood concentrations have been estimated to be 10^{-5} M (Packer *et al.*, 1988), but comparisons between the *in vitro* bathing medium concentration and the *in vivo* blood levels may be misleading and may not reflect the concentration at the receptor level.

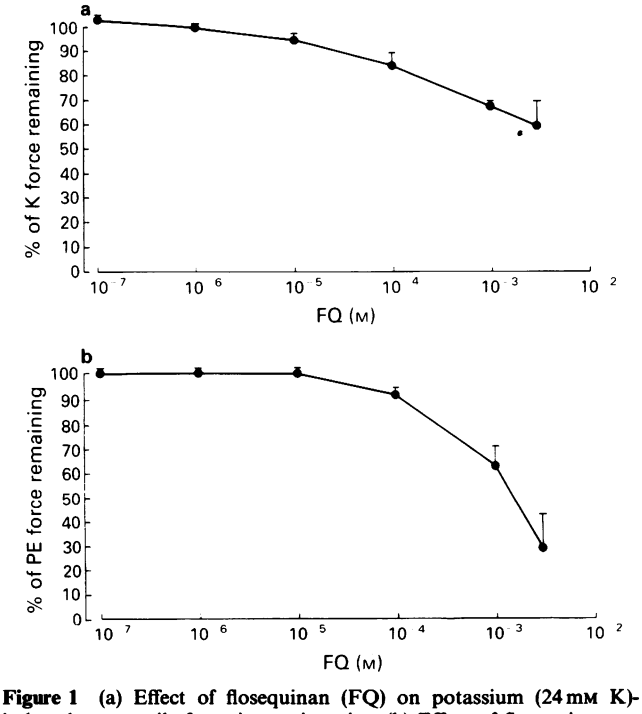

Figure 1 (a) Effect of flosequinan (FQ) on potassium (24 mM K)-induced contractile force in aortic strips. (b) Effect of flosequinan on phenylephrine (PE) (10^{-5} M)-induced contractile force in aortic strips. In both panels, each point shows the mean of 7 strips from 6 ferrets; s.e. shown by vertical bars. The control potassium contraction had a mean force of 18 ± 1.9 mN and the control phenylephrine contraction had a mean force of 22 ± 1.9 mN.

Effect of flosequinan on $[Ca^{2+}]_i$

Resting $[Ca^{2+}]_i$ in the ferret aorta at 37°C was 233 ± 10 nM (mean ± s.e.mean, n = 28 strips, 17 animals). Resting intrinsic tone measured as the active steady state tone generated on warming the muscle from 2°C to 37°C was 16.2 ± 0.9 mN (n = 12 strips, 9 animals). The addition of a physiological saline solution in which 24 mM KCl replaced an equal amount of NaCl (24 mM K), resulted in an increase in $[Ca^{2+}]_i$ to a steady state level of 318 ± 22 nM (n = 14 strips, 14 animals) and an increase in force of 14.1 ± 1.0 mN (n = 17 strips, 14 animals). The addition of 10^{-3} M flosequinan to a 24 mM K-contracted preparation resulted in a decrease in $[Ca^{2+}]_i$ and force (Figure 2). (In a group of 12 experiments (9 animals), where zero force was defined as the level of resting force at body temperature, a mean value of 207 ± 16 nM (n = 9 strips, 9 animals) and −2.5 ± 1.2 mN (n = 12 strips, 9 animals) were obtained for $[Ca^{2+}]_i$ and force, respectively in the presence of 10^{-3} M flosequinan and 24 mM K.)

As previously reported (Morgan & Morgan, 1984a), the addition of the α-adrenoceptor agonist, phenylephrine, to the ferret aorta results in an initial large spike-like increase in $[Ca^{2+}]_i$ during the development of the contraction, but during tone maintenance, $[Ca^{2+}]_i$ returns to near-basal levels (Figure 3). The addition of flosequinan to a phenylephrine-contracted preparation resulted in a decrease in contractile force [from 22.4 ± 1.2 mN (n = 15 strips, 11 animals) to 6.3 ± 2.1 mN (n = 5 strips, 5 animals) where zero force is defined as the level of resting tone at 37°C] and a small, but significant decrease ($P < 0.05$ by a paired t test for n = 5 strips, 5 animals), in $[Ca^{2+}]_i$ levels from 291 ± 18 nM (n = 15 strips, 11 animals) to 240 ± 27 nM (n = 5 strips, 5 animals). It can be seen from Figure 3, that typically, during the action of flosequinan in the presence of phenylephrine, both $[Ca^{2+}]_i$ and force return to approximately baseline levels.

Flosequinan was also found to decrease intrinsic tone. As mentioned above, the ferret aorta generated an average of 16 mN of force on warming from 2°C to 37°C. This tone is stable and persists in the presence of blockade of neurotransmitters with phentolamine and tetrodotoxin. However, it is sensitive to blockade with the relatively selective protein kinase-C inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydro-chloride (H-7) (Ruzycky & Morgan, 1989). The addition of 10^{-3} M flosequinan to the ferret aorta at body temperature in the absence of vasoconstrictors results in a significant drop in tone of 4.4 ± 0.1 mN (n = 5 animals, 5 strips)

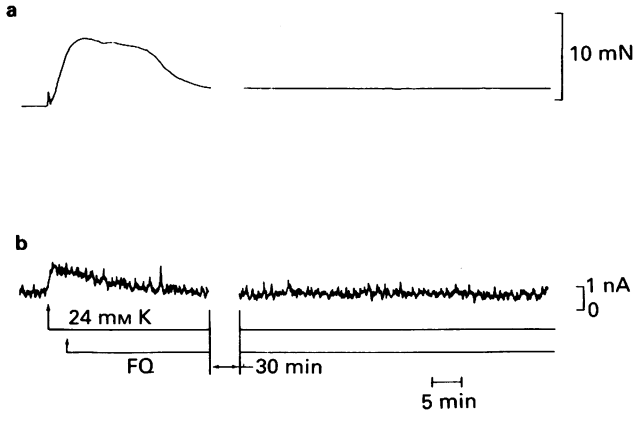

Figure 2 Trace (a): force; (b): luminescence. Effect of flosequinan (FQ) (10^{-3} M) in relaxing a potassium-induced contraction. Zero light is determined by dark current from the photomultiplier tube with the shutter closed.

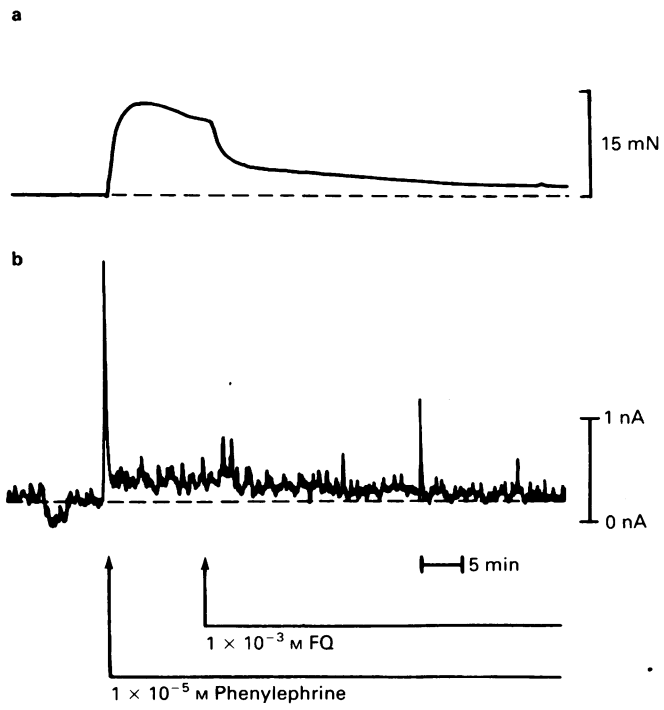

Figure 3 Trace (a): force; (b): luminescence. Effect of flosequinan (FQ) in relaxing a phenylephrine-induced contraction. Zero light is determined with the shutter closed. The closing of the shutter is indicated by the asterisk. The dashed lines mark the resting levels of force and luminescence.

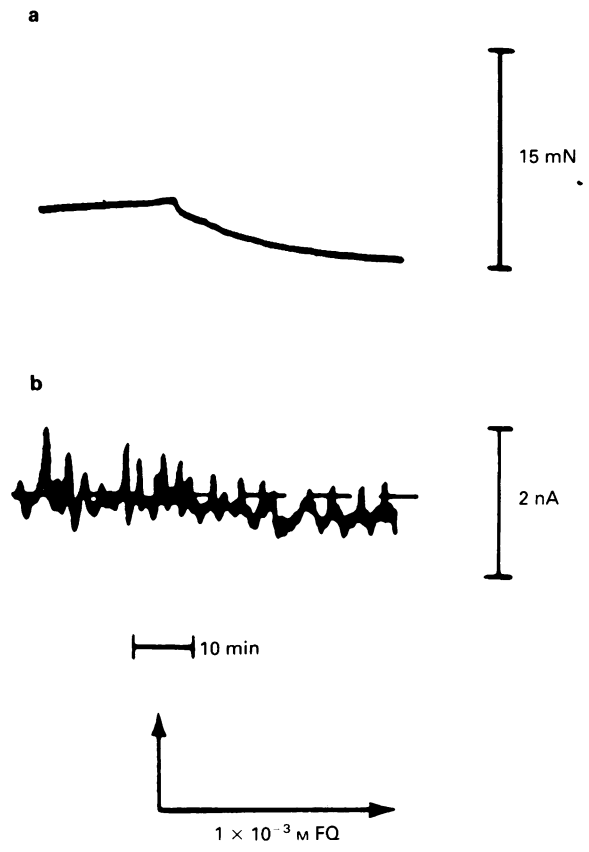

Figure 4 Trace (a): force; (b): luminescence. Effect of flosequinan (FQ) in relaxing intrinsic tone (the tone developed on warming from 2°C to 37°C). Zero light is the light level with the shutter closed. Dashed line marks the position of resting luminescence.

and $[Ca^{2+}]_i$ to 220 ± 26 nM (n = 5 animals, 5 strips). Both the decrease in $[Ca^{2+}]_i$ and force were statistically significant ($P < 0.05$ by a paired Student's t test). A typical data trace is shown in Figure 4.

Effect of flosequinan on $[Ca^{2+}]_i$-force relationships

In Figure 5, steady state $[Ca^{2+}]_i$ levels have been calibrated and plotted against the corresponding steady state force level. All forces have been normalized as a percentage of the force in response to an initial challenge to 22 mM K performed at the beginning of each experiment. The right hand curve (K curve) was obtained either by increasing levels (15 mM or 24 mM) of potassium depolarization or decreasing the extracellular $[Ca^{2+}]$ surrounding the potassium-depolarized (24 mM K) muscle. The extracellular $[Ca^{2+}]$ was decreased by using a nominally Ca^{2+}-free solution containing 2 mM EGTA. It has previously been reported that the removal of extracellular Ca^{2+} from the depolarized ferret aorta results in a decrease in $[Ca^{2+}]$ and force below baseline levels (Ruzycky & Morgan, 1989). The K curve probably reflects the true sensitivity of the contractile apparatus to calcium since no pharmacological manipulations were performed.

The phenylephrine curve was obtained in the presence of 10^{-5} M phenylephrine and varying (2.5, 0.5 or nominally Ca-free plus 2 mM EGTA) extracellular $[Ca^{2+}]$. This concentration of phenylephrine has previously been found to be maximally effective with respect to force production in ferret aorta (Ruzycky & Morgan, 1989). As previously reported (Morgan & Morgan, 1984a), and confirmed quantitatively here, phenylephrine appears to increase the Ca^{2+} sensitivity of the contractile apparatus.

The effect of flosequinan on a potassium-depolarized muscle as well as the resting muscle was to cause parallel changes in $[Ca^{2+}]_i$ and force such that flosequinan did not appear to cause a shift in the potassium-generated $[Ca^{2+}]_i$-force relationship. The effect of flosequinan on the phenylephrine-contracted muscle is also shown in Figure 5. Flosequinan

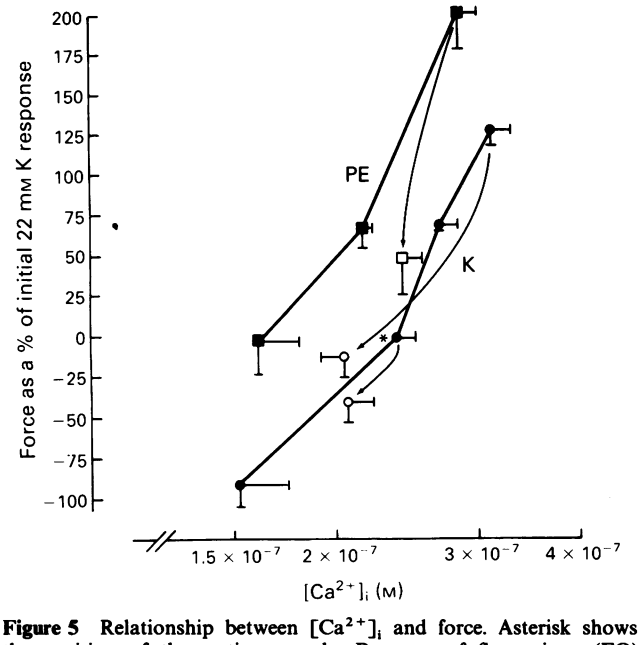

Figure 5 Relationship between $[Ca^{2+}]_i$ and force. Asterisk shows the position of the resting muscle. Presence of flosequinan (FQ) (10^{-3} M) shown by (○) and (□). Arrows connect the control to the appropriate flosequinan-induced relaxation. Potassium curve (K) (●) generated by increasing the [K] in the bathing medium or decreasing [Ca] in the bathing medium surrounding the depolarized muscle. Phenylephrine curve (PE) (■) obtained by removing extracellular Ca from the bathing medium in the presence of 10^{-5} M PE. 100% force is the level produced by an initial challenge to 22 mM K and to which data for each individual strip were normalized. The response to 22 mM K was 12.0 ± 1.2 mN (n = 21 strips) for the experiments for the K curve, 12.7 ± 1.1 mN (n = 15 strips) for the experiments for the PE curve and 11.6 ± 0.9 (n = 15 strips) for the experiments for the FQ points. Data points represent 5–16 experiments from 5–14 animals.

caused a small, but statistically significant (see above) decrease in $[Ca^{2+}]_i$ but it caused a much larger decrease in contractile force and it appears that flosequinan shifts the phenylephrine $[Ca^{2+}]_i$-force relationship to the right.

Comparison with other vasodilators

The effect of sodium nitroprusside (10^{-5} M) on $[Ca^{2+}]_i$-force relationships during either phenylephrine (10^{-5} M)- or potassium (24 mM K)-induced contractions is shown in Figure 6. In both cases, parallel decreases in $[Ca^{2+}]_i$ and force were observed as the muscle relaxed. In both cases, the magnitude of the decrease in $[Ca^{2+}]_i$ appeared to be sufficient to explain the magnitude of the decrease in force and there was no evidence for a shift in the $[Ca^{2+}]_i$-force relationship. Thus, sodium nitroprusside differs from flosequinan in that even at 10^{-5} M it was not capable of reversing the calcium sensitizing actions of phenylephrine.

The effects of milrinone were also determined for comparison. Milrinone has been reported to increase cyclic AMP levels by inhibiting phosphodiesterase activity (Opie, 1986). As is shown in Figure 7, milrinone had a very different effect from that of flosequinan on basal tone in that it decreased intrinsic tone in the absence of a significant ($P > 0.05$) drop in $[Ca^{2+}]_i$. The effect of a range of concentrations of milrinone (10^{-3}–10^{-6} M) displayed an unusual concentration-response relationship. With increasing concentrations, basal tone decreased progressively, but basal $[Ca^{2+}]_i$ increased. We have previously reported similar actions of other agents capable of increasing cyclic AMP levels, such as forskolin, papaverine and isoprenaline (Morgan & Morgan, 1984b); but this effect was not seen even at 10^{-3} M flosequinan. This action of uncoupling $[Ca^{2+}]_i$ from force was apparently lost when milrinone was added to the depolarized muscle. Although milrinone caused relaxation of the potassium-induced contraction, it apparently did this in the absence of a shift in the $[Ca^{2+}]_i$-force relationship (Figure 7). In this regard, it differs from the previously described actions of other phosphodiesterase inhibitors and stimulants of adenylate cyclase (Morgan & Morgan, 1984b; DeFeo & Morgan, 1989); thus, it appears that milrinone has a complex mechanism of action. Milrinone also relaxed the phenyleprine-induced contraction in the absence of a shift in the phenylephrine-generated $[Ca^{2+}]_i$-force curve. In this regard, it also differed in mechanism of action from flosequinan.

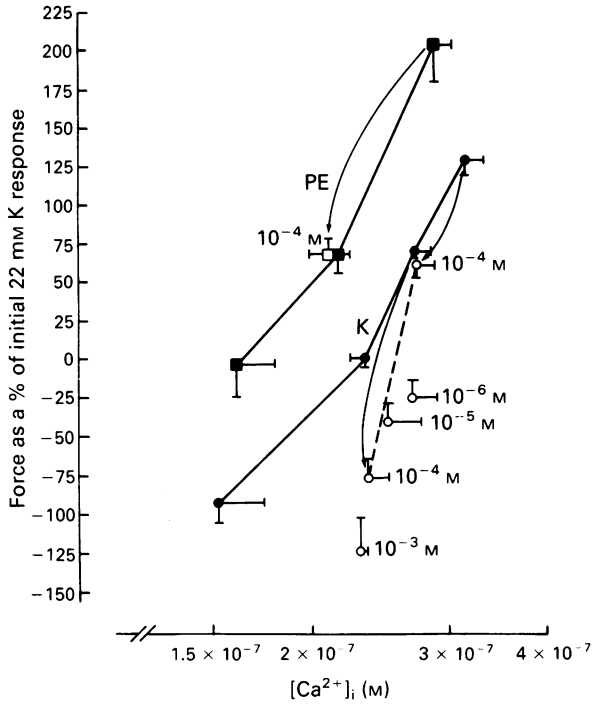

Figure 7 Effect of milrinone on the calcium-force relationship: (●) K curve; (■) phenylephrine curve; effect of milrinone: (□) and (○). All milrinone points are labelled with the concentration of milrinone used. The appropriate control contraction is connected to the milrinone-induced relaxation by the arrows. 100% force was 9.8 ± 0.1 mN (n = 19 strips). Data points represent 5–28 experiments from 5–17 animals.

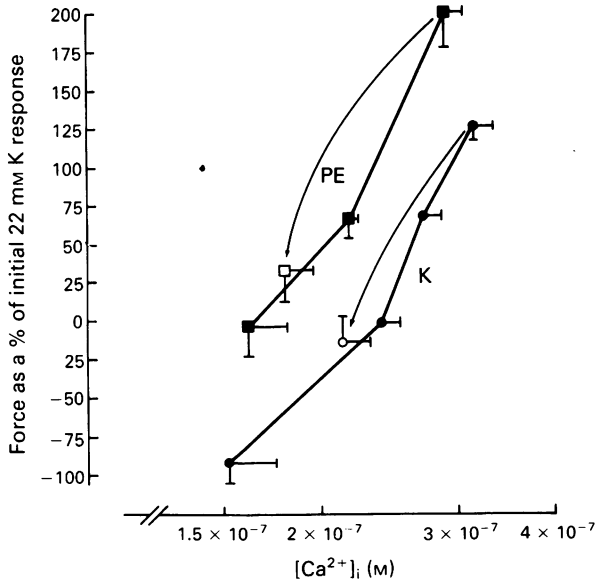

Figure 6 Relationship between intracellular ionized calcium levels and contractile force during relaxation of the muscle by sodium nitroprusside. Control potassium (●) and phenylephrine-induced (■) calcium-force relationships shown; (○) and (□) demonstrate the effect of 10^{-5} M sodium nitroprusside. The appropriate control contraction is connected to the nitroprusside-induced relaxation by the arrows. 100% force was 10.1 ± 1.8 mN (n = 11 strips) for the nitroprusside experiments. Data points represent 5–16 experiments from 5–14 animals.

Discussion

The main conclusion of this study is that flosequinan differs qualitatively in its mechanism of action both from agents that increase cyclic GMP and those which increase cyclic AMP. Although sodium nitroprusside has been previously reported to decrease intracellular ionized calcium levels in vascular smooth muscle cells (Morgan & Morgan, 1984b; Karaki *et al.*, 1988), the possibility of an additional effect involving change in sensitivity of the contractile apparatus to Ca^{2+} has not previously been investigated. In the present study we have determined quantitatively the effect of maximally effective concentrations of sodium nitroprusside on $[Ca^{2+}]_i$-force relationships. We found no effect of this agent on the $[Ca^{2+}]_i$-force relationship either when the muscle is potassium-depolarized or when the Ca^{2+} sensitivity is increased by the presence of phenylephrine. Thus, sodium nitroprusside causes relaxation in this muscle solely by decreasing $[Ca^{2+}]_i$ levels.

Milrinone has been referred to as an 'inodilator' because of its actions as both a positive inotropic agent on cardiac muscle as well as its vasodilator actions (Opie, 1986). Both of these actions are due at least in part, to the ability of this agent to inhibit phosphodiesterase. In a ferret model, studies of Falotico *et al.* (1989) have suggested that flosequinan might have similar properties. When applied to the resting muscle, milrinone is capable of causing a decrease in intrinsic tone in the absence of any decrease in $[Ca^{2+}]_i$. At the lower concentrations, an increase in $[Ca^{2+}]_i$ is actually observed. We have previously reported similar results in ferret aorta with another agent capable of increasing cyclic AMP levels, forskolin

(DeFeo & Morgan, 1989). We have also seen similar results in ferret portal vein with papaverine (Morgan & Morgan, 1984b) and adenosine (Bradley & Morgan, 1985), two additional agents capable of increasing cyclic AMP levels. The mechanism of this apparent uncoupling of Ca^{2+} from force production is unknown. Possible mechanisms include the phosphorylation of myosin light chain kinase by cyclic-A dependent kinase (Adelstein *et al.*, 1978; Silver & DiSalvo, 1979) or the phosphorylation of the myosin light chains by cyclic A-kinase (Walsh *et al.*, 1981). Flosequinan has been reported to be capable of inhibiting a non-specific phosphodiesterase in cardiac muscle with an IC_{10} of 1.0×10^{-4} M (Frodsham *et al.*, 1989) which could result in an increased cyclic AMP level in vascular smooth muscle as well. However, in the intact cell, even at the concentration of 10^{-3} M used in this study, we saw no evidence of a rightward shift in the potassium-generated $[Ca^{2+}]_i$-force curve including the values from the resting muscles.

Milrinone appears to be capable of vasodilator actions separate from an increase in cyclic AMP levels since it did not cause a shift in the $[Ca^{2+}]_i$-force relationship in the potassium-depolarized muscle. This is different from the previously reported actions of forskolin, adenosine, isoprenaline and papaverine in ferret aorta and portal vein (Morgan & Morgan, 1984b; Bradley & Morgan, 1985; DeFeo & Morgan, 1989). Milrinone also did not shift the phenylephrine $[Ca^{2+}]_i$-force relationship, but the action of agents capable of increasing cyclic AMP levels on this relationship has not been determined.

Flosequinan appears to fit the pattern of neither a vasodilator which increases cyclic GMP levels nor a vasodilator which increases cyclic AMP levels. Thus, the question arises as to the actual mechanism of vasodilatation produced by this compound. In the case of relaxation of potassium-contracted muscles, the relaxation can be explained solely on the basis of the decrease in $[Ca^{2+}]_i$, since no shift in the $[Ca^{2+}]_i$-force relationship was observed under these conditions. Surprisingly, flosequinan did appear to be capable of shifting the phenylephrine-induced $[Ca^{2+}]_i$-force relationship to the right. Thus, flosequinan appeared to be reversing the action of phenylephrine in sensitizing the contractile apparatus to Ca^{2+}. We and others have previously discussed the possibility that phenylephrine responses might involve the activation of protein kinase-C (Ruzycky & Morgan, 1989; Jiang & Morgan, 1987; Exton, 1985; Danthuluri & Deth, 1986). Although not all data are consistent with this interpretation, if tone maintenance during phenylephrine-induced contractions depends on C-kinase activation, this raises the interesting possibility that flosequinan might exert at least part of its vasodilator actions through an antagonism of protein kinase-C.

Financial support was provided by USPHS grant HL31704 and a research grant from Boots Pharmaceuticals. K.G.M. was an Established Investigator of the American Heart Association.

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(Received November 14, 1990
Accepted November 28, 1990)

Br. J. Pharmacol. (1991), **102**, 979–985

The contribution of Rb-permeable potassium channels to the relaxant and membrane hyperpolarizing actions of cromakalim, RP49356 and diazoxide in bovine tracheal smooth muscle

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1 Cromakalim (1 and 10 μM), RP49356 (5 and 50 μM) and diazoxide (100 and 300 μM) produced full relaxation of smooth muscle strips pre-contracted with 25 mM KCl. These agents caused membrane hyperpolarization and increased ^{42}K and ^{86}Rb efflux. The time taken to achieve the maximum change in each of these parameters (t_{max}) was less for the higher concentration levels of cromakalim, RP49356 and diazoxide than for the lower concentration levels.

2 Calculation of permeability (P) changes showed that cromakalim (1 and 10 μM) produced a greater rise in PK than PRb, although the PRb:PK ratio was similar at both concentration levels. Similarly RP49356 produced a greater change in PK than PRb. However, in contrast to cromakalim, this difference was more marked at the higher concentration (50 μM) and was reflected by a differential effect of the two concentrations of RP49356 on the PRb:PK ratio. Diazoxide (100 and 300 μM) produced similar changes in PK and PRb.

3 For cromakalim (1 and 10 μM) the t_{max} for the electrical and mechanical effects and also the profile of change in these parameters corresponded to changes in both PK and PRb. For RP49356 (5 μM), changes in tension and membrane potential were related to both changes in PK and PRb, whereas at 50 μM these responses more closely corresponded to changes in PK. For diazoxide (100 and 300 μM) the electrical and mechanical effects corresponded to changes in both PK and PRb.

4 The results show that changes in ^{42}K and ^{86}Rb efflux induced by cromakalim, RP49356 and diazoxide are good indicators of changes in membrane PK and PRb evoked by these agents. Furthermore, it is concluded that the K channels involved in the mechanical and electrical effects of cromakalim are represented by the opening of a single population through which Rb can pass less easily than K, whilst the K channels associated with actions of diazoxide are equally permeable to both K and Rb. In contrast, the relaxant and membrane hyperpolarizing actions of RP49356 may involve the opening of more than one group of K channels which differ in their permeability to Rb.

Keywords: K-channels; RP49356; diazoxide; cromakalim; rubidium; hyperpolarization; ion flux; tracheal smooth muscle

Introduction

Changes in ^{42}K efflux from smooth muscle tissues evoked by potassium (K) channel opening drugs such as cromakalim are generally regarded as satisfactory indicators of changes in membrane K permeability (PK). A number of studies have measured the effects of K channel openers on K efflux using ^{86}Rb as a substitute for K, since this radionuclide has a more convenient half-life and is available with a higher specific activity (e.g. Hamilton *et al.*, 1986; Quast, 1987). Although Rb has similar chemical and physical properties to K (Ussing, 1960), patch-clamp studies have shown that some K channels can discriminate between these two ions (e.g. mouse exocrine acinar cells: large conductance Ca-activated K channel; Gallacher *et al.*, 1984; rat pancreas; ATP-sensitive K channels; Ashcroft *et al.*, 1989).

In smooth muscle tissues there are several reports that K channels opened by cromakalim-like drugs may also distinguish between K and Rb. In some vascular tissues, cromakalim and pinacidil evoke larger increases in K efflux than Rb efflux and the ratio of K:Rb flux varies with concentration of K channel opener used (Quast & Baumlim, 1988; Videbaek *et al.*, 1988; Bray & Weston, 1989). In guinea-pig bladder and rat aorta, cromakalim and minoxidil sulphate respectively produce marked increases in K efflux with no detectable effect on the loss of Rb (Foster *et al.*, 1989; Newgreen *et al.*, 1990) and in rat bladder, cromakalim-stimulated increases in Rb efflux, but not K efflux, show 'desensitisation' on a second exposure to this drug (Edwards & Weston, 1989). In rabbit aorta, cromakalim (10 μM)-induced changes in Rb efflux are more sensitive to the effects of glibenclamide than K efflux (Bray & Weston, 1989) whilst in guinea-pig bladder replacement of extracellular KCl with RbCl abolishes the relaxant effect of cromakalim (Foster *et al.*, 1989).

Assessment of the effects of K channel openers on K permeability (PK) using the data derived from ^{42}K/^{86}Rb efflux experiments is complicated by the ability of these agents to produce membrane hyperpolarization. Such a change in membrane potential increases the electrical force retaining K in the cell with a resultant underestimation of changes in membrane PK. Furthermore, if the channel is less permeable to Rb than K, then measurement of Rb flux will produce a greater error in the estimation of the effects of K channel openers on PK.

The aims of the present study were to determine firstly whether the K channels involved in the actions of cromakalim, RP49356 and diazoxide were permeable to Rb and/or K, and secondly whether there were any differences in the groups of K channels opened by the three K channel openers. Therefore, we examined the effects of cromakalim, RP49356 and diazoxide on K and Rb permeability (PK and PRb respectively) in bovine tracheal smooth muscle. We also investigated whether changes in PRb and/or PK corresponded to the changes in membrane potential and tension evoked by these K channel openers. Finally, we assessed whether measurement of changes in K and Rb efflux were good indicators of changes in PK and PRb respectively.

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A preliminary account of some of this work has been presented to the British Pharmacological Society (Longmore et al., 1990a).

Methods

Fresh specimens of bovine trachea were obtained from Manchester Abbatoir and were placed in cold (4°C) physiological salt solution (PSS) for transport to the laboratory (approximately 10 min). Each trachea was opened along its longitudinal axis and the smooth muscle was isolated and cleaned of any connective tissue. Strips of smooth muscle, each approximately 4 mm in width, were then prepared. In all experiments the appropriate time- and vehicle-matched controls were employed.

Tissue bath experiments

Strips of smooth muscle were mounted for isometric tension recording in a 20 ml organ bath containing PSS, pH 7.4, aerated with 95% O_2 and 5% CO_2. The tissues were suspended under 1 g tension and allowed to equilibrate for 60 min after which time tension was readjusted to 1 g. A further 30 min equilibration period was allowed before experiments started.

Calculation of IC_{50} values

Tissues were pre-contracted with 25 mM KCl and when the contractile response had reached a plateau (approximately 10–20 min), the relaxant effects of cromakalim, RP49356 and diazoxide were assessed by cumulative addition to the tissue baths at 5 min intervals. The IC_{50} values for the K channel openers were then calculated. In the case of cromakalim and RP49356 concentrations corresponding to approximately 5 and 50 times the IC_{50} values were used in all subsequent experiments (i.e. 1 or 10 μM for cromakalim and 5 or 50 μM for RP49356). In the case of diazoxide, it was only possible to use concentrations corresponding to 5 and 15 times the IC_{50} values (i.e. 100 and 300 μM) since at concentrations of diazoxide greater than 300 μM substantial effects of its vehicle (DMSO) were seen.

Time-course of mechano-inhibitory effects

Tissues were pre-contracted with 25 mM KCl and when the contractile response to this agent had reached a plateau each tissue was exposed to one of the following drugs for a period of 24 min: cromakalim (1 or 10 μM), RP49356 (5 or 50 μM) or diazoxide (100 or 300 μM). This contact time (24 min) was used in order to make the relaxant experiments comparable with the electrophysiological and ion flux studies. For the low concentrations of the K channel openers used it took up to 17 min for some responses to achieve their maximum (see Table 2). Therefore a contact time of 24 min was employed in all experiments in order to measure the responses at their plateau.

Electrophysiological measurements

A strip of smooth muscle was placed in a recording chamber (15 ml) through which PSS was flowing at the rate of 5 ml min^{-1}. The tissue was fixed to the Sylgard floor of the chamber with fine pins and allowed to equilibrate for 1 h. Glass microelectrodes (resistance 40–85 MΩ) were inserted into the smooth muscle cells and when a stable intracellular recording of membrane potential of at least 1 min duration had been obtained, cromakalim (1 and 10 μM), RP49356 (5 and 50 μM) or diazoxide (100 and 300 μM) were added to the PSS reservoir. Recordings were then made over a period of at least 10 min or until the electrode became dislodged. Results were discarded if the resistance of electrode, when dislodged, markedly differed from that prior to impalement.

Ion flux studies

Strips of smooth muscle were mounted on a syringe needle and each was suspended in a plastic vial containing 3 ml PSS. After 10 min the tissues were transferred to vials containing a modified PSS in which KCl had been replaced with $^{42}K_2CO_3$ (1.57 μCi ml^{-1}) and/or to which ^{86}RbCl (5 μCi ml^{-1}; final concentration <50 μM) had been added. After a 3 h loading period, ^{42}K and/or ^{86}Rb were allowed to efflux from the tissues by transferring them, at 4 min intervals, to a series of vials containing normal PSS (for further details see Hamilton et al., 1986). Each tissue was exposed to one of the following drugs: cromakalim (1 or 10 μM), RP49356 (5 or 50 μM) or diazoxide (100 or 300 μM) with appropriate vehicle controls. The ^{42}K and ^{86}Rb content of the tissues and efflux samples were measured with a gamma counter. The samples were then stored at 20°C for 5–6 days and the remaining ^{86}Rb component was counted again, using a half-life correction factor. The ^{42}K counts were calculated by subtraction of the second counts (^{86}Rb only) from the first count (^{42}K and ^{86}Rb combined) and the data expressed in terms of the efflux rate coefficient (fractional loss of ^{42}K or ^{86}Rb from the tissue) over a 1 min period. In some experiments, for each individual time point, the fractional loss of ^{42}K or ^{86}Rb from strips of smooth muscle exposed to the drugs was expressed as a percentage of that from strips obtained from the same trachea but exposed to the vehicle control alone. Expression of the efflux data in this fashion eliminates differences in the basal loss of K and Rb and also reduces any decline in the rate of efflux throughout the course of the experiment which may occur as a result of depletion of the intracellular pool of K or Rb (Jones, 1980).

Drugs and solutions

The following substances were used: (±)-cromakalim (Beecham); (±)-N-methyl-2-(3-pyridinyl)-tetrahydrothiopyran-2-carbothioamide-1-oxide (RP49356) (Rhône-Poulenc); diazoxide (Glaxo); glibenclamide (Sigma); $^{42}K_2CO_3$ (University of Manchester Reactor); ^{86}RbCl (Amersham). Stock solutions were prepared by dissolving cromakalim (10 mM) and RP49356 (10 mM) in 70% v/v ethanol:distilled water; diazoxide (10 mM) in DMSO (dimethyl sulphoxide; Sigma); glibenclamide (1 mM) in absolute ethanol. The PSS had the following composition (mM); NaCl 120, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1 and glucose 11. When KCl was used as the spasmogen the stated concentration excludes the KCl (5.9 mM) already present in the PSS.

Analysis of data

Data are expressed as mean ± s.e.mean and statistical comparisons were made with Student's t test (unpaired comparison). An *a priori* criterion of $P < 0.05$ was used to identify significant effects. The relationship between K efflux and PK is described by the following equation (adapted from Jones, 1980):

$$Jc = PK \cdot K_i \cdot \left(\frac{u}{1 - e^{-u}}\right) \quad (1)$$

where Jc = efflux (mol cm^{-1} s^{-1}), PK = K permeability (cm s^{-1}), K_i = intracellular K concentration, u = VF/RT (V is membrane potential (mV); F is Faraday's constant (96500 C/mol); R is the gas constant (8.3 J/mol/°); T is the absolute temperature (310°K at 37°C).

In the present experiments the effects of cromakalim, RP49356 and diazoxide on K and Rb efflux and membrane potential were used to determine the effects on PK and PRb respectively. The calculation of PK and PRb (expressed as arbitrary units; U) was based on a modification of Equation

(1):

$$\text{PK (or PRb)} = \frac{\text{efflux (\% control) at time } t \text{ min}}{K_i} \times \frac{1 - e^{-u}}{u} \text{ at time } t \text{ min} \quad (2)$$

Firstly, for each concentration of each drug, the mean membrane potential change obtained from $n = 2$ or 3 cells was measured at time points corresponding to those used in the efflux studies (i.e. 0, 4, 8, 12, 16, 20 and 24 min). The difficulty in maintaining impalement of smooth muscle cells with microelectrodes limited the number of cells which could be successfully studied over the full 24 min period. These data were used to calculate the $(1 - e^{-u}$ divided by u) term and the efflux data obtained from each individual tissue were multiplied by this factor and divided by K_i (105 mM from Kirkpatrick, 1981) to give PK or PRb (measured in arbitrary units U). Finally, for each individual tissue the ratio of the maximum changes in PRb and PK evoked by each drug was calculated (i.e. PRb_{max}/PK_{max}).

Results

Tissue bath studies

Figure 1 shows the relaxant concentration-effect curves for cromakalim, RP49356 and diazoxide in bovine tracheal smooth muscle pre-contracted wtih 25 mM KCl. The IC_{50} values for cromakalim, RP49356 and diazoxide were $0.19 \pm 0.02\,\mu M$, $1.65 \pm 0.30\,\mu M$ and $21.3 \pm 3.6\,\mu M$ respectively ($n = 4$–6). The higher concentrations of each K channel opener (cromakalim, $10\,\mu M$; RP49356, $50\,\mu M$; diazoxide, $300\,\mu M$) produced complete relaxation of the 25 mM KCl-induced contractions. The time taken to achieve a maximal effect (t_{max}) ranged between 7 and 9 min. The lower concentrations of each K channel opener (cromakalim, $1\,\mu M$; RP49356, $5\,\mu M$; diazoxide, $100\,\mu M$) also caused complete relaxation, although it took longer for this effect to be achieved (11–18 min). For details see Tables 1 and 2. DMSO (the vehicle for diazoxide) itself produced a marked relaxation although this effect occurred over a longer time-course than the effects of diazoxide itself.

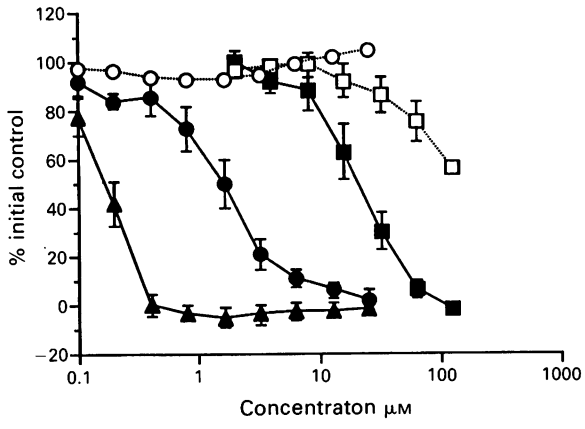

Figure 1 The effects of cromakalim (▲), RP49356 (●), diazoxide (■) and vehicle control (○, ethanol; □, dimethyl sulphoxide) on 25 mM KCl-induced contractions in bovine tracheal smooth muscle. Ordinate scale: % initial 25 mM KCl-induced contraction; abscissa scale: concentration of K channel opener (μM) on a logarithmic scale. Points show mean values ($n = 4$–6). Vertical bars signify ±s.e.mean.

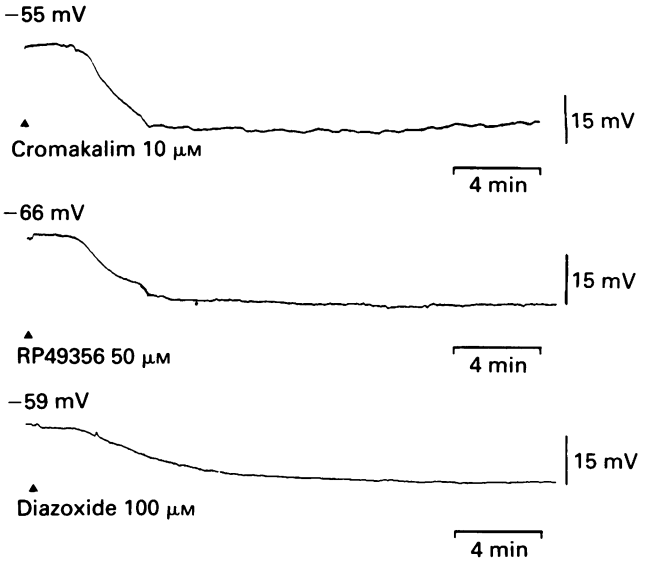

Figure 2 Typical examples of the effect of (a) cromakalim $10\,\mu M$, (b) RP49356 $50\,\mu M$ and (c) diazoxide $100\,\mu M$ on membrane potential in bovine tracheal smooth muscle. The (●) indicates the point at which the drugs were added to the PSS reservoir and the values refer to the resting membrane potential (mV).

Electrophysiological measurements

The mean resting membrane potential of bovine tracheal smooth muscles cells was $-61.6\,mV \pm 1.5\,mV$ ($n = 28$ cells obtained with 18 different trachea) and showed little spontaneous fluctuation during an experiment. The effects of cromakalim, RP49356 and diazoxide on membrane potential and the time taken to attain the maximum effect (t_{max}) are summarised in Tables 1 and 2. Each agent produced membrane hyperpolarization which was relatively well maintained throughout the period of impalement (10–24 min). Figure 2 shows typical examples of the effects of cromakalim ($10\,\mu M$), RP49356 ($50\,\mu M$) and diazoxide ($100\,\mu M$) on membrane potential. The solvents used (ethanol or DMSO) had no apparent effect on the level of membrane potential, but membrane noise was increased in the presence of DMSO.

Ion flux experiments

Figure 3 shows the effects of cromakalim ($10\,\mu M$) on ^{42}K efflux from tissues which were loaded in a solution containing either ^{42}K alone or ^{42}K and ^{86}Rb. The loss of ^{42}K was similar in both experimental groups indicating that at the concentrations used, Rb itself did not influence the loss of K from the

Table 1 Maximum responses in bovine isolated tracheal muscle observed in presence of K channel openers

		Tension (%)	*Membrane potential* (mV)	*K efflux* (% control)	*Rb efflux* (% control)	*PK* (U × 10^2)	*PRb* (U × 10^2)	*PRb/PK*
Cromakalim	1 μM	97.0 ± 6.9	−74.4 ± 1.6	233 ± 32	156 ± 13	120 ± 77	79 ± 4	0.61 ± 0.07
Cromakalim	10 μM	97.0 ± 4.0	−79.2 ± 1.3	337 ± 43	235 ± 18	168 ± 21	111 ± 7	0.66 ± 0.08
RP49356	5 μM	95.6 ± 3.5	−76.6 ± 1.3	203 ± 28	185 ± 13	108 ± 7	88 ± 8	0.81 ± 0.05
RP49356	50 μM	105.3 ± 7.4	−82.5 ± 1.1	419 ± 56	249 ± 13	251 ± 24	136 ± 7	0.57 ± 0.06
Diazoxide	100 μM	97.9 ± 12.5	−71.9 ± 2.2	173 ± 24	154 ± 20	75 ± 11	65 ± 7	0.98 ± 0.18
Diazoxide	300 μM	100.7 ± 5.2	−80.9 ± 1.0	212 ± 17	190 ± 28	107 ± 10	91 ± 14	0.84 ± 0.16

Values are mean ± s.e.mean; $n = 3$–7.

Table 2 t_{max} (min) values i.e. time taken to achieve maximal effect in bovine isolated tracheal muscle

		Tension	*Membrane potential*	*K efflux*	*Rb efflux*	*PK*	*PRb*
Cromakalim	1 μM	11.2 ± 3.1	17.0 ± 2.7	16–20	>24	16–20	20–24
Cromakalim	10 μM	8.7 ± 1.9	6.2 ± 0.5	8–12	>24	8–12	8–12
RP49356	5 μM	12.2 ± 3.9	12.3 ± 0.7	20–24	20–24	20–24	20–24
RP49356	50 μM	8.3 ± 3.1	5.5 ± 0.5	8–12	20–24	8–12	20–24
Diazoxide	100 μM	12.7 ± 0.8	14.5 ± 2.8	>24	16–20	20–24	16–20
Diazoxide	300 μM	7.4 ± 1.1	7.0 ± 0.8	>24	16–20	16–20	16–20

Values are mean ± s.e.mean; n = 3–7.

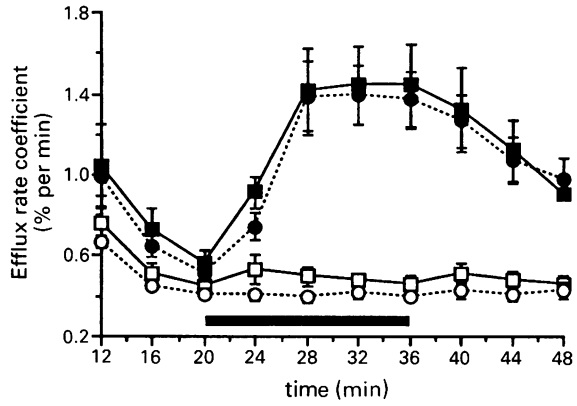

Figure 3 Effect of cromakalim on ^{42}K efflux from bovine tracheal smooth muscle. Square symbols show the loss of ^{42}K from tissues loaded with ^{42}K alone and round symbols show ^{42}K efflux from tissues loaded with ^{42}K and ^{36}Rb. The filled symbols show the effects of cromakalim (10 μM) and the open symbols show the effects of vehicle (ethanol) control. Ordinate scale: K efflux rate coefficient (% per min); abscissa scale: time into efflux (min). Points show mean values (n = 6) and vertical bars signify ± s.e.mean. The horizontal bar indicates the period of time over which the tissues were exposed to the modifying agents.

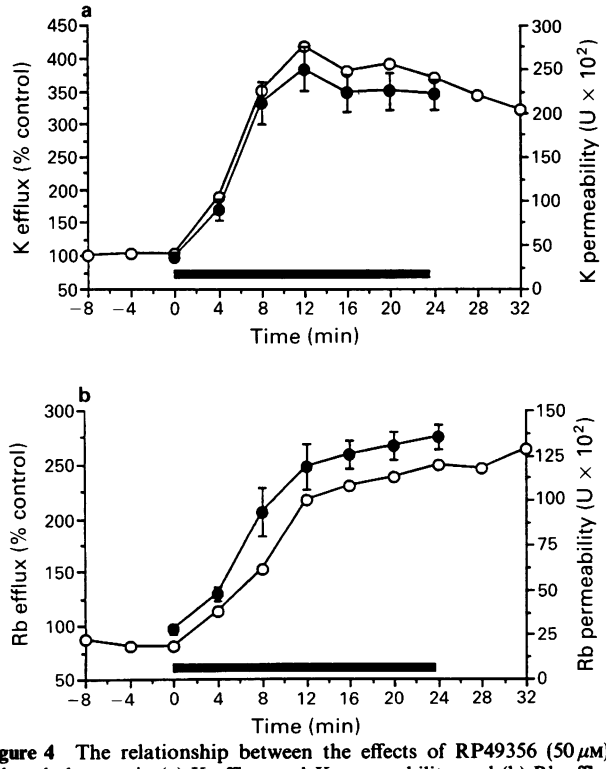

Figure 4 The relationship between the effects of RP49356 (50 μM)-induced changes in (a) K efflux and K permeability and (b) Rb efflux and Rb permeability: (○) show effects on ion flux and (●) show effects on permeability. Points show mean values (n = 4–5) and vertical bars signify ± s.e.mean. The horizontal bar indicates the period of time over which the tissues were exposed to RP49356 (50 μM).

tissues. For both concentrations of cromakalim, RP49356 and diazoxide the profile of changes in K and Rb efflux parallelled the profile of change in PK and PRb respectively. This is illustrated in Figure 4 which shows the relationship between RP49356 (50 μM)-evoked changes in K and Rb effluxes and permeabilities.

The resting PK (U × 10^2) and PRb (U × 10^2) were 36.6 ± 1.4 and 32.1 ± 1.9 respectively (n = 31–37). Cromakalim (1 and 10 μM) produced larger increases in PK than PRb and the PRb/PK ratio did not differ significantly at the two concentration levels. RP49356 (5 and 50 μM), similarly to cromakalim, produced larger increments in PK than PRb; however, in contrast to cromakalim, there was a significant difference in the PRb/PK ratio for each concentration used. Diazoxide (100 and 300 μM) produced similar changes in PK and PRb. For details see Table 1.

At the lower concentration levels all three K channel openers produced similar changes in PRb (range 109% to 148%). Cromakalim (1 μM) and RP49356 (5 μM) produced comparable rises in PK whereas diazoxide (100 μM), which represents a similar effective relaxant concentration (i.e. 5 times the IC_{50} value), produced a significantly smaller change in PK. Comparison of the effects of the higher concentrations showed that RP49356 (50 μM) produced a significantly greater increase in PK than cromakalim (10 μM), which in turn was more effective than diazoxide (300 μM). The smaller effect of diazoxide (300 μM) on PK is likely to reflect the lower effective relaxant concentration of this K channel opener.

The effects of cromakalim, RP49356 and diazoxide on PK and PRb, tension and membrane potential are summarised in Figures 5, 6 and 7 respectively.

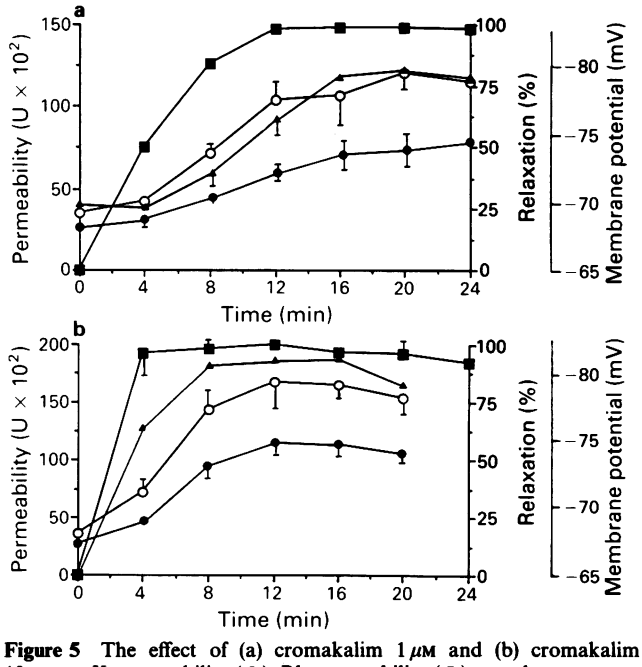

Figure 5 The effect of (a) cromakalim 1 μM and (b) cromakalim 10 μM on K permeability (○), Rb permeability (●), membrane potential (▲) and tension (■). Points show mean values (n = 3–7) and the vertical bars signify ± s.e.mean.

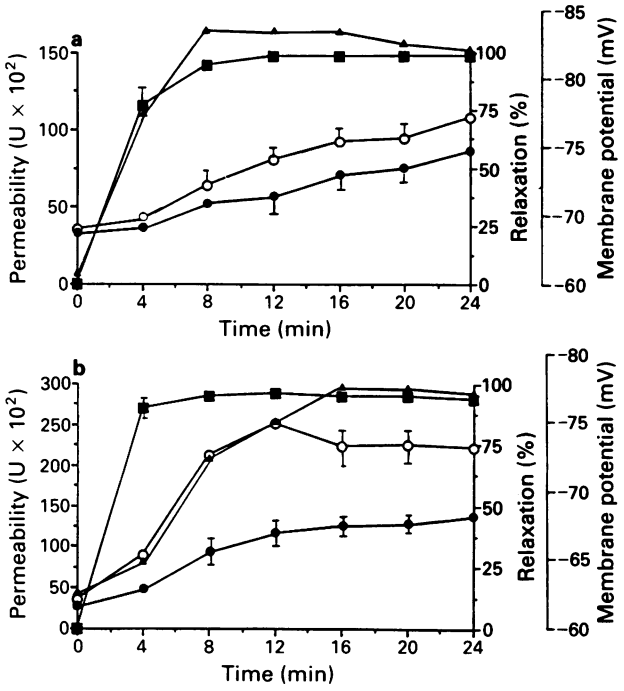

Figure 6 The effect of (a) RP49356 5 μM and (b) RP49356 50 μM on K permeability (○), Rb permeability (●), membrane potential (▲) and tension (■). Points show mean values (n = 3–7) and the vertical bars signify ±s.e.mean.

Discussion

Bovine tracheal smooth muscle has little if any spontaneous tone and therefore, the relaxant effects of cromakalim, RP49356 and diazoxide were studied in strips of smooth muscle pre-contracted with 25 mM KCl. All three K channel openers produced full relaxation and the rank order of potency was cromakalim > RP49356 > diazoxide with potency ratios of 1:9:112 when IC_{50} values were compared. These values are comparable to those reported for cromakalim and RP49356 in guinea-pig pulmonary artery (Eltze, 1989) and in rat portal vein (Longmore *et al.*, 1990b) and for cromakalim and diazoxide in rat aorta (Quast & Cook, 1989; Newgreen *et al.*, 1990) and in portal vein (Quast & Cook, 1989; Longmore *et al.*, 1990b).

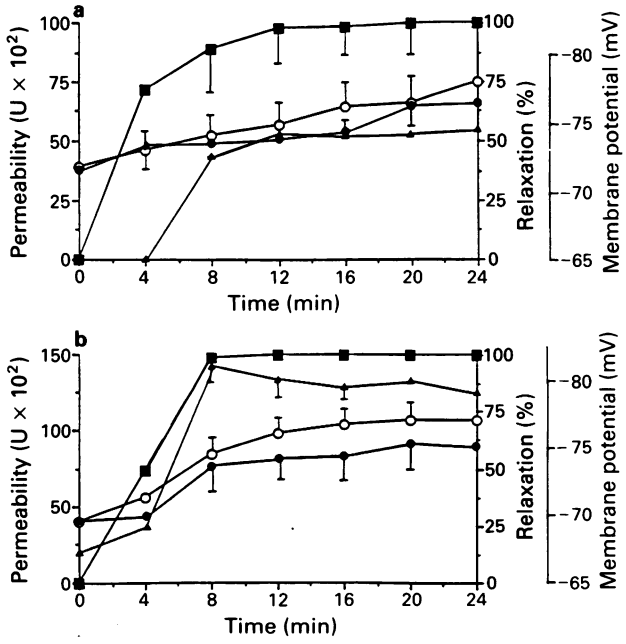

Figure 7 The effect of (a) diazoxide 100 μM and (b) diazoxide 300 μM on K permeability (○), Rb permeability (●), membrane potential (▲) and tension (■). Points show mean values (n = 3–7) and the vertical bars signify ±s.e.mean.

Cromakalim, RP49356 and diazoxide each produced marked membrane hyperpolarization towards the calculated K equilibrium potential (E_K) in this tissue (−77 mV, assuming an intracellular K concentration of 105 mM, Kirkpatrick, 1981). Although the electrophysiological studies were carried out in the absence of 25 mM KCl, there was a good relationship between the mechanical and electrical effects observed in the different experimental series. There were considerable similarities in the profile of changes in tension and changes in membrane potential (see Figures 5–7) and the t_{max} values for the mechanical and electrical effects were similar for all drugs except in the case of cromakalim (1 μM) where the t_{max} value for the relaxant effect was less than that for the membrane potential effect. This may reflect the possibility that low concentrations of cromakalim produce smooth muscle relaxation via a mechanism which is independent of membrane hyperpolarization (Bray *et al.*, 1991).

The close correspondence between the electrical and mechanical effects, despite the mechanical studies being carried out in tissues pre-contracted with 25 mM KCl, most likely reflects the concentration of KCl used. Under these conditions the contractile effects of 25 mM KCl in bovine tracheal smooth muscle are associated with membrane depolarization to approximately −33 mV (Longmore *et al.*, 1991). Recent work in our laboratory (unpublished observations) suggests that within this tissue voltage-operated calcium channels are opened within the range −33 to −41 mV. Therefore full relaxation, mediated via closure of voltage-operated calcium channels, would not be predicted to occur until a K channel opener hyperpolarized the membrane potential to a value approximately equal to or greater than −41 mV. The calculated new E_K with an extracellular K concentration of 31 mM is −39 mV and this value would be predicted to be the maximum membrane potential observed in the presence of a K channel opener. Indeed, it has been shown that in the presence of 25 mM KCl, lemakalim (BRL 38227, the active enantiomer of cromakalim) does hyperpolarize the membrane to a value close to that of the calculated new E_K (Longmore *et al.*, 1991). Thus maximum relaxation would coincide with maximum changes in membrane potential.

In the present study cromakalim, RP49356 and diazoxide increased ^{42}K and ^{86}Rb efflux. It has been reported that for some K channels, Rb ions move through the channel more slowly than K and effectively block the channel or impair the movement of K (Gallacher *et al.*, 1984; Ashcroft *et al.*, 1989). Indeed, it has been shown that replacement of extracellular KCl with RbCl in guinea-pig bladder abolishes the relaxant effect of cromakalim (Foster *et al.*, 1989) and in guinea-pig tracheal smooth muscle a transient rather than sustained mechano-inhibitory action of cromakalim is observed (Morris & Taylor, 1989). However, in the present ion flux experiments it is unlikely that the presence of Rb (maximum concentration < 50 μM) caused channel blockade since cromakalim (10 μM)-stimulated ^{42}K efflux was similar from tissues loaded with solutions containing ^{42}K alone or ^{42}K and ^{86}Rb.

The three K channel openers produced substantial membrane hyperpolarization and thus measurement of changes in ^{42}K (or ^{86}Rb) efflux would be predicted to underestimate the effects of these agents on K permeability. The relationship between changes in K efflux and changes in PK is defined by Equation (2) shown above. The correction factor by which efflux was multiplied to derive permeability ranged between 37.4 and 63.0 at −61.6 mV (resting membrane potential) and −82.5 mV (the highest membrane potential seen in the presence of the potassium channel openers), respectively. Thus measurement of changes in K efflux induced by the K channel openers would underestimate the change in PK by a maximum of 1.74 fold. Nevertheless, it can be seen from Figure 3 that in the case of RP49356 (50 μM), the profile of

changes in ^{42}K or ^{86}Rb efflux closely corresponded to the profile of changes in K or Rb permeability. This was also the case for cromakalim (1 and 10 μM), RP49356 (5 μM) and diazoxide (100 μM and 300 μM). Thus changes in K efflux appear to give a good indication of changes in PK.

It is well established that the basal loss of Rb from smooth muscle tissues is less than that of K (for example see Smith *et al.*, 1986; Videbaek *et al.*, 1988; Quast & Baumlim, 1988) and that K channels involved in the action of K channel openers may also differ in their permeability to Rb (see Introduction). In the present experiments, the flux data were expressed as a percentage of control, effectively eliminating differences in the basal loss of K and Rb. All three K channel openers tested produced increases in both PK and PRb. However, there were some differences. Cromakalim (1 and 10 μM) caused greater increases in PK than PRb and the PK:PRb ratio was similar at both concentrations tested (0.61 and 0.66 respectively). These results are similar to, although not entirely consistent with, the previously reported effects of cromakalim in rat and rabbit aorta and in guinea-pig portal vein (Quast & Baumlim, 1988; Bray & Weston, 1989) and those of pinacidil in rat mesenteric artery (Videbaek *et al.*, 1988). These previous studies showed that the magnitude of the PRb/PK ratio was dependent on the concentration of K channel opener used and it was suggested that different concentrations of either cromakalim or pinacidil open groups of K channels which differ in their permeability to Rb. However, the present results suggest that in bovine tracheal smooth muscle cromakalim (1 and 10 μM) opens either a single group of K channels through which Rb can pass less easily than K or that cromakalim opens two groups of channels one of which is permeable to both K and Rb and one which is relatively Rb-impermeable. At present it is not possible to distinguish between these two possibilities.

RP49356, like cromakalim, also caused a greater increase in PK than PRb. However RP49356 differed from cromakalim since the two concentrations of RP49356 (5 and 50 μM) had differential effects on the PRb/PK ratio (see Table 1). At the lower concentration (RP49356 5 μM) similar increases in both PK and PRb were observed whereas at the higher concentration (RP49356 50 μM) a great stimulatory effect on PK than PRb was seen. These observations suggest that RP49356 can open at least two groups of K channels, the channel open at the 5 μM level being permeable to both K and Rb whereas at the 50 μM level there is an increased proportion of 'K-selective' channels open (i.e. channels which allow K to pass through more readily than Rb). This effect of RP49356 contrasts with the previously reported effects of pinacidil which at low concentrations opens 'K'-selective channels and at higher concentrations opens 'Rb-permeable' K channels (Videbaek *et al.*, 1988). Diazoxide (100 μM and 300 μM) produced similar changes in both PK and PRb, indicating that K channels involved in the action of this drug are equally permeable to both K and Rb. This effect of diazoxide is similar to that found by Newgreen (personal communication) in rat aorta. Thus in the present experiments the effects of the K channel openers on Rb:K permeability ratio show some qualitative similarities to those found previously in other tissue types particularly vascular smooth muscle, although some discrepancies exist. These may reflect differences between the sensitivity or density of 'K-selective' and 'Rb-permeable' K channels in bovine tracheal smooth muscle and vascular tissues.

The present study also examined the relationship between the effects of cromakalim, RP49356 and diazoxide on tension, membrane potential, PK and PRb. In the case of cromakalim (1 and 10 μM) the mechanical and electrical effects appear to be related to changes in both PK and PRb, as all four curves rise together and reach their peak at approximately the same time point (see Figure 4 and Table 2). In the case of RP49356 (50 μM) tension and membrane potential responses appear to correspond more closely to increases in PK since the maximum changes in these parameters temporally coincide whereas PRb continues to rise throughout the period of exposure to RP49356 (50 μM; see Figure 4). For RP49356 (5 μM) the mechanical and electrical effects appear to be related both to changes in PK and PRb since there was little difference in the profile of K and Rb permeability changes. Finally in the case of diazoxide (100 and 300 μM) there was little difference in the profile of change in PK and PRb. However, in contrast to the effects of cromakalim and RP49356 where t_{max} values for the mechanical, electrical and PK effects closely corresponded, the t_{max} values for diazoxide-induced changes in tension and membrane potential were less than those for PK and PRb. This separation of the mechanical and electrical effects from the effects on ion flux was more marked at the higher concentration of diazoxide (300 μM). It is possible that at least part of the actions of diazoxide may be mediated by an additional action other than K channel opening. Indeed, Thorens & Haeusler (1979) showed that diazoxide (at concentrations > 300 μM) exerted effects consistent with calcium channel blockade. Such an action may contribute to the relaxant effects seen in the present study but it would not account for the effects of this drug on membrane potential. Thus a further mechanism (e.g. decrease in chloride permeability) may also be involved.

In conclusion, the present results show that in bovine tracheal smooth muscle there are differences in the permeability of the channels involved in the actions of cromakalim, RP49356 and diazoxide to K and Rb. Furthermore, under the experimental conditions employed in this study, measurement of K efflux is a satisfactory indicator of changes in membrane K permeability evoked by K channel opening drugs. However, measurement changes in PRb may not always reflect changes in PK.

J.L. was supported by a grant from Rhône-Poulenc and K.M.B. was supported by a Ciba-Geigy Studentship.

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(Received October 23, 1990
Revised November 29, 1990
Accepted December 12, 1990)

Br. J. Pharmacol. (1991), **102**, 986–990

Facilitation of amphetamine-induced hypothermia in mice by GABA agonists and CCK-8

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1 Amphetamine-induced hypothermia in mice is facilitated by dopaminergic stimulation and 5-hydroxytryptaminergic inhibition. The present study was designed to investigate: (a) the involvement of other neuronal systems, such as the γ-aminobutyric acid (GABA), the opioid and the cholecystokinin (CCK-8) systems; (b) the possible contribution of hydroxylated metabolites of amphetamine to the hypothermia; (c) the capacity of dopamine itself to induce hypothermia and its mechanisms, in order to clarify the resistance of amphetamine-induced hypothermia to certain neuroleptics.

2 Pretreatment with the GABA antagonists, bicuculline and picrotoxin, did not inhibit amphetamine-induced hypothermia. The $GABA_B$ agonist, baclofen (2.5 mg kg^{-1}, i.p.) potentiated this hypothermia, whereas the $GABA_A$ agonist, muscimol, did not. γ-Butyrolactone (GBL) (40 mg kg^{-1}, i.p.) and the neuropeptide CCK-8 (0.04 mg kg^{-1}, i.p.) also induced potentiation. The opioid antagonist, naloxone, was without effect.

3 Dopamine itself (3, 9, 16 and 27 μg, i.c.v.) induced less hypothermia than the same doses of amphetamine. Sulpiride did not block dopamine-induced hypothermia, but pimozide (4 mg kg^{-1}, i.p.), *cis*(z)flupentixol (0.25 mg kg^{-1}, i.p.) and haloperidol (5 μg, i.c.v.) did. The direct dopamine receptor agonist, apomorphine, did not alter the hypothermia. Neither the 5-hydroxytryptamine (5-HT) receptor blocker, cyproheptadine, nor the inhibitor of 5-HT synthesis, *p*-chlorophenylalanine (PCPA), modified dopamine-induced hypothermia. Fluoxetine, an inhibitor of 5-HT reuptake, had no effect, whereas quipazine (6 mg kg^{-1}, i.p.), a 5-HT agonist, totally prevented the hypothermia. Hypothermia was unaffected by pretreatment with CCK-8.

4 These data indicate that the hypothermia induced by amphetamine involves not only dopaminergic and 5-hydroxytryptaminergic systems which are functionally antagonistic, but is also facilitated by direct or indirect GABA and CCK-8 receptor stimulation. This facilitation could result, in part, from modulation of dopaminergic neurotransmission. This may explain the apparent resistance of amphetamine-induced hypothermia to some neuroleptics, while dopamine-induced hypothermia is not resistant. The possible action of hydroxylated metabolites of amphetamine may also help to explain these differences.

Keywords: Amphetamine; hypothermia in mice; GABA systems; CCK-8; dopamine; hydroxylated metabolites

Introduction

Our earlier studies on amphetamine-induced hypothermia in mice demonstrated that this effect has a central origin and occurs when (+)-amphetamine is injected either intraventricularly, or directly into the hypothalamus or dopamine-containing areas, such as the nucleus caudatus, the olfactory tubercle or the nucleus accumbens (Boschi & Rips, 1982). The latter area appears to be the most responsive. Interaction studies have shown that dopaminergic and 5-hydroxytryptaminergic mechanisms are at least partially involved in this hypothermia and that these two systems display a functional antagonism (Boschi & Launay, 1985). Thus, stimulation of dopaminergic or inhibition of 5-hydroxytryptaminergic neurotransmission facilitates the hypothermic response to amphetamine.

However, our findings suggested that the mechanism underlying this hypothermia is more complex. As the nucleus accumbens is generally considered to be a major target in amphetamine-induced hypothermia, we have examined, in addition to the contributions of the dopaminergic and 5-hydroxytryptaminergic systems, the possible contributions of other neurotransmitter or neuromodulator systems which are involved in this brain area. We have therefore investigated the effects of pretreatment with γ-aminobutyric acid (GABA)-related substances, the neuropeptide cholecystokinin (CCK-8), and drugs acting on opioid systems, such as naloxone, on the hypothermia elicited by intraventricular amphetamine in mice.

Finally, we checked that the hypothermia-inducing effects of dopamine were similar to those previously described for amphetamine under identical experimental conditions. This was necessary for any attempt at explaining why this hypothermia is resistant to the dopamine receptor antagonist effect of some of the neuroleptics used in our previous study (Boschi & Launay, 1985). We therefore evaluated the effects of intraventricular dopamine on the rectal temperature of mice and determined, as in the study of the hypothermic action of amphetamine, the possible implication of the dopaminergic and 5-hydroxytryptaminergic systems, and CCK-8 neurones on dopamine-induced hypothermia.

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Methods

Animals

Male OF1 mice (Iffa Credo), 7 weeks old and weighing 30–32 g on the day of the experiment were maintained on a 12 h light-dark cycle in a temperature-regulated room (22 ± 1°C). Each mouse was used only once.

Injections

Peripheral injections were given intraperitoneally in a volume of 0.1 ml 10 g^{-1} body weight. Central injections were given via a guide cannula implanted into the right lateral ventricle one week prior to the experiment (Boschi *et al.*, 1981). The drugs were injected in a volume of 0.5 μl over a period of 50 s. Following the experiments, the location of the cannula was verified histologically after an injection of methylene blue into the cannula.

Control mice were given the same volume of vehicle alone.

Measurement of rectal temperature

Rectal temperature was measured with a thermocouple probe (Bailey Instruments), inserted to a depth of 1.5 cm. Readings were taken before (60, 30, 0 min) and after (20, 30, 60 min) the central injection of amphetamine. Mice were kept in groups of 3–4 and were free to move in their cages except during the brief time required for temperature measurement. Experiments were carried out between 10 h 00 min and 15 h 00 min in a temperature-regulated room (22 ± 1°C).

Interaction studies

The hypothermic effects occurred with doses of 3–27 μg/mouse for amphetamine and dopamine given intraventricularly. Doses of 9 μg amphetamine/mouse and 16 μg dopamine/mouse were selected for investigating antagonist actions and 3 μg/mouse of both drugs was used to investigate potentiation. Preliminary experiments determined the maximal dose of the different agonists and antagonists used which, alone, induced no change in rectal temperature. Each interaction study was performed at least twice.

Drugs

For central injections, (+)-amphetamine sulphate (De Laire) and dopamine hydrochloride (Fluka) were dissolved in sterile pyrogen-free 0.9% w/v NaCl solution (saline). Controls were given an equal volume of saline. Haloperidol (Janssen) was taken from commercially available ampoules. Controls were given distilled water. All doses refer to the free base.

For systemic injections, the following drugs were dissolved in water: apomorphine hydrochloride (Sigma), *cis*(z)flupentixol dihydrochloride (Lundbeck), cyproheptadine hydrochloride (Merck Sharp and Dohme), DL-*p*-chlorophenylalanine ethyl ester (PCPA) (Sigma), γ-butyrolactone (GBL) (Sigma), muscimol (Sigma), cholecystokinin (CCK-8S) (Sigma), naloxone hydrochloride (Endo). Solutions of pimozide (Janssen) and (±)-sulpiride (Delagrange) were prepared in water from the commercially available solutions. Fluoxetine (Lilly) and quipazine maleate (Miles) were suspended in an aqueous solution of Tween 80 (2%). (±)-Baclofen (Ciba-Geigy), picrotoxin (Sigma) and bicuculline (Sigma) were dissolved in 1 M HCl, adjusted to pH 5–6 with 5 M NaOH and diluted in water. Controls were given the appropriate vehicle. Doses of drugs are expressed as the weight of the salt.

Statistical analysis

Each experiment included two groups of animals, a drug-treated group and the appropriate control group. They were compared by the Mann-Whitney U test.

Results

Effects of pretreatment with GABA-related drugs on amphetamine-induced hypothermia

The results are shown in Figure 1. The hypothermia elicited by intraventricular (i.c.v.) amphetamine (9 μg/mouse) was not abolished by intraperitoneal (i.p.) injections of the GABA antagonists, bicuculline (2 mg kg^{-1}) or picrotoxin (0.5 mg kg^{-1}), 15 min prior to amphetamine injection.

Pretreatment for 15 min with the $GABA_A$ agonist, muscimol (0.5 mg kg^{-1}, i.p.), did not significantly potentiate the amphetamine-induced hypothermia, whereas the $GABA_B$ agonist, baclofen (2.5 mg kg^{-1}, i.p.), potentiated ($P < 0.05$) the effect of amphetamine (3 μg i.c.v.) at 30 min, which is the time of the maximal amphetamine activity. The concomitant administration of the GABA analogue, γ-butyrolactone (GBL) (40 mg kg^{-1}, i.p.) and amphetamine (3 μg, i.c.v.) also potentiated ($P < 0.05$) the hypothermia at 30 min. None of the GABA drugs caused a significant change in body temperature when given alone.

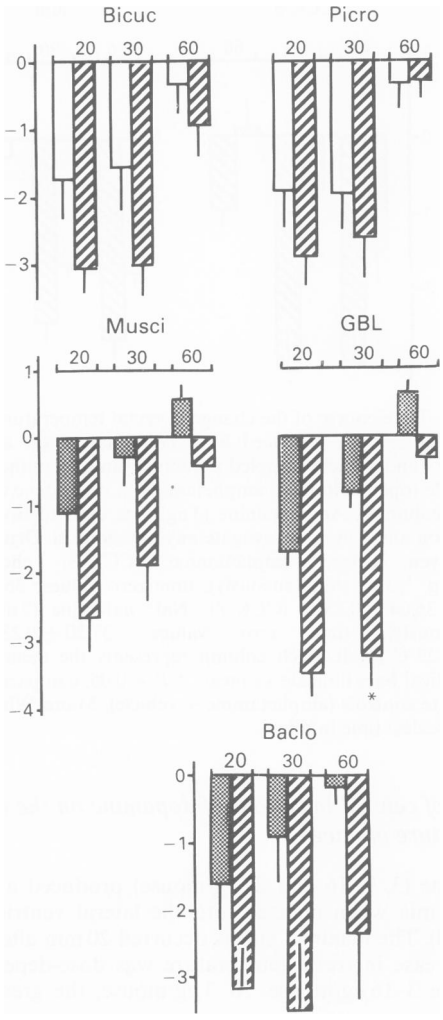

Figure 1 Time course of the change in rectal temperature (Δ°C, ordinate scales) in mice produced by a combination of: amphetamine (9 μg, i.c.v.) and vehicle (open columns); amphetamine (3 μg, i.c.v.) and vehicle (stippled columns); amphetamine (9 or 3 μg, i.c.v.) and γ-aminobutyric acid (GABA) drugs (hatched columns). Amphetamine (9 μg) was used to investigate any antagonism and 3 μg to investigate any potentiation. GABA-related drugs or vehicle were given before amphetamine. Bicuc: bicuculline (2 mg kg^{-1}, i.p., 15 min), time zero values: 36.11 ± 0.14°C (vehicle), 36.11 ± 0.19°C (Bicuc); Picro: picrotoxin (0.5 mg kg^{-1}, i.p., 15 min), time zero values: 36.31 ± 0.14°C (vehicle), 36.40 ± 0.24°C (Picro); Musci: muscimol (0.5 mg kg^{-1}, i.p., 15 min), time zero values: 36.25 ± 0.19°C (vehicle), 36.40 ± 0.17°C (Musci); GBL: γ-butyrolactone (40 mg kg^{-1}, i.p., simultaneously), time zero values: 36.37 ± 0.34°C (vehicle), 36.67 ± 0.28°C (GBL); Baclo: baclofen (2.5 mg kg^{-1}, i.p., 15 min), time zero values: 36.44 ± 0.23°C (controls), 36.68 ± 0.18°C (Baclo). Each column represents the mean of 7 to 12 mice. Vertical bars indicate s.e.mean. * $P < 0.05$, comparison with the appropriate controls (amphetamine + vehicle), Mann-Whitney U test. Abscissa scales: time in min.

Effects of pretreatment with other drugs on amphetamine-induced hypothermia

The effects of a neuropeptide, CCK-8, and naloxone, an opioid antagonist, on amphetamine-induced hypothermia are shown in Figure 2. Simultaneous injections of CCK-8 (0.04 mg kg^{-1}, i.p.) and amphetamine (3 μg, i.c.v.) caused potentiation ($P < 0.05$) 20 and 30 min after amphetamine injection. Naloxone (2 mg kg^{-1}, i.p.) did not prevent amphetamine-induced hypothermia (9 μg, i.c.v.).

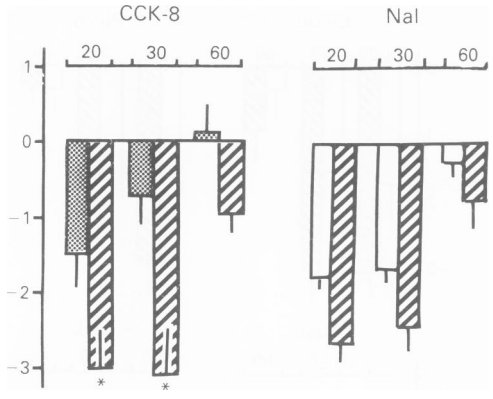

Figure 2 Time course of the change in rectal temperature (Δ°C, ordinate scales) in mice produced by a combination of: amphetamine (3 μg, i.c.v.) and vehicle (stippled columns); amphetamine (9 μg, i.c.v.) and vehicle (open columns); amphetamine (3 or 9 μg, i.c.v.) and drugs (hatched columns). Amphetamine (3 μg) was used to investigate any potentiation and 9 μg to investigate any antagonism. Drugs or vehicle were given before amphetamine. CCK-8: cholecystokinin (0.04 mg kg^{-1}, i.p., simultaneously), time zero values: 36.27 ± 0.22°C (vehicle), 36.04 ± 0.25°C (CCK-8); Nal: naloxone (2 mg kg^{-1}, i.p., simultaneously), time zero values: 37.20 ± 0.23 (vehicle), 36.72 ± 0.22°C (Nal). Each column represents the mean of 6 to 11 mice. Vertical bars indicate s.e.mean. * $P < 0.05$, comparison with the appropriate controls (amphetamine + vehicle), Mann-Whitney U test. Abscissa scales: time in min.

Effects of central injections of dopamine on the rectal temperature of mice

Dopamine (3, 9, 16 and 27 μg/mouse) produced a significant hypothermia when injected into the lateral ventricle of mice (Figure 3). The maximal effects occurred 20 min after injection. The decrease in rectal temperature was dose-dependent over the range 3–16 μg/mouse. At 3 μg/mouse, the greatest fall in rectal temperature was −1.08 ± 0.14°C ($P < 0.05$). Doses of 9 and 16 μg/mouse caused mean maximal changes in body temperature of −2.52 ± 0.18°C ($P < 0.01$) and −3.12 ± 0.26°C ($P < 0.01$) respectively. A dose of 27 μg/mouse produced the same degree of hypothermia (−2.90 ± 0.24°C, $P < 0.01$) as did 16 μg/mouse.

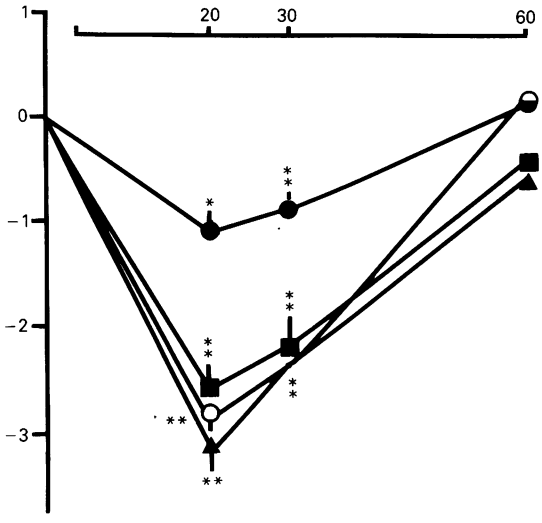

Figure 3 Time course of the change in rectal temperature (Δ°C, ordinate scale) in mice produced by increasing doses of dopamine given i.c.v. The effect is expressed as the difference between pre- and post-injection temperature corrected for the effect of 0.9% NaCl. Dopamine (●) 3, (■) 9, (▲) 16 and (○) 27 μg. Each dose was studied with its own control (saline) on separate days. Time zero values: 36.50 ± 0.15°C (dopamine 3), 36.45 ± 0.12°C (saline); 36.36 ± 0.18°C (dopamine 9), 36.90 ± 0.29°C (saline); 36.13 ± 0.21°C (dopamine 16), 36.24 ± 0.25°C (saline); 36.01 ± 0.15°C (dopamine 27), 36.29 ± 0.19°C (saline). Each point represents the mean of 7 to 11 mice. In order to simplify the figure, the vertical lines indicating the s.e.mean are shown only for the significant values. * $P < 0.05$; ** $P < 0.01$, comparison with the corresponding saline controls (not shown), Mann-Whitney U test. Abscissa scale: time in min.

Effects of pretreatment with drugs acting on dopamine receptors on dopamine-induced hypothermia

Neuroleptics were tested for their ability to block dopamine-induced hypothermia (Figure 4). The hypothermia induced by dopamine (16 μg, i.c.v.) was not abolished by injection of sulpiride (40 mg kg^{-1}, i.p.) 2 h prior to dopamine. In contrast, pretreatment with pimozide (4 mg kg^{-1}, i.p., 2 h) and haloperidol (5 μg, i.c.v., 45 min) inhibited ($P < 0.05$ and $P < 0.01$,

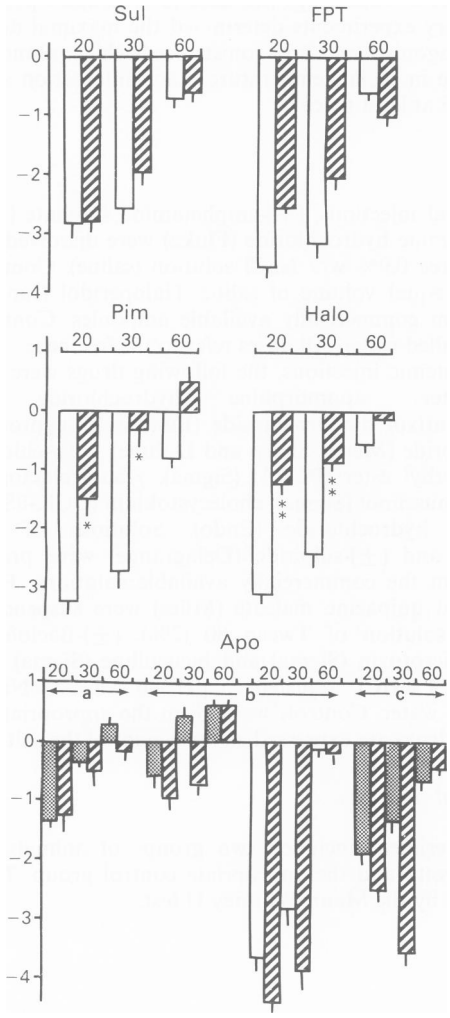

Figure 4 Time course of the change in rectal temperature (Δ°C, ordinate scales) in mice produced by the combination of: dopamine (16 μg, i.c.v.) and vehicle (open columns); dopamine (3 μg, i.c.v.) and vehicle (stippled columns); dopamine (16 or 3 μg, i.c.v.) and dopamine-related drugs (hatched columns). Dopamine (16 μg) was used to investigate any antagonism and 3 μg to investigate any potentiation. Dopamine drugs or vehicle were given before dopamine. Sul: sulpiride (40 mg kg^{-1}, i.p., 2 h), time zero values: 36.99 ± 0.19°C (vehicle), 36.41 ± 0.26°C (Sul); FPT: *cis*(z)flupentixol (0.25 mg kg^{-1}, i.p., 2 h), time zero values: 37.37 ± 0.18°C (vehicle), 36.77 ± 0.14°C (FPT); Pim: pimozide (4 mg kg^{-1}, i.p., 2 h), time zero values: 36.86 ± 0.12°C (vehicle), 35.69 ± 0.48°C (Pim); Halo: haloperidol (5 μg, i.c.v., 45 min), time zero values: 36.54 ± 0.22°C (vehicle), 36.66 ± 0.22°C (Halo). Apo: apomorphine (0.025 (a), 0.1 (b), 0.4 (c) mg kg^{-1}, i.p., 10 min). Each column represents the mean of 7 to 12 mice. Vertical bars indicate s.e.mean. * $P < 0.05$; ** $P < 0.01$, comparison with the appropriate controls (dopamine + vehicle), Mann-Whitney U test. Abscissa scales: time in min.

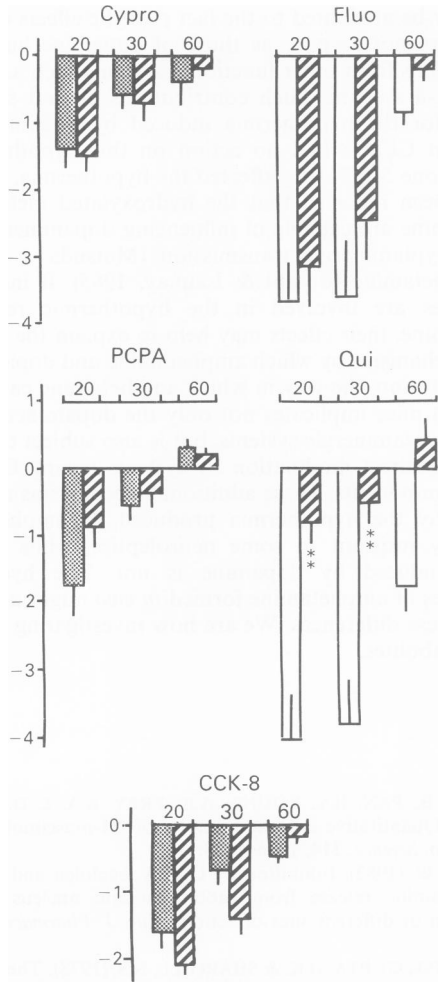

Figure 5 Time course of the change in rectal temperature (Δ°C, ordinate scales) in mice produced by the combination of: dopamine (3 μg, i.c.v.) and vehicle (stippled columns); dopamine (16 μg, i.c.v.) and vehicle (open columns); dopamine (3 or 16 μg, i.c.v.) and 5-hydroxytryptamine-related drugs or cholecystokinin (CCK-8) (hatched columns). Dopamine (3 μg) was used to investigate any potentiation and 16 μg to investigate any antagonism. Drugs or vehicle were given before dopamine. Cypro: cyproheptadine (0.5 mg kg^{-1}, i.p., 30 min), time zero values: 36.97 ± 0.26°C (vehicle), 36.36 ± 0.13°C (Cypro); Fluo: fluoxetine (10 mg kg^{-1}, i.p., 30 min), time zero values: 36.94 ± 0.18°C (vehicle), 36.77 ± 0.23°C (Fluo); PCPA: *p*-chlorophenylalanine (300 mg kg^{-1}, i.p., 48 and 24 h), time zero values: 36.48 ± 0.24°C (vehicle), 36.40 ± 0.23°C (PCPA); Qui: quipazine (6 mg kg^{-1}, i.p., 10 min), time zero values: 37.15 ± 0.12°C (vehicle), 36.18 ± 0.24°C (Qui). CCK-8: cholecystokinin (0.04 mg kg^{-1}, i.p., simultaneously), time zero values: 36.68 ± 0.18°C (vehicle), 36.20 ± 0.09°C (CCK-8). Each column represents the mean of 7 to 14 mice. Vertical bars indicate s.e.mean. ** $P < 0.01$, comparison with the appropriate controls (dopamine + vehicle), Mann-Whitney U test. Abscissa scales: time in min.

respectively) the hypothermia. *Cis*(z)flupentixol (0.25 mg kg^{-1}, i.p.), 2 h prior to dopamine, slightly reduced ($P < 0.05$) this hypothermia. Pretreatment for 10 min with the direct dopamine receptor agonist, apomorphine (0.025, 0.1, 0.4 mg kg^{-1}, i.p.), did not alter dopamine-induced hypothermia (3 and 16 μg, i.c.v.). At these doses, these drugs alone had no effect on body temperature in mice.

Effects of pretreatment with drugs acting on 5-HT receptors and CCK-8 on dopamine-induced hypothermia

Neither the 5-HT receptor blocker, cyproheptadine (0.5 mg kg^{-1}, i.p.) given 30 min before dopamine, nor the inhibitor of 5-HT synthesis, PCPA (300 mg kg^{-1}, i.p.), administered 48 and 24 h before dopamine (3 μg, i.c.v.), modified dopamine-induced hypothermia (Figure 5). A 30 min pretreatment with fluoxetine (10 mg kg^{-1}, i.p.), which inhibits the reuptake of 5-hydroxytryptamine, had no effect on dopamine-induced hypothermia (16 μg, i.c.v.). In contrast, the 5-HT receptor agonist, quipazine (6 mg kg^{-1}, i.p.) given 10 min prior to dopamine, totally prevented ($P < 0.01$) the hypothermia. Simultaneous injections of CCK-8 (0.04 mg kg^{-1}, i.p.) and dopamine (3 μg, i.c.v.) did not potentiate dopamine-induced hypothermia (Figure 5).

Discussion

We have previously shown that the nucleus accumbens may be one of the sites involved in amphetamine-induced hypothermia in mice (Boschi & Rips, 1982) and that this hypothermia is mediated by dopaminergic and 5-hydroxytryptaminergic mechanisms (Boschi & Launay, 1985). These findings confirm and extend the results of other authors (Yehuda & Wurtman, 1975; Wirtshafter *et al.*, 1978) showing that the dopamine release from the mesolimbic system, especially from the nucleus accumbens, may underlie the effects of amphetamine on thermoregulation. However, the nucleus accumbens receives not only dopaminergic (Ungerstedt, 1971) and 5-hydroxytryptaminergic (Conrad *et al.*, 1974) projections, but also contains a high concentration of GABA (Balcom *et al.*, 1975) corresponding to very dense GABA projections (Walaas & Fonnum, 1979). The present study was undertaken to obtain further information on the mechanisms of the hypothermic action of amphetamine by use of drugs which alter the function of the GABA systems in the brain. Pretreatment with the $GABA_A$ agonist muscimol (Penney *et al.*, 1981) did not potentiate the hypothermia, whereas the $GABA_B$ agonist baclofen (Bowery *et al.*, 1983; 1985) did. This facilitation of the action of amphetamine may result from GABA release induced by baclofen (Kerwin & Pycock, 1978; Roberts *et al.*, 1978). Biochemical and behavioural studies have shown that GABA exerts an inhibitory control on dopaminergic systems (Davidson, 1976; Scheel-Krüger, 1986). An attenuation of the activity of dopaminergic neurones by GABA would result in decreased dopamine release and intraneuronal catabolism, which in turn would increase the neuronal concentration of dopamine. More endogenous dopamine could then be available at the presynaptic nerve endings to be released by amphetamine. However, baclofen has also been reported to reduce the evoked release of dopamine (Reimann, 1983), to increase the dopamine in the mesolimbic dopaminergic terminals (Kelly & Moore, 1978) and to inhibit the firing rate of dopaminergic nerves (Fuxe *et al.*, 1975). All these mechanisms may be implicated in the potentiation of hypothermia by baclofen, using the explanation given above. In the same way, the combined treatment with amphetamine and γ-butyrolactone (GBL), a GABA congener which blocks nerve impulses at dopaminergic neurones (Roth *et al.*, 1973), also facilitated hypothermia. This facilitation may result from a marked increase in dopamine synthesis caused by the blockade of the impulse flow (Roth *et al.*, 1973). Nevertheless, our results disagree with a previous report indicating that GABA drugs, such as baclofen and GBL, antagonize the locomotor activity produced by such diverse stimulants as morphine, apomorphine and amphetamine (Cott & Engel, 1977). The reasons for this discrepancy remain to be elucidated, but they may result from differences in the doses of drugs used. The doses used in the present work were generally smaller than theirs (at least for GBL), so as not to modify the rectal temperature by pretreatment.

The hypothermia induced by amphetamine may also be modified by the cholecystokinin (CCK-8) and opioid systems. CCK-8 is present in high concentration in a subpopulation of dopamine-containing neurones (Hökfelt *et al.*, 1980). There is considerable controversy as to whether CCK-8 potentiates or decreases dopaminergic transmission. In our study, CCK-8 clearly potentiated the hypothermia induced by amphetamine

at a dose which, given alone, did not produce a hypothermia (Zetler, 1982). This potentiation may depend, at least partially, on dopaminergic mechanisms, since it has been suggested that CCK-8 potentiates the effect of dopamine on dopamine receptors (Studler *et al.*, 1985). Consequently, the activity of amphetamine would be enhanced.

Taken together, these results seem to indicate that endogenous dopamine modulates the hypothermia produced by amphetamine via GABA and CCK-8 interactions.

There is some evidence to suggest that endogenous opiates can alter body temperature (Rosow *et al.*, 1980) and may mediate part of the hypothermic response to dopamine receptor agonists (Weiss *et al.*, 1984). The failure to demonstrate any action of naloxone on amphetamine-induced hypothermia indicates that the naloxone-sensitive opioid systems are probably not implicated in this response.

Finally, we compared the effects of dopamine itself with the effects previously described of amphetamine so as to obtain more information on the resistance of the hypothermia induced by amphetamine to some neuroleptics. The hypothermia induced by dopamine was less intense and shorter than that produced by amphetamine. It was reduced by three neuroleptics, pimozide, *cis*-flupentixol and haloperidol, whereas the hypothermia induced by amphetamine was only blocked by haloperidol (Boschi & Launay, 1985). These differences may be attributed to the fact that the effects of amphetamine were not as pure as those of dopamine but included contributions from other functional systems such as the 5-HT and CCK-8 systems. Such contributions do not seem to be required for the hypothermia induced by dopamine, as we found that CCK-8 had no action on this hypothermia and that only one 5-HT drug affected the hypothermia. It has also recently been reported that the hydroxylated metabolites of amphetamine are capable of influencing dopaminergic and 5-hydroxytryptaminergic transmission (Matsuda *et al.*, 1989), like amphetamine (Boschi & Launay, 1985). If indeed these metabolites are involved in the hypothermic response to amphetamine, their effects may help to explain the differences in the mechanisms by which amphetamine and dopamine act.

In conclusion, the way in which amphetamine causes hypothermia in mice implicates not only the dopaminergic and 5-hydroxytryptaminergic systems, but is also subject to a further direct or indirect modulation by systems having GABA and CCK-8 components. These additional mechanisms may be the reason why the hypothermia produced by amphetamine is apparently resistant to some neuroleptics, while the hypothermia induced by dopamine is not. The hydroxylated metabolites of amphetamine formed *in vivo* might also help to explain these differences. We are now investigating the role of these metabolites.

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(Received July 31, 1990
Revised October 30, 1990
Accepted November 29, 1990)

Br. J. Pharmacol. (1991), **102**, 991–997

Blood pressure and vascular reactivity changes in spontaneously hypertensive rats fed fish oil

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1 To examine possible mechanisms of antihypertensive effects of feeding fish oil rich in n-3 fatty acids, we have studied vascular reactivity of aortic rings and perfused mesenteric resistance vessels of spontaneously hypertensive rats (SHR) given such a diet.

2 In two experiments, rats were fed a semi-synthetic diet containing either 'fish oil' (10 and 20% by weight) or hydrogenated coconut oil (control) (10 and 20%) for 4 weeks.

3 Blood pressure rose significantly less in the fish oil group than in controls in both experiments.

4 Aortic rings from control rats showed endothelium-dependent relaxations to low concentrations of acetylcholine (ACh) but relaxed less at higher concentrations. In contrast, rings from the fish oil group had relaxations which increased through the range of concentrations used. Indomethacin (10 μM) also increased the relaxation responses seen in rings from control rats, suggesting that fish oil inhibits a contractile cyclo-oxygenase product. This contractile substance may be thromboxane A_2 (TxA_2) or its endoperoxide precursor, prostaglandin H_2 (PGH_2) as aortic incubates and serum levels of TxB_2 (the stable product of TxA_2) were greatly reduced in fish oil-fed rats, and the decrease of relaxant responses to high concentrations of ACh were also blocked by a TxA_2/PGH_2 receptor blocker (SQ 29548).

5 In contrast to aortic rings, perfused preconstricted mesenteric resistance vessels of control rats relaxed to ACh in a similar fashion to tissues from fish oil-fed rats. However, in this preparation, fish oil feeding enhanced relaxations to sodium nitroprusside (SNP) and contractile responses to noradrenaline were less than controls. After removal of endothelium with 0.05% saponin, contractile responses to noradrenaline increased in both groups but responses from fish oil-treated rats were still attenuated. This suggests that fish oil feeding alters reactivity of mesenteric resistance vessels at the level of the smooth muscle.

6 The results indicate that fish oil feeding may reduce blood pressure by decreasing vascular smooth muscle reactivity to noradrenaline in resistance vessels. The effect may be enhanced by inhibition of an endothelium-derived cyclo-oxygenase product, such as TxA_2 or PGH_2 in conduit vessels.

Keywords: Fish oil; vascular reactivity; spontaneously hypertensive rats; blood pressure

Introduction

Dietary fish oils rich in eicosapentaenoic acid (EPA) have diverse effects. In man there is evidence that fish oil supplementation can lower blood pressure in subjects with essential hypertension (Norris *et al.*, 1986; Knapp & Fitzgerald, 1989; Bonaa *et al.*, 1990). The effects of dietary fish oils in rat models of hypertension are variable. Some reports have claimed that fish oil feeding lowers blood pressure (Schoene & Fiore, 1981) while others have shown no effect on blood pressure in spontaneously hypertensive rats (SHR) on a normal sodium intake but exacerbation of hypertension after salt loading (Codde *et al.*, 1987). Fish oil feeding has been reported to prevent dexamethasone induced hypertension (Codde & Beilin, 1985) but had no effect on Goldblatt 1-kidney, 1-clip hypertensive rats (Codde *et al.*, 1985).

Changes in blood pressure after fish oil feeding may be related to alterations in vascular reactivity. Fish oil supplementation has been reported to facilitate endothelium-dependent relaxations to bradykinin, adenosine diphosphate (ADP) and 5-hydroxytryptamine (5-HT) in porcine coronary arteries (Shimokawa *et al.*, 1987). Aortae of SHR treated with pure EPA also have augmented endothelium-dependent relaxation to acetylcholine (ACh) (Yin *et al.*, 1988). In this study we have further examined the effects of fish oil feeding on vascular reactivity of aortic rings and investigated the role of cyclo-oxygenase products in modulating relaxations to ACh in these vessels. In addition we have examined the effect of fish oil feeding on endothelium-dependent and independent reactivity of isolated perfused mesenteric resistance vessels of SHR.

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Methods

Male SHR (220–265 g) were studied in two separate experiments in each of which, rats were randomly divided into 2 equal groups matched for blood pressure and weight. In the first experiment, rats were fed a semi-synthetic diet containing 20% (by weight) of either 'Max EPA' fish oil (fish oil) or hydrogenated coconut oil containing 3% safflower oil (control).

In the second experiment, rats were fed 10% (by weight) of these two oil diets. The higher levels of oils were used initially to maximize effects on vascular reactivity but as rats on the diets with 20% fish oil gained less weight than controls (Table 1), 10% oils were used in the second experiment. The major fatty acid composition of these oils was analysed by gas chromatography. 'Max EPA' fish oil contained C16:0 (16%), C18:0 (3%), C18:1 (12%), C20:1 (4%), C20:5 (18%), C22:6 (12%). Safflower oil contained 16:0 (8%), 18:0 (2%), 18:1 (13%), 18:2 (77%). The hydrogenated coconut oil was free of unsaturated fatty acids and safflower oil was added so that the mixture contained 3% safflower oil to prevent essential fatty acid deficiency. The dry component of the feed consisted of (by weight): casein (22.2%), macrominerals (2.2%), vitamins and trace minerals (0.83%), cellulose (7.8%), choline chloride (0.28%), cornflour (50%) and sucrose (16.7%). The feed was mixed in our laboratories before being baked into small biscuits at 120°C for 20 min. Rat feed was kept at 4°C and made up weekly. The dietary period lasted 4 weeks. Systolic blood pressures were taken by tail-cuff sphygmomanometry on rats warmed to a temperature of 39°C for 15 min. Blood pressures were taken as an average of 3 readings before the start of the dietary period and at the end of 4 weeks. At the

Table 1 Summary of weight and blood pressure changes in both 10% and 20% oil fed spontaneously hypertensive rats (SHR)

	Control (20%) ($n = 20$)		*Fish oil* (20%) ($n = 20$)	
Expt. 1.	*start*	*end*	*start*	*end*
Weight (g)	247 ± 4	315 ± 3	250 ± 4	295 ± 4**
Blood pressure (mmHg)	195 ± 5	220 ± 5	190 ± 4	205 ± 3**
	Control (10%) ($n = 25$)		*Fish oil* (10%) ($n = 25$)	
Expt. 2	*start*	*end*	*start*	*end*
Weight (g)	237 ± 3	326 ± 4	237 ± 2	321 ± 3
Blood pressure (mmHg)	178 ± 2	231 ± 2	179 ± 2	222 ± 2**

Values are mean ± s.e.mean of rats at the start and the end of the 4 week dietary period.
Significantly less than control values ** $P < 0.01$.

end of the dietary period, the rats were anaesthetized with 0.1 ml 100 g^{-1} Nembutal (60 mg ml^{-1}) administered intraperitoneally. Two ml of blood was taken from the inferior vena cava for haematocrit, plasma electrolytes, serum fatty acid and serum thromboxane B_2 (TxB_2) analysis. In experiment 1 (20% oils), the reactivity of aortic rings alone was examined while in experiment 2, responses of both the aortic rings and perfused mesenteric resistance vessels were investigated.

Vascular reactivity in aortic rings

For experiment 1, a segment of thoracic aorta approximately 1.5 cm long was removed and cut into two rings of 5 mm length with a double bladed scalpel. One ring had its endothelium removed by gentle rubbing with a cotton probe. In histological studies carried out before this study it was seen that rubbing had clearly removed the endothelium from its adjacent smooth muscle ($n = 5$). Aortic rings were connected to force transducers (Grass FTO3) with platinum wires and mounted in 25 ml organ baths. The tissues were allowed to equilibrate for 1 h at 2 g tension in Krebs solution. Composition of the Krebs solution was (mM) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.1 and calcium EDTA 0.026. The tissues were maintained at 37°C and bubbled continuously with 95% O_2:5% CO_2. The rings were then challenged twice with priming concentrations of 30 nM noradrenaline to evaluate the viability of the tissues. A recovery period of 15 min was allowed between challenges.

Cumulative concentration-effect curves to ACh and sodium nitroprusside (SNP) were constructed on rings precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (1–7 μM) that produced a tension of approximately 1.3 g which was 50–70% of maximum contraction of the aortic rings to $PGF_{2\alpha}$.

For experiment 2, the procedure for investigation of vascular reactivity in aortic rings was the same as in experiment 1, but in addition, the role of cyclo-oxygenase products on the endothelium-dependent responses to ACh were evaluated by constructing concentration-effect curves to ACh before and after addition of indomethacin (10 μM). The inhibitor was added 15 min before and during construction of a 2nd ACh concentration-effect curve. After maximum relaxation to ACh had been achieved, methylene blue (10 μM) an inhibitor of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Rapoport & Murad, 1983; Martin *et al.*, 1985) was added to assess the role of endothelium-derived relaxing factor (EDRF) in these vessels.

In a separate experiment, to investigate the possible role of thromboxane in ACh-stimulated responses in aortic rings of SHR, SQ 29548, a (TxA_2/PGH_2) receptor blocker (Ogletree *et al.*, 1985) was used. In this experiment, ACh concentration-effect curves were constructed with and without addition of SQ 29548 (1 μM) to the organ bath. This concentration of TxA_2/PGH_2 receptor blocker was used because it has been shown to inhibit platelet aggregation and contractile responses of rat aortic strips to the thromboxane-mimetic, 11,9-epoxymethano PGH_2 (Ogletree *et al.*, 1985). The precontractile agent used in these experiments was phenylephrine (30–100 nM) to give a contraction of 1.3 g. $PGF_{2\alpha}$ was not used because SQ 29548 inhibited contractile responses to this prostaglandin.

Vascular reactivity in the perfused mesenteric bed preparation

The superior mesenteric artery was isolated and cannulated with a polyethylene cannula of dimensions 0.5 mm (internal diameter) and 1.5 mm (outer diameter), (SP 40). Warmed Krebs solution (37°C) was immediately perfused at a flow rate of 2.8 ml min^{-1}. The Krebs solution used was of the same composition as that described above except that EDTA was not added. The thoracic aorta was then quickly removed for organ bath studies (procedure is described above). The small intestine was carefully dissected away from the mesenteric vessels and the preparation mounted in a water jacketed organ bath maintained at 37°C. Krebs solution was pumped by a 3-channel peristaltic pump (Pharmacia P-3) through polyethylene tubing (SP 70) connected to a debubbler and to the cannula (Figure 1). The debubbler was of 2 ml volume where at least 1 ml was always filled with perfusion fluid. Perfusion pressure was measured by a pressure transducer (Gould-Statham P 23ID) via a side arm and connected to a Grass polygraph (model 7B). All tubing was immersed in the water bath at 37°C.

All three channels of the peristaltic pump were working simultaneously to deliver solutions at a constant flow rate. When drug solutions were to be perfused, tubing running through channel B was simply removed from the Krebs solution and placed in a tube containing the drug. Similarly, if two drugs were to be perfused simultaneously, then channel B and C were used. This enabled us to perfuse two drug solutions simultaneously without altering the flow rate.

Basal pressure of mesenteric preparations of fish oil-fed rats (15 ± 0.6 mmHg, $n = 15$) were similar to that of control preparations (16 ± 0.8 mmHg, $n = 15$). After 30 min equilibration, the preparation was perfused with 5 μM phenylephrine to ensure the viability of the preparation. This perfusion was repeated before concentration-effect curves to ACh, SNP and noradrenaline were constructed in all tissues. The order was as mentioned and was strictly maintained. When examining the relaxation responses to ACh and SNP, the tissues were preconstricted with phenylephrine (10–40 μM) to obtain a

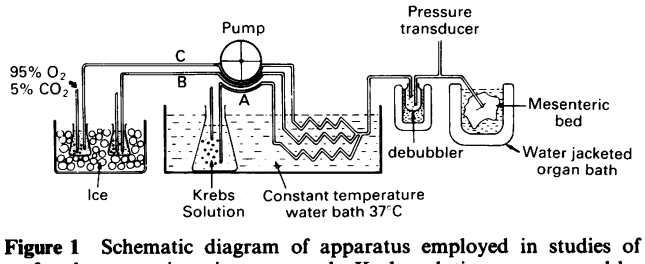

Figure 1 Schematic diagram of apparatus employed in studies of perfused mesenteric resistance vessels. Krebs solution was pumped by a 3-channel peristaltic pump connected to a debubbler and to the cannula. Perfusion pressure was measured by a pressure transducer via a side arm. To maintain a constant temperature all tubing was immersed in the water bath at 37°C. Krebs solution was pumped through all three channels simultaneously to deliver solutions at a constant flow rate of 2.8 ml min^{-1}. When drugs were to be perfused, the end of the tubing running through either channel B or channel C was simply placed in a flask containing the specific drug dissolved in Krebs solution. This method allowed the perfusion of several drugs simultaneously without alteration in flow rate.

background perfusion pressure of 70 mmHg above baseline. This was between 50–70% of maximum contraction response to phenylephrine. After construction of the relaxation and constrictor curves mentioned above, 0.05% saponin was perfused through the preparation for 90 s to remove the endothelium. Saponin has previously been reported to remove endothelium without damaging the underlying smooth muscle (Chiba & Tsukada, 1984; DeMey & Gray, 1985). In preliminary experiments, it was seen that the preparations did not relax to ACh after saponin perfusion but relaxations to SNP were unimpaired ($n = 4$). In histological sections stained with Factor VIII, saponin had significantly damaged the endothelium ($n = 3$). Basal pressure after saponin perfusion was slightly raised but was not significantly different between groups (fish oil: 21 ± 0.8 mmHg, $n = 15$; control: 21 ± 1 mmHg, $n = 15$). Lastly, a second concentration-effect curve to noradrenaline was constructed.

Serum thromboxane B_2 and fatty acid analysis

Blood samples were allowed to clot by incubation for 30 min at 37°C and were then centrifuged at 2500 r.p.m. for 15 min. Aliquots of 100 μl for serum TxB_2 and 200 μl were taken for fatty acid analysis and stored at −20°C. Serum TxB_2 was measured by direct radioimmunoassay (RIA). The relative fatty acid composition of serum was determined by gas chromatography of the corresponding methyl esters. Fatty acid methyl esters were prepared by treatment of serum extracts with 4% H_2SO_4 in methanol at 100°C for 20 min. Methyl esters were analysed by gas chromatography (Hewlett-Packard 5890A) on a 10 m × 0.53 m Superox 11 Column (Alltech), temperature programmed from 190°C to 245°C at 5° per min with nitrogen carrier gas and a split ratio of 30:1. Peak areas were calculated automatically with a Hewlett-Packard 3393A computing integrator.

Aortic thromboxane B_2 and 6-keto prostaglandin $F_{1\alpha}$ analysis

Segments of abdominal aorta (1 cm long) were also removed from the rats for analysis of eicosanoid production. The segments of aorta were cleared of fat and connective tissue before being incubated in Krebs solution (without EDTA) for 1 h at 37°C. The segments were then removed, blotted dry and weighed. Tissue incubates were stored at −20°C until analysis by direct RIA (Codde *et al.*, 1984).

Haematocrit and plasma electrolytes

Part of the plasma collected was placed in microhaematocrit tubes and centrifuged in a microfuge. The rest of the plasma was placed in heparin-lined tubes and sent for electrolyte analysis at the Biochemistry Department of Royal Perth Hospital.

Drugs and solutions

Acetylcholine chloride, noradrenaline (arterenol) bitartrate, sodium nitroprusside, $PGF_{2\alpha}$ and indomethacin were all purchased from Sigma chemicals. Saponin was from BDH chemicals and methylene blue was from AJAX chemicals. A stock solution of $PGF_{2\alpha}$ (10 mg ml^{-1}) was made up in ethanol and stored at −20°C. On the days of the experiment, $PFG_{2\alpha}$ was made up fresh by drying down the required amount with nitrogen and reconstituting in Krebs solution. Indomethacin was made up fresh everyday by dissolving in equimolar sodium carbonate. The TxA_2/PGH_2 receptor blocker, SQ 29548 ([1S-[1α,2β(5Z),3β,4α]]-7-[3-[[2-(phenylamino) carbonyl] hydrazino]methyl]-7- oxabicyclo[2.2.1] hept-2-yl]-5-heptenoic acid) was a donation from Squibb. A solution of 10 mg ml^{-1} was made up in 95% ethanol and then diluted down to the required concentration with 2 mM sodium carbonate. Concentration of ethanol in the organ bath was 0.0095%. All other drugs were made up in Krebs solution. Concentrations mentioned in the text are final bath concentrations.

Statistics

Statistical differences between concentration-effect curves of responses from different dietary groups were ascertained by calculating mathematically the area under the curve by the methods outlined by Matthews *et al.* (1990). The areas were then tested by either unpaired or paired t tests. Relaxations are expressed as the percentage of the precontractile agent. EC_{50} is taken as the mean of 50% of maximum responses of individual concentration-effect curves. Means were then compared by either paired or unpaired t tests. All results are expressed as mean ± s.e.mean with n = number of rats. P values of less than 0.05 were considered significant.

Results

Blood pressure

In the first experiment, systolic blood pressure of both groups increased over the 4 week dietary period but rats fed 20% fish oil had 15 mmHg lower systolic pressure (Table 1) compared to control rats ($P < 0.01$). Body weights also increased less with fish oil feeding (Table 1). In the second experiment when the rats were given 10% oils, there was a smaller (9 mmHg) but significant difference ($P < 0.01$) in the blood pressure rise between the two groups while body weights of fish oil-fed rats increased to a similar extent compared to controls.

Vascular reactivity

Aortic rings: Rings with intact endothelium from control rats fed 20% saturated fats relaxed to 0.1 μM ACh (Figure 2) but relaxed less to higher concentrations. Responses were significantly different between 0.1–3 μM ACh ($P < 0.01$) with the highest concentration causing a relaxation of $41 \pm 6\%$ ($n = 14$) in rings from control SHR while rings from fish

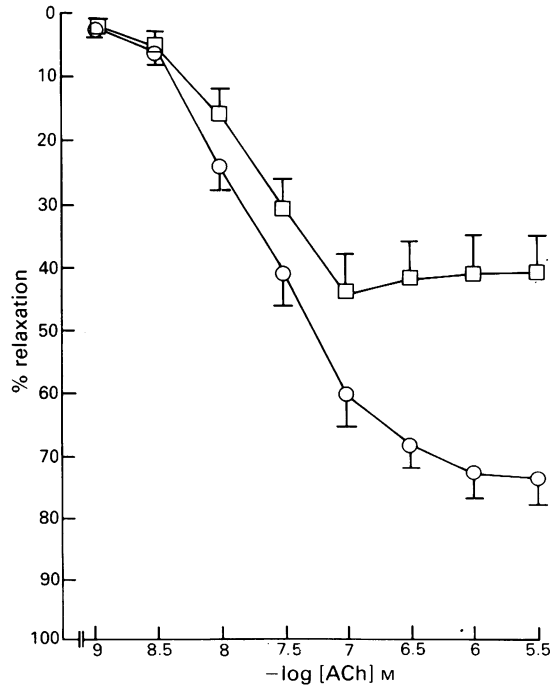

Figure 2 Effects of fish oil (20%) feeding on endothelium-dependent relaxation of aortae to acetylcholine (ACh). Cumulative concentration-effect curves were constructed on rings from fish oil-fed rats (○; $n = 19$) and control (□; $n = 14$) rats. Aortic rings were precontracted with prostaglandin $F_{2\alpha}$ (1–7 μM) to a tension of 1.3 g.

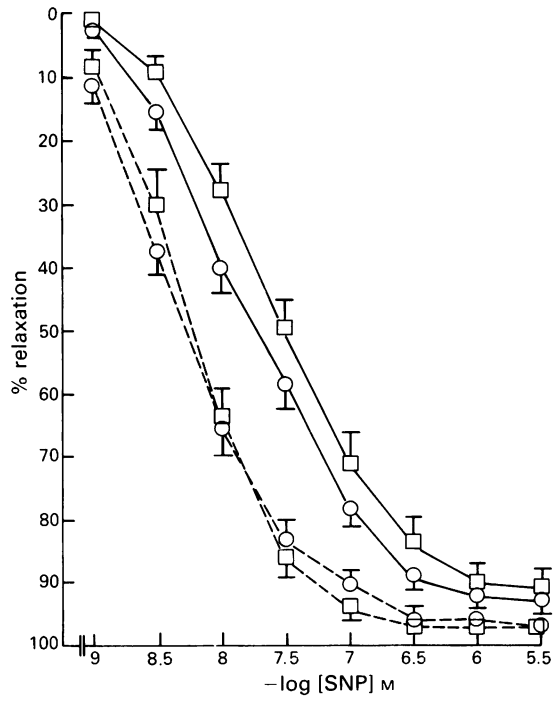

Figure 3 Cumulative concentration-effect curves to sodium nitroprusside (SNP) of aortae from 20% fish oil [(○), continuous lines = with endothelium ($n = 15$); broken lines = without endothelium ($n = 17$)] and control rats, [(□), solid lines = with endothelium ($n = 15$), broken lines = without endothelium ($n = 15$)]. Aortic rings were precontracted with prostaglandin $F_{2\alpha}$ (1–7 μM) to a tension of 1.3 g before cumulative concentrations of sodium nitroprusside were added.

oil-fed rats relaxed to 74 ± 4% of $PGF_{2\alpha}$ precontraction ($n = 19$). Rings denuded of endothelium did not relax or contract in response to ACh. Endothelium-dependent relaxations to SNP were similar in both groups of rats (Figure 3). The rings without endothelium were more sensitive to SNP than aortae with endothelium. The EC_{50}s were as follows: control (with endothelium), 70.4 ± 20.1 nM ($n = 15$), fish oil (with endothelium), 20.7 ± 5 nM ($n = 15$); control (without endothelium), 6.9 ± 1.1 nM ($n = 17$; $P < 0.01$ as compared to rings with endothelium), fish oil (without endothelium), 8.0 ± 2.0 nM ($n = 15$; $P < 0.01$ as compared to rings with endothelium).

In the second experiment, intact aortic rings from control rats fed 10% saturated fat, showed responses similar to those seen in rats fed 20% saturated fats (Figure 4). Rings from fish oil-fed rats relaxed more than controls between 0.3 μM–30 μM ACh ($P < 0.01$). At 0.3 μM, rings from fish oil-fed rats (53 ± 3%) relaxed to a greater extent than controls (42 ± 4%). At higher concentrations of ACh, control rings relaxed less but rings from fish oil-fed rats continued to relax. Preincubation with indomethacin (10 μM) increased the relaxations to high concentrations of ACh in control tissues. Indomethacin enhanced relaxations to ACh in rings from fish oil-fed rats (68 ± 3% at 30 μM) compared to control rings preincubated with indomethacin (58 ± 4% at 30 μM; $P < 0.05$). Methylene blue (10 μM) completely reversed the relaxations in rings from both groups of rats (data not shown). Rings without endothelium did not relax to ACh.

In the presence of the TxA_2/PGH_2 receptor blocker SQ 29548, relaxations to 0.3–30 μM ACh continued to increase in aortic rings taken from SHR ($n = 7$; $P < 0.01$) (Figure 5).

Perfused mesenteric resistance vessels Tissues from fish oil-fed rats were more sensitive (2 fold shift in EC_{50} vs controls) and relaxed more to concentrations of SNP between 1 nM and 10 μM (Figure 6a) as compared to control tissues. In contrast to the aortic rings, control preparations showed no enhancement of relaxation to ACh by fish oil (Figure 6b).

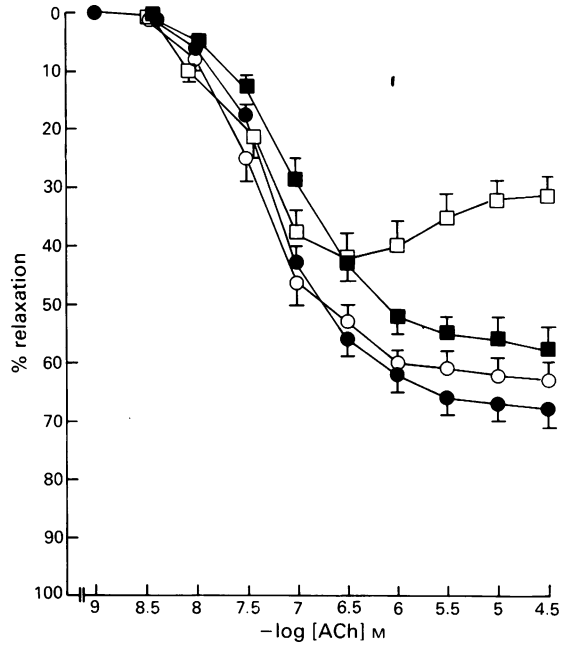

Figure 4 Endothelium-dependent relaxations to acetylcholine (ACh) of aortic rings from 10% fish oil (○)-fed ($n = 14$) and control (□) rats ($n = 16$). Cumulative concentration-effect curves were constructed on rings precontracted with prostaglandin $F_{2\alpha}$ (1–7 μM) to a tension of 1.3 g. Indomethacin (10 μM) was incubated with rings from fish oil-fed (●) rats ($n = 15$) and control (■) rats ($n = 15$), 15 min before and throughout construction of a 2nd concentration-effect curve to ACh. Areas under the curve were calculated and statistical significance tested by unpaired t tests when assessing differences between aortic rings. Paired t tests were only used when assessing the reactivity to ACh within the same tissue before and after incubation with indomethacin.

Tissues with intact endothelium from fish oil-fed rats contracted less to noradrenaline between 3–30 μM ($P < 0.05$) than tissues from control rats. Contractile responses to 30 μM noradrenaline in fish oil-fed rats (163 ± 18 mmHg) were almost

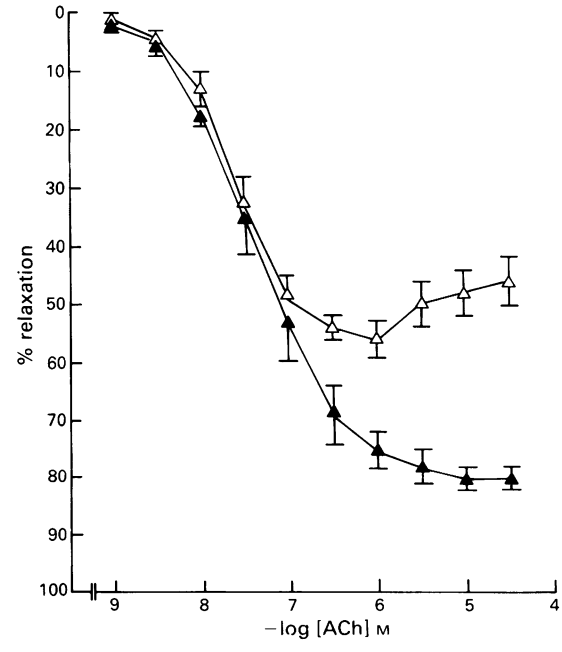

Figure 5 Concentration-effect curves to acetylcholine (ACh) of aortic rings in spontaneously hypertensive rats (SHR) with (▲) and without (△) the thromboxane A_2/prostaglandin H_2 receptor blocker, SQ 29548. Cumulative concentration-effect curves were constructed in rings precontracted by phenylephrine (30–100 nM) to give a contraction of 1.3 g. Values are mean of $n = 7$ rats in each group; s.e.mean shown by vertical bars.

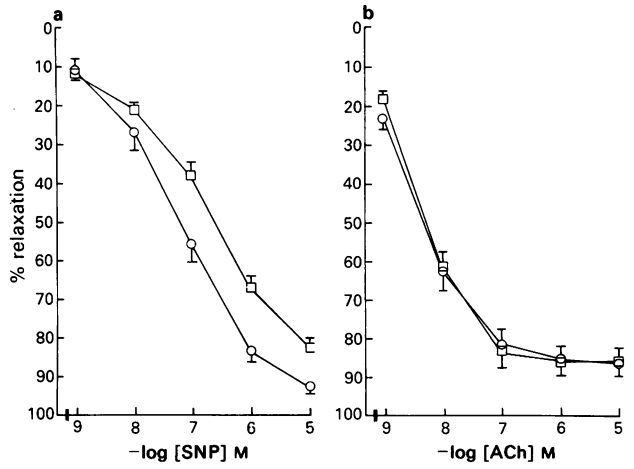

Figure 6 (a) Concentration-effect curves to sodium nitroprusside (SNP) of perfused mesenteric resistance vessels from 10% fish oil-fed spontaneously hypertensive rats (SHR, ○) and controls (□), $n = 15$ in both groups. EC_{50} of tissues from fish oil-fed rats ($0.09 \pm 0.02\,\mu M$) was significantly less than control tissues ($0.2 \pm 0.03\,\mu M$; $P < 0.01$). Mesenteric resistance vessel preparations were perfused with phenylephrine (10–40 μM) to achieve a perfusion pressure of 70 mmHg above baseline before construction of a cumulative concentration effect curve to SNP. (b) Concentration-effect curves to acetylcholine (ACh) of perfused mesenteric resistance vessels from 10% fish oil-fed SHR (○) and controls (□). Points are mean of 15 rats in both groups; s.e.mean shown by vertical bars.

half that of controls (92 ± 15 mmHg; Figure 7) but the EC_{50} was not different between groups. This lack of difference in EC_{50} could have been due to the large difference in contractile responses to the maximum concentration of 30 μM noradrenaline used. Both sensitivity and contractions to 30 μM noradrenaline were increased in endothelium-denuded preparations as compared to endothelium-intact preparations of the two dietary groups (Figure 7). Nevertheless, fish oil-fed tissues still contracted less in response to noradrenaline (between 1–10 μM; $P < 0.01$) than control preparations. Sensitivity of preparations from fish oil-fed rats to noradrenaline was significantly less than (2 fold rightward shift in EC_{50} vs controls) controls in tissues denuded of endothelium.

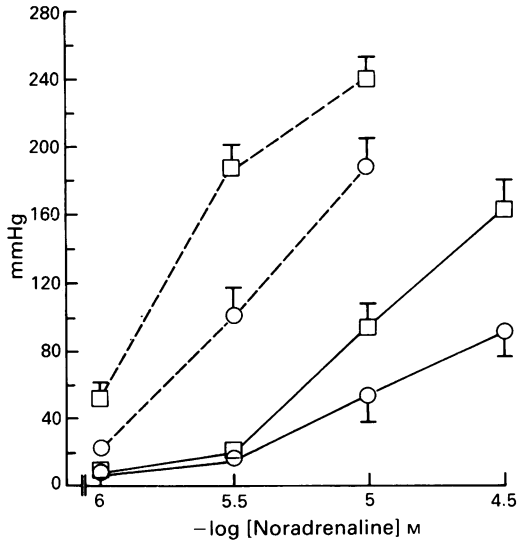

Figure 7 Concentration-effect curves to noradrenaline of perfused mesenteric resistance vessels from 10% fish oil-fed spontaneously hypertensive rats (SHR). [(○), solid lines = with endothelium, broken lines = without endothelium)] and controls [(□), solid lines = with endothelium, broken lines = without endothelium)], $n = 15$ for all groups. To investigate the role of endothelium in modulating the responses of these preparations to noradrenaline, the endothelium was removed by perfusion of 0.05% saponin for 90 s before construction of a second concentration-effect curve to noradrenaline. Areas under the curve were analysed statistically by unpaired t tests for preparations from different dietary groups. Paired t tests were only used to assess significant differences within the same preparation before and after saponin perfusion. EC_{50} of tissues with intact endothelium from fish oil-fed rats ($9.2 \pm 0.9\,\mu M$) was similar to control tissues ($9.2 \pm 0.6\,\mu M$). After saponin perfusion, sensitivity to noradrenaline was increased in tissues from both groups (fish oil, EC_{50}: $2.0 \pm 0.1\,\mu M$; control, EC_{50}: $4.7 \pm 0.6\,\mu M$) as compared to intact preparations but EC_{50} of control tissues was more than that from fish oil-fed rats ($P < 0.01$ for both comparisons).

Table 2 Serum fatty acids of 10% oil-fed spontaneously hypertensive rats (SHR)

	Control ($n = 8$)	*Fish oil* ($n = 10$)
16:0	29.0 ± 0.8	33.0 ± 1.5*
18:0	22.5 ± 0.8	24.0 ± 1.6
18:1	18.0 ± 0.8	12.9 ± 0.7**
18:2	9.6 ± 0.8	6.6 ± 0.7**
20:4	15.3 ± 1.2	8.2 ± 0.6**
20:5	trace	6.3 ± 1.1**
22:6	1.0 ± 0.2	5.6 ± 0.7**

Results expressed as % serum fatty acid composition.
Significantly different from control values. * $P < 0.05$; ** $P < 0.01$.

The following biochemical analyses were carried out in animals fed 10% fish oil or hydrogenated coconut oil.

Serum fatty acids

Arachidonic acid (20:4) levels were 54% lower in serum from 10% fish oil-fed rats than in controls (Table 2). Eicosapentaenoic acid (20:5) levels were much greater in the serum of fish oil-fed rats than in control rats.

Aortic and serum eicosanoid production

Aortae of 10% fish oil-fed rats had a reduced capacity to generate TxB_2 and 6-keto $PGF_{1\alpha}$ than controls (Table 3) by 25% and 43% respectively. TxB_2 levels of serum from fish oil-fed rats were approximately 3 times lower than controls.

Plasma electrolytes and haematocrit

There were no differences in plasma Na, urea, creatinine or haematocrit between dietary groups.

Discussion

These results demonstrate two possible mechanisms by which fish oil may have an antihypertensive effect and attenuate the blood pressure rise in SHR. Firstly, there was a reduction in vascular reactivity to noradrenaline of resistance vessels and secondly enhanced endothelium-dependent relaxation to ACh in conduit vessels.

In perfused mesenteric resistance vessels the major findings were that fish oil feeding (i) reduced the contractile responses to noradrenaline by a mechanism independent of endothelium, (ii) facilitated the relaxations to SNP and (iii) did not affect the relaxations to ACh. Contractile responses to 30 μM noradrenaline were decreased in fish oil-fed animals. After the

Table 3 Eicosanoid production in tissues of 10% oil-fed spontaneously hypertensive rats (SHR)

	Control	*Fish oil*
Aortic TxB_2 ($ng\,mg^{-1}$)	0.48 ± 0.08 ($n = 16$)	0.12 ± 0.02** ($n = 16$)
Serum TxB_2 ($ng\,ml^{-1}$)	769 ± 60.9 ($n = 18$)	213 ± 13.5** ($n = 18$)
Aortic 6-keto $PGF_{1\alpha}$ ($\mu g\,mg^{-1}$)	17.5 ± 2.5 ($n = 16$)	7.6 ± 1.0** ($n = 16$)

Significantly less than control values; $P < 0.01$.

endothelium was removed, the contractile responses to noradrenaline increased in both groups, while both the sensitivity and contractions to 30 μM noradrenaline were still reduced in tissues from fish oil-fed rats. The increased reactivity to noradrenaline after endothelium removal is similar to that reported in deoxycorticosterone acetate (DOCA) hypertensive rats (King & Webb, 1988) and may be attributable to stimulation by noradrenaline of endothelium-derived relaxing factor (EDRF) release (Cocks & Angus, 1983) or the basal release of EDRF may inhibit the contractile response to noradrenaline (Bullock *et al.*, 1986).

The mechanism by which the fish oil diet decreased mesenteric vascular reactivity to noradrenaline is unclear at present. It is interesting to speculate that its effect might be due to changes at the receptor level, alterations to the excitation/contraction coupling or changes to the contractile process. When the α_1-adrenoceptor is occupied, inositol-1,4,5 trisphosphate is released into the cytoplasm and stimulates the release of calcium from the sarcoplasmic reticulum (Abdel-Latiff *et al.*, 1986). The inositol-1,4,5 trisphosphate-stimulated calcium release is large enough to cause myosin light chain kinase activation (Van Breemen *et al.*, 1986) and hence smooth muscle contraction. Locher and co-workers (1988) reported that vascular smooth muscle cells treated for 4 weeks with either 'Max EPA' fish oil or pure eicosapentaenoic acid significantly reduced low-density lipoprotein stimulated synthesis of inositol-1,4,5 trisphosphate. Thus it could be possible that fish oil decreased the reactivity of resistance vessels to noradrenaline by reducing phosphoinositide activity and hence inositol-1,4,5 trisphosphate-stimulated calcium release from the sarcoplasmic reticulum. It is also possible that fish oil feeding could affect the structure and alter the phosphorylation/dephosphorylation regulation of the α_1-adrenoceptors. Investigation into whether the decreased responses were specific to noradrenaline may help to confirm this hypothesis.

The increased relaxations after fish oil feeding of resistance vessels to SNP which acts directly on the smooth muscle, are difficult to explain but may be linked to the mechanism involved in the decreased reactivity of these vessels to noradrenaline. SNP is believed to cause relaxation through the generation of nitric oxide via a cysteine-dependent enzyme system at the smooth muscle level (Moncada *et al.*, 1988). It is possible that fish oil feeding enhanced the production of nitric oxide through this cysteine-dependent enzyme system.

In contrast to aortic rings, perfused mesenteric resistance vessels of controls did not exhibit a decrease in relaxation at high concentrations of ACh. This indicates that there is less (if any) production of the contractile cyclo-oxygenase metabolite in the resistance vessels than in the aortic rings. As ACh stimulates the production/release of EDRF from the mesenteric resistance vessels, it appears that fish oil did not affect the stimulated production/release of EDRF from these vessels.

It was recently reported that isolated segments (3rd order) of mesenteric resistance vessels of SHR with intact endothelium contract in response to high concentrations of ACh and that this effect is abolished by indomethacin (Watt & Thurston, 1989) suggesting that the contractions are caused by a cyclo-oxygenase product. In another study in which resistance vessels were taken just proximal to the gut wall of the stroke-prone SHR, the results were similar to ours in that no decrease in relaxation to high concentrations of ACh was observed (Tesfamariam & Halpern, 1988). A possible explanation for these discrepancies could be the size of the vessels used, with larger vessels producing more of the contractile factor.

Aortic rings (with intact endothelium) of control SHR in both experiments (20% and 10% oils) relaxed to 0.1 and 0.3 μM ACh respectively but relaxed less to higher concentrations. These rings preincubated with indomethacin relaxed to 0.1 μM ACh and continued relaxing at higher concentrations of ACh. A similar effect of indomethacin has been reported in precontracted aortae of adult SHR (Luscher & Vanhoutte, 1986) and old WKY rats (Koga *et al.*, 1989). Indomethacin reduced ACh-induced contractions in intact quiescent rings of canine basilar arteries (Katusic *et al.*, 1988), rabbit pulmonary arteries (Altiere *et al.*, 1986) and aortae of SHR (Luscher & Vanhoutte, 1986). Our results confirm the hypothesis that ACh stimulates the production of a contractile cyclo-oxygenase substance that decreases relaxation of aortic rings in response to ACh.

Rings from fish oil-fed rats relaxed to ACh in an essentially similar fashion to control rings preincubated with indomethacin. This would suggest that fish oil enhanced endothelium-dependent relaxation to ACh by inhibiting the production of a contractile cyclo-oxygenase product. That the contractile cyclo-oxygenase product may be TxA_2 or an endoperoxide precursor is supported by the demonstration of a reduced capacity of serum and aortae of fish oil-fed rats to produce TxB_2 as has been demonstrated in other rat tissues (Croft *et al.*, 1985). A previous report has shown that both basal and ACh-stimulated production of TxB_2 is greater in aortae of SHR than normotensive WKY controls (Luscher *et al.*, 1986). Whereas the use of imidazole as a thromboxane-synthetase inhibitor failed to block the ACh-induced contractions (Luscher & Vanhoutte, 1986), the thromboxane synthetase inhibitor, CV-4151 significantly reduced the ACh-induced contractions in aortae of old WKY rats (Koga *et al.*, 1989). In our hands, the TxA_2/PGH_2 receptor blocker, SQ 29548 increased the endothelium-dependent relaxations to ACh and stopped the decrease in relaxant responses of aortic rings to high concentrations of ACh. Recently, it has been suggested that the endothelium-derived contracting factor released by ACh in aortae of SHR may be PGH_2 (Kato *et al.*, 1990) because the TxA_2/PGH_2 receptor blocker SQ 29548 inhibited the contractile responses to ACh but the thromboxane synthetase inhibitor OKY 046 did not. This provided indirect evidence that the contractile cyclo-oxygenase product is PGH_2 but direct evidence is required to prove this hypothesis. Thus our results lend support to the hypothesis that the aortic endothelium of SHR produces a contractile cyclo-oxygenase product in response to high concentrations of ACh. We postulate that this contractile product may be TxA_2 or PGH_2 and that their synthesis is inhibited by fish oil feeding.

The active component of dietary fish oils that is involved in mediating these possible changes in cyclo-oxygenase is probably EPA. Dietary EPA has been reported to depress the production of TxA_2 by reducing platelet phospholipid arachidonic acid stores and by competitively inhibiting cyclo-oxygenase (Needleman *et al.*, 1979). Another fatty acid found in fish oils is docosahexaenoic acid (DHA) but this fatty acid is probably not as active as EPA. Croft and co-workers reported that a diet rich in DHA did not alter arachidonic acid content of either liver or kidney phospholipids (Croft *et al.*, 1987). Whole blood thromboxane and vascular prostacyclin was also unchanged in rats fed a DHA-enriched diet.

It is unlikely that the enhanced relaxations to ACh could be attributed to the increased production of vasodilator prostaglandins, as indomethacin would have blocked the production of such prostanoids and one would then expect to have even less relaxation occurring when adding indomethacin to aortic rings of fish oil-fed rats. Another alternative is that fish oil feeding increased the production/release of EDRF. This was postulated as the mechanism for augmented endothelium-dependent relaxations in coronary arteries (Shimokawa *et al.*, 1987) and microvessels (Shimokawa *et al.*, 1988) of pigs fed fish oil. This explanation may be plausible in our experiment since methylene blue which inhibits guanylate cyclase and hence the action of EDRF (Martin *et al.*, 1985) completely reversed the endothelium-dependent relaxations to ACh of all tissues. However, this finding does not provide any information on whether the enhanced endothelium-dependent relaxations seen in aortae of fish oil-fed rats was due to increased production/release of EDRF, it simply indicates that ACh caused endothelium-dependent relaxations which were mediated by EDRF. One way to resolve the issue of the role of

EDRF in aortae of fish oil-fed rats is to assay the release of the substance.

Relaxations to ACh are probably due primarily to the production/release of EDRF (Peach *et al.*, 1985) and to a much lesser extent prostacyclin which has been shown to be the main vasodilator prostanoid released from ACh stimulation (Luscher *et al.*, 1986; Luscher & Vanhoutte, 1986). In our experiment, fish oil-treated rings relaxed more between 0.1 and 0.3 μM ACh than control rings with indomethacin. This could be caused by fish oil increasing EDRF production/release or by indomethacin inhibiting both the cyclo-oxygenase derived contractile factor as well as prostacyclin.

In summary, our results suggest that fish oil feeding could attenuate the blood pressure rise in SHR by a decrease in reactivity of resistance vessels to noradrenaline. The blood pressure lowering effect could be enhanced by an effect on compliance of conduit vessels caused by the inhibition of an endothelium generated contractile cyclo-oxygenase product such as TxA_2 or PGH_2.

These results may help explain the antihypertensive effect of fish oils in man and in some forms of experimental hypertension.

We would like to thank Michael Jorgensen for his excellent technical assistance and Dr Bruce Latham of the Department of Pathology, Royal Perth Hospital for doing the histology. This work was supported by the National Heart Foundation of Australia and the Medical Research Foundation of Royal Perth Hospital.

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(Received July 31, 1990
Revised December 19, 1990
Accepted December 20, 1990)

Br. J. Pharmacol. (1991), **102**, 998–1002

The mechanism of the sympathoinhibitory action of urapidil: role of 5-HT_{1A} receptors

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1 An investigation was carried out to determine if the sympathoinhibition caused by urapidil is due to activation of 5-HT_{1A} receptors by investigating whether it could be reversed by the non-selective 5-HT_{1A} receptor antagonist spiperone. To control for the possibility of functional antagonism by spiperone, the ability of spiperone to reverse the sympathoinhibition caused by clonidine was also investigated. These experiments were carried out in anaesthetized prazosin-pretreated cats to prevent the α_1-adrenoceptor antagonist action of urapidil and spiperone from masking any effects observed.

2 Cats were anaesthetized with α-chloralose and simultaneous recordings were made of whole cardiac, splanchnic and renal nerve activities, blood pressure, heart rate and femoral arterial flow (from which conductance was derived). All animals were initially pretreated with prazosin (1 mg kg^{-1}, i.v.) given in divided doses (0.75 followed 10 min later by 0.25 mg kg^{-1}), then either urapidil (0.75 mg kg^{-1}, i.v.) or clonidine (10 μg kg^{-1}, i.v. in two divided doses) followed by 3 separate injections of spiperone (1 mg kg^{-1}, i.v.). In another set of experiments urapidil was given followed by injections of the appropriate vehicle for spiperone, while in another set urapidil was replaced with an injection of the appropriate vehicle followed by injections of spiperone. In the experiments with clonidine, the α_2-adrenoceptor antagonist Wy 26392 (0.3 mg kg^{-1}) was given after the last injection of spiperone.

3 The prazosin pretreatment caused a fall in blood pressure associated with femoral vasodilatation, a small bradycardia and little change in cardiac, splanchnic or renal nerve activities. Urapidil or clonidine injection after prazosin caused sympathoinhibition associated with an additional bradycardia. However, only urapidil caused an additional fall in blood pressure. Spiperone injections reversed the sympathoinhibition caused by urapidil but not that caused by clonidine. The sympathoinhibition caused by clonidine was reversed by the α_2-adrenoceptor antagonist Wy 26392.

4 These results show that the sympathoinhibition caused by urapidil in prazosin-pretreated cats can be reversed by spiperone. The reversal of this sympathoinhibition is not due to functional antagonism. It is concluded that urapidil can cause sympathoinhibition by activation of 5-HT_{1A} receptors.

Keywords: 5-HT_{1A} receptors; sympathoinhibition; blood pressure; urapidil; clonidine; prazosin; spiperone; anaesthetized cats

Introduction

The central sympathoinhibitory action of urapidil cannot simply be explained by its ability to block central α_1-adrenoceptors (Kellar *et al.*, 1984; Sanders & Jurna, 1985; Van Zwieten *et al.*, 1985; Ramage, 1986b). Instead, the similarity of the cardiovascular and central sympathoinhibitory actions of urapidil to those of the 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptor agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), have led to the suggestion that it may be activation of 5-HT_{1A} receptors that is responsible for the central sympathoinhibition caused by urapidil (Ramage, 1986b). Urapidil has since been shown to bind to 5-HT_{1A} receptors (Fozard & Mir, 1987; Groß *et al.*, 1987) and to show agonist activity at this receptor (Fozard & Mir, 1987; Schoeffter & Hoyer, 1988) supporting such a hypothesis.

However, evidence from *in vivo* experiments that urapidil lowers blood pressure by a 5-HT_{1A} receptor agonist action is contradictory. Doods *et al.* (1988) were unable to prevent the central hypotensive action caused by urapidil using (−)-pindolol, which is known to block 5-HT_{1A} receptors (Schoeffter & Hoyer, 1988; Hoyer, 1988). On the other hand, spiroxatrine, another putative 5-HT_{1A} receptor antagonist (Nelson & Taylor, 1986) was found to attenuate the hypotensive effect of urapidil (Sanders *et al.*, 1988; Kolassa *et al.*, 1989). However, spiroxatrine alone caused a fall in blood pressure which may have contributed to the apparent antagonism of the hypotension caused by urapidil, while in the experiments of Doods *et al.* (1988) it is possible that the α_1-adrenoceptor antagonist action of urapidil was masking the ability of (−)-pindolol to attenuate the hypotensive action of urapidil.

The present experiments were designed to overcome these problems by trying to reverse the sympathoinhibitory action of urapidil in prazosin pretreated animals with the non-selective 5-HT_{1A} antagonist spiperone (see Hoyer, 1988), which has been reported to reverse the sympathoinhibitory action of the 5-HT_{1A} agonist 8-OH-DPAT (McCall *et al.*, 1987). Prazosin pretreatment has the advantage of preventing any haemodynamic effects, caused by the ability of both urapidil (Sanders *et al.*, 1984) and spiperone (Feniuk *et al.*, 1985) to block α_1-adrenoceptors, from masking any of the responses observed. Further, as there are many pathways involved in the central control of sympathetic outflow (Coote, 1988), the reversal of any sympathoinhibition caused by urapidil could be due to spiperone causing disinhibition in an unrelated central sympathoinhibitory pathway i.e. spiperone causing functional antagonism. To overcome this problem, the ability of spiperone to reverse the sympathoinhibitory action of clonidine in prazosin pretreated cats was also investigated. Finally, as 5-HT_{1A} receptor agonists cause differential sympathoinhibition (Ramage & Wilkinson, 1989), recordings were made from more than one sympathetic outflow.

A preliminary account of some of these observations has been published (Ramage, 1989).

Methods

Experiments were performed on 20 male adult cats (2.5–4.2 kg) anaesthetized with a mixture of α-chloralose (70 mg kg^{-1}, i.v.)

and pentobarbitone sodium (12 mg per animal). Supplementary doses of α-chloralose (10–15 mg kg^{-1}) were given as required. The animals were artificially ventilated using positive pressure after neuromuscular blockade with vecuronium bromide (200 μg kg^{-1}). Arterial blood gases, pH, body temperature, blood pressure, heart rate and femoral arterial conductance were monitored as previously described (Ramage, 1984). Drugs were given by bolus injection into the jugular vein.

Simultaneous recordings were made of whole nerve activity from the inferior cardiac, splanchnic and renal nerves as previously described (Ramage & Wilkinson, 1989). Nerve activity was quantified by rectifying and integrating the signal above noise over 5 s using solid state electronic integrators. The validity of the threshold setting was verified at the end of the experiment by administration of sodium pentobarbitone (60 mg per animal) or by crushing the nerve to block all activity. The outputs of the integrators were displayed on a Grass polygraph. Sympathetic nerve activity was tested to ensure that it was under baroreceptor modulation, by checking that activity in these nerves was increased by a fall in blood pressure caused by sodium nitroprusside (2 μg kg^{-1}) and decreased by a rise in blood pressure caused by noradrenaline (0.25 μg per animal).

In all 20 experiments variables were recorded over a 20 min period before injection of prazosin (0.75 mg kg^{-1}) followed 10 min later by a second injection of prazosin (0.25 mg kg^{-1}). In one group of 10 experiments, urapidil (0.75 mg kg^{-1}) was injected 5 min later followed, in 5 of these experiments, 7 min later by 3 separate injections of spiperone (1 mg kg^{-1}) at 2–3 min intervals while in the other 5 experiments the vehicle for spiperone (vehicle 2; 0.4 M lactic acid plus 10% 1 M bicarbonate) was given instead of spiperone over the same time intervals. Of the remaining 10 experiments, in 5, the vehicle for urapidil (vehicle 1; 0.04 M lactic acid) was given instead of urapidil after prazosin, while in the other 5 experiments clonidine (10 μg kg^{-1}) was given instead of urapidil followed, in both cases, by 3 separate injections of spiperone (1 mg kg^{-1}) at 2–3 min intervals. Clonidine was administered in divided doses (5 μg kg^{-1}) approximately 5 min apart. In these experiments spiperone was given between 11–16 min after the first injection of clonidine, followed, 3 min after the last dose of spiperone, by the α_2-adrenoceptor antagonist Wy 26392 (0.3 mg kg^{-1}). The doses of urapidil and clonidine used were chosen as the doses that were found to cause an approximately equal degree of sympathoinhibition, which was submaximal, in preliminary experiments. The time between the injections represents the time taken for the change in blood pressure caused by these drugs to stabilize. All drugs except prazosin, urapidil and spiperone were dissolved in saline. Prazosin and urapidil were dissolved in 0.04 M lactic acid (vehicle 1) while spiperone was dissolved in 0.4 M lactic acid then 10% (by volume) of 1 M bicarbonate was added to bring the pH to approximately 4 (vehicle 2). All doses except noradrenaline, prazosin and urapidil refer to the salts of the drug.

Analysis of data

All results are expressed as changes from baseline values. Changes caused by prazosin are taken from the first set of baseline values i.e. the values obtained prior to injection of the first dose of prazosin, while changes caused by remaining treatments are taken from the second set of baseline values i.e. just prior to the injection of urapidil, clonidine or vehicle 1. All data are presented as changes (mean ± s.e.mean) from either of these sets of baseline values. Responses for all variables were measured just prior to the next injection. The changes that occurred after the final injection were measured 3 min after spiperone or vehicle and between 2 and 6 min after Wy 26392. These times represent the times when the effects on the above variables had stabilized. Nerve activity was taken as the mean level over 1 min in arbitrary units. Comparison of changes caused by prazosin with that of initial baseline values and changes caused by the second dose of prazosin from the first dose were carried out by Student's paired t test on the absolute values obtained before and after the injections of prazosin. Comparisons of the data obtained in the urapidil and clonidine experiments were carried out by two way analysis of variance and the least significant difference for comparisons between the means (Sokal & Rohlf, 1969). The effect of spiperone on vehicle 1 (0.04 M lactic acid) pretreatment was analysed by a one way analysis of variance and again the least significant difference for comparison between the means (Sokal & Rohlf, 1969). Differences were considered significant when $P < 0.05$.

Drugs

α-Chloralose and sodium nitroprusside were purchased from Sigma and pentobarbitone sodium from May & Baker Ltd. The following drugs were kindly donated by the companies indicated, prazosin (Pfizer U.K. Ltd.), urapidil (Byk Gulden Pharmazeutika), clonidine HCl (Boehringer Ingelheim), spiperone (Janssen, Belgium) and Wy 26392 (N-((2β, 11bα)-1, 3,4,6,7,11b - hexahydro - 2H - benzo - (a) - quinolizin - 2 - yl) - N - methylpropanesulphonamide, HCl; Wyeth Lab. U.K.).

Results

Effects of prazosin pretreatment

The initial injection of prazosin (0.75 mg kg^{-1}) caused a significant fall in blood pressure along with a significant increase in femoral arterial conductance and a small bradycardia. This was not associated with a significant change in nerve activity (see Table 1). A further injection (i.v.) of prazosin (0.25 mg kg^{-1}) had no significant additional action on the variables being recorded (see Table 1). Baseline values for all groups of experiments after prazosin pretreatment are shown in Table 2.

Table 1 Changes caused by divided doses of prazosin (0.75 mg kg^{-1} followed by 0.25 mg kg^{-1}) given i.v. on: mean blood pressure (BP), heart rate (HR), femoral arterial conductance (FAC), cardiac nerve activity, splanchnic nerve activity and renal nerve activity in anaesthetized cats

	Baseline value	*Prazosin* (0.75 mg kg^{-1})	*Prazosin* (0.25 mg kg^{-1})
BP (mmHg)	125 ± 4	−44 ± 3***	−42 ± 3
HR (beats min^{-1})	235 ± 6	−16 ± 4**	−22 ± 5
FAC (ml mmHg^{-1} min^{-1} × 10^{-3})	85 ± 10	+49 ± 12***	+49 ± 11
Cardiac nerve activity (%)		−13 ± 12	−17 ± 12
Splanchnic nerve activity (%)		−10 ± 9	−18 ± 8
Renal nerve activity (%)		+3 ± 7	−6 ± 6

Baseline values for BP, HR and FAC are also shown. All results are shown as the mean ± s.e.mean; $n = 20$. **$P < 0.01$; ***$P < 0.001$. Although data are presented as the mean changes from baseline values, statistical analysis was performed on the absolute values measured before and after administration of prazosin.

Table 2 Anaesthetized cats: baseline values of mean blood pressure (BP), heart rate (HR) and femoral arterial conductance (FAC) after prazosin pretreatment for each group of experiments: vehicle 1 plus spiperone, urapidil plus spiperone, urapidil plus vehicle 2 and clonidine plus spiperone

Experimental groups:	*Vehicle 1 + spiperone*	*Urapidil + spiperone*	*Urapidil + vehicle 2*	*Clonidine + spiperone*
BP (mmHg)	77 ± 8	82 ± 4	86 ± 6	91 ± 10
HR (beats min^{-1})	227 ± 11	231 ± 13	205 ± 10	189 ± 8
FAC (ml $mmHg^{-1}$ min^{-1} × 10^{-3})	120 ± 23	138 ± 19	214 ± 37	91 ± 10

All results are shown as the mean ± s.e.mean; $n = 5$.

Effects of urapidil and clonidine in prazosin pretreated animals

Urapidil (0.75 mg kg^{-1}) or clonidine (10 μg kg^{-1}; in divided doses) caused significant ($P < 0.05$) sympathoinhibition in all nerves being recorded of between 50 and 96% (Figures 1 and 2). This was associated with a significant bradycardia for both drugs. In the urapidil groups of experiments, both in those which went on to receive spiperone and those which went on to receive vehicle alone, there were significant falls in blood pressure of 12 ± 4 and 12 ± 3 mmHg, respectively. The sympathoinhibition caused by clonidine was associated with a significant decrease in femoral arterial conductance but no change in blood pressure (Figure 2).

Effects of divided doses of spiperone on the changes caused by urapidil and clonidine in prazosin pretreated cats

From Figure 1 it can be seen that spiperone given in divided doses (i.v.) of 1 mg kg^{-1} reversed the sympathoinhibition caused by urapidil but not that caused by clonidine. The latter was reversed by Wy 26392. The combined results are shown in Figure 2. Comparison of the abilities of spiperone and vehicle (2) control to increase nerve activity show that spiperone began to increase cardiac and splanchnic nerve activity significantly ($P < 0.05$) after the first dose but renal nerve activity was not significantly increased until after the second dose of spiperone (data not illustrated). The additional fall in blood pressure caused by urapidil, in prazosin pretreated animals, was nearly maximally reversed by the first dose of spiperone (data not illustrated). Further, spiperone caused a significant increase in femoral arterial conductance only in the urapidil group, again reaching near maximum after the first dose. In this respect the vehicle used to dissolve spiperone also caused a significant increase in femoral arterial conductance but that caused by spiperone was significantly larger at all doses.

Effects of divided doses of spiperone in prazosin pretreated cats which had received vehicle instead of urapidil

Divided doses of spiperone caused no significant increases in sympathetic nerve activity. However, spiperone did cause a

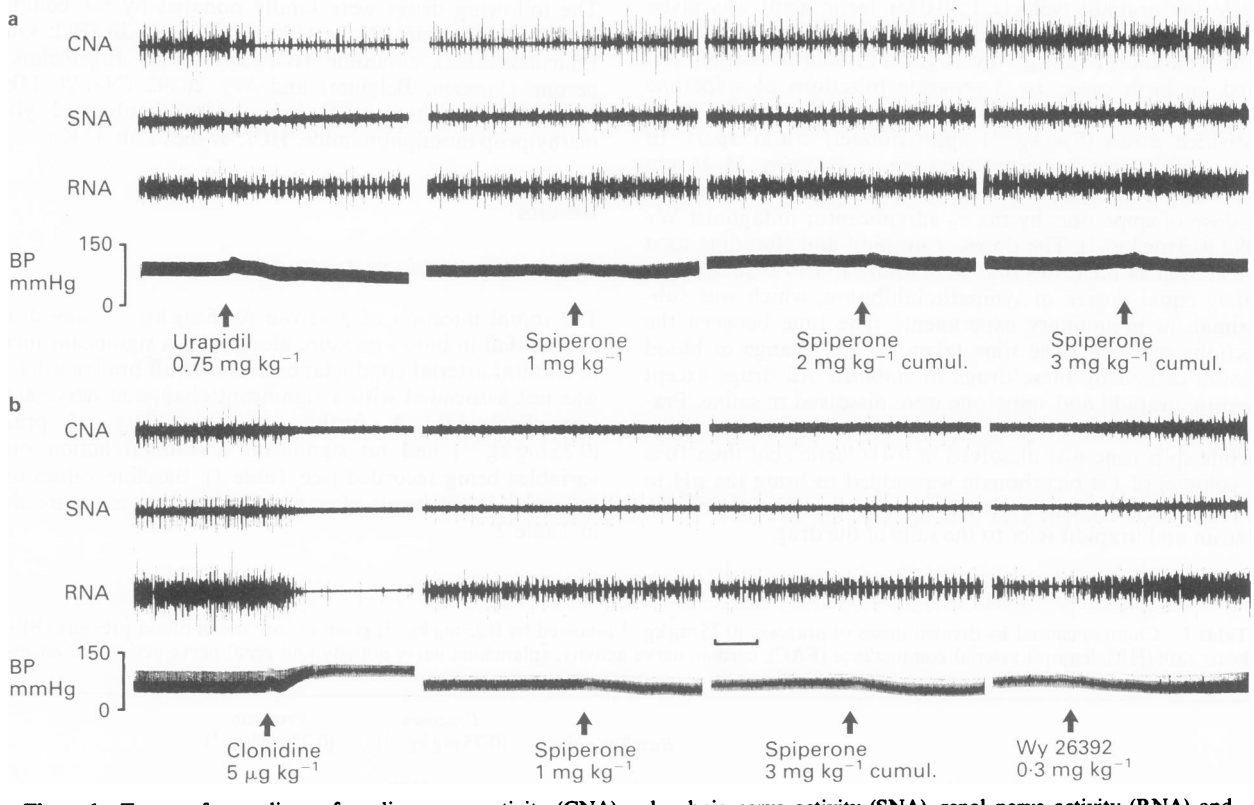

Figure 1 Traces of recordings of cardiac nerve activity (CNA), splanchnic nerve activity (SNA), renal nerve activity (RNA) and blood pressure (BP) from two separate anaesthetized prazosin (1 mg kg^{-1}) pretreated cats. Trace (a) shows the effect of injection (i.v.) of urapidil followed by 3 consecutive injections (i.v.) of spiperone on these variables. Trace (b) shows the effect of the first i.v. injection of clonidine followed by that of first injection of spiperone. It should be noted that this injection of spiperone is after a second injection of clonidine (5 μg kg^{-1}) which has been omitted for clarity as has the part of the trace showing the second injection of spiperone. The last portion of trace (b) shows the effect of a single (i.v.) injection of the α_2-adrenoceptor antagonist, Wy 26392, which was given after the last dose of spiperone. cumul. = cumulative. It should be noted that the initial sympathoinhibition observed (first part of trace b) on injection of clonidine is baroreceptor-mediated due to the rise in blood pressure caused by the peripheral vasoconstrictor action of clonidine.

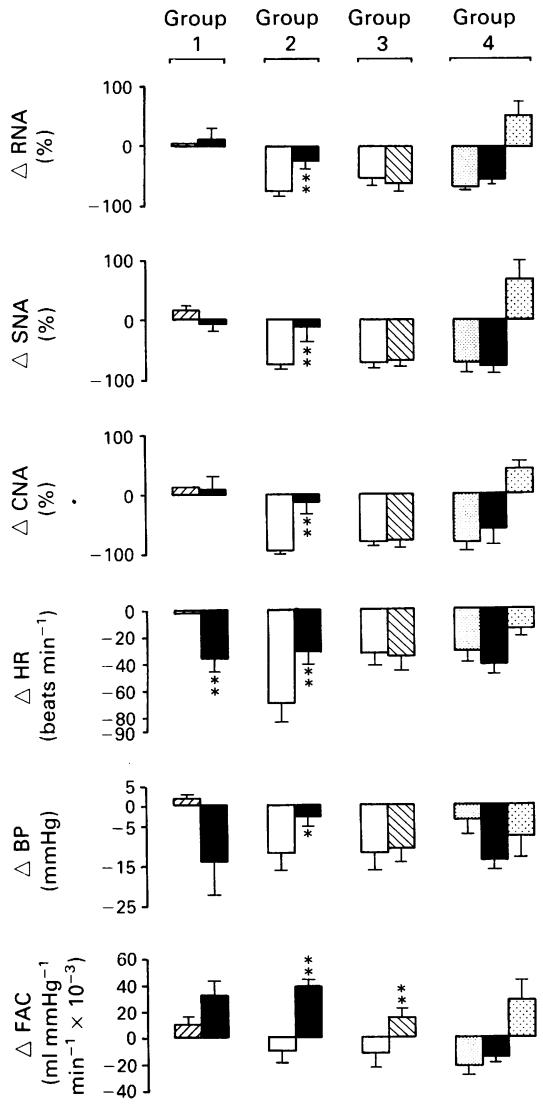

Figure 2 Prazosin (1 mg kg^{-1}) pretreated anesthetized cats showing changes in renal (RNA), splanchnic (SNA) and cardiac nerve activity (CNA), heart rate (HR), blood pressure (BP) and femoral arterial conductance (FAC) caused by the following injections (i.v.). Group 1 shows the effect of 3 mg kg^{-1} (cumulative) of spiperone (closed columns) on changes caused by vehicle 1 (urapidil vehicle; cross hatched columns). Group 2 shows the effect of 3 mg kg^{-1} (cumulative) of spiperone (closed columns) on changes caused by urapidil (0.75 mg kg^{-1}, open columns). Group 3 shows the effect of vehicle 2 (spiperone vehicle, cross hatched columns) on changes caused by urapidil (0.75 mg kg^{-1}, open columns). Group 4 shows the effect of 3 mg kg^{-1} (cumulative) of spiperone (closed columns) on changes caused by clonidine (10 μg kg^{-1}, heavily stippled columns) plus the effect of the α_2-adrenoceptor antagonist Wy 26393 (0.3 mg kg^{-1}, lightly stippled columns) given after 3 mg kg^{-1} spiperone on the above variables. All changes (Δ) are from the prazosin pretreatment values. All values are mean ($n = 5$) with s.e.mean shown by vertical bars above the columns. The effects of spiperone on vehicle 1 pretreatment (Group 1) were analyzed by using a one way analysis of variance and the least significance difference test to compare the means. The effects of spiperone on the above variables in the urapidil and clonidine groups (Groups 2 & 4) have been compared with those of vehicle 2 (Group 3) using a two way analysis of variance and the least significance difference test to compare the means, $*P < 0.05$; $**P < 0.01$. The data for each single injection of spiperone have been omitted for the sake of clarity.

dose-related fall in heart rate, which was significant after the last dose, plus an increase in femoral arterial conductance although this was only significant at the second dose of spiperone (not illustrated). There was a tendency for blood pressure to decline but this was not significant.

Discussion

These experiments demonstrate that spiperone can reverse the sympathoinhibitory action of urapidil in prazosin-pretreated, anaesthetized cats. However, spiperone failed to reverse the sympathoinhibition caused by clonidine in these prazosin-pretreated cats, the latter being reversed by the α_2-adrenoceptor antagonist Wy 26392 (Paciorek *et al.*, 1984). Spiperone also did not cause sympathoexcitation in cats pretreated with prazosin alone. These latter two observations make it highly unlikely that spiperone is causing the reversal of urapidil by functional antagonism. Both urapidil and spiperone have in common an affinity for α_1-adrenoceptors (Schoetensack *et al.*, 1977; Eltze, 1984; Sanders *et al.*, 1984; Feniuk *et al.*, 1985) and 5-HT$_{1A}$ receptors (see Hoyer, 1988). Any interaction between spiperone and urapidil at α_1-adrenoceptors is extremely unlikely as these receptors would seem to be completely blocked by the prazosin pretreatment, as indicated by the second dose of prazosin failing to cause any further changes in the variables being recorded. Thus, an interaction of spiperone and urapidil at 5-HT$_{1A}$ receptors is the more likely explanation for the ability of spiperone to reverse the sympathoinhibitory action of urapidil. In this respect urapidil has been shown to act as a partial agonist at the 5-HT$_{1A}$ receptors on the guinea-pig ileum (Fozard & Mir, 1987) and on forskolin-stimulated adenylate cyclase activity (Schoeffter & Hoyer, 1988) while spiperone is a silent antagonist in this latter system (Schoeffter & Hoyer, 1988). Thus these combined observations support the view that the sympathoinhibition caused by urapidil is due to activation of 5-HT$_{1A}$ receptors.

The rostral ventrolateral medulla has been identified as a site of action at which urapidil, as well as the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT, act to cause central sympathoinhibition (Gillis *et al.*, 1988; Laubie *et al.*, 1989; Mandel *et al.*, 1989). Local administration of spiperone can prevent the hypotension and bradycardia induced when urapidil is applied to this area and application of spiperone to this area completely reverses the hypotension and bradycardia caused by i.v. urapidil (Mandel *et al.*, 1989), thus supporting the conclusion from the present observations. However, as Mandel *et al.* (1989) point out, their experiments suggest that α_1-adrenoceptor blockage is not important in the hypotensive action of urapidil, although the dose given in this study (Mandel *et al.*, 1989) is known to block α_1-adrenoceptors. This could be due to some functional antagonism caused by spiperone.

The observation that spiperone alone had no effect on nerve activity in prazosin pretreated animals suggests that central 5-HT$_{1A}$ receptors, at least in anaesthetized, prazosin pretreated cats, are not under tonic activation. In this respect application of spiperone alone to the ventral surface of the medulla has also been demonstrated to have no effect on blood pressure or heart rate (Mandel *et al.*, 1989). It is conceptually possible that blockade by spiperone of central 5-HT$_2$ receptors may have masked such an action as central 5-HT$_2$ receptors are considered to have a sympathoexcitatory action (McCall *et al.*, 1987). However, this seems doubtful as a selective 5-HT$_2$ receptor antagonist, cinanserin, failed to have any effect on nerve activity in prazosin pretreated, anaesthetized cats (Ramage, 1988a). The increase in femoral arterial conductance caused by spiperone in the present experiments may be related to blockade of central 5-HT$_2$ receptors as this is a characteristic action of 5-HT$_2$ receptor antagonists (Ramage, 1985; 1988b). The bradycardic effect of spiperone is not due to an increase in vagal tone as it is not blocked by atropine methonitrate (unpublished observations) and is therefore a peripheral action of unknown mechanism.

The present experiments also demonstrate that α_1-adrenoceptor antagonists, in addition to causing a fall in blood pressure, prevent the expected reflex rise in sympathetic nerve activity; this would be expected from their ability to have a central inhibitory action on sympathetic outflow

(McCall & Humphrey, 1981; Persson *et al.*, 1981; McCall & Schuette, 1984; Ramage, 1984; 1986a,b).

In conclusion the present results demonstrate that urapidil can activate 5-HT_{1A} receptors to cause sympathoinhibition. This explains why urapidil differs in its action on the cardiovascular system compared to other α_1-adrenoceptor antagonists (Ramage, 1986b).

I wish to thank Mr S. Wilkinson for technical assistance and Byk Gulden Pharmazeutika for support.

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(Received August 20, 1990
Revised November 19, 1990
Accepted December 3, 1990)

Br. J. Pharmacol. (1991), **102**, 1003–1007

5-Hydroxytryptamine-induced bronchoconstriction in the guinea-pig: effect of 5-HT_2 receptor activation on acetylcholine release

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1 The bronchoconstrictor responses to 5-hydroxytryptamine (5-HT) were studied in the guinea-pig to establish whether they are partly attributable to parasympathetic activation within the airways. 5-HT dose-response curves were constructed in anaesthetized and ventilated guinea-pigs pretreated with saline, or by bilateral cervical vagotomy or vagotomy plus atropine 3 mg kg^{-1}, i.v. Vagotomy had no effect on 5-HT-induced bronchoconstriction but vagotomy plus atropine significantly reduced it.

2 To determine whether parasympathetic activation within the airways resulted from pre- or postganglionic stimulation, 5-HT dose-response curves were constructed for two groups of vagotomized guinea-pigs treated with hexamethonium 2 mg kg^{-1}, or hexamethonium 2 mg kg^{-1}, plus atropine 3 mg kg^{-1}. Guinea-pigs treated with hexamethonium plus atropine experienced significantly less 5-HT-induced bronchoconstriction than those treated with hexamethonium alone.

3 To characterize the subtype of 5-HT receptors involved in the activation of the parasympathetic system by 5-HT, dose-response curves to 5-HT were constructed for four groups of vagotomized guinea-pigs treated with saline, 1 mg kg^{-1} of the 5-HT_3 antagonist ICS 205-930, or either 0.01 or 0.1 mg kg^{-1} of the 5-HT_2 antagonist ketanserin. ICS 205-930 enhanced 5-HT-induced bronchoconstriction but 0.01 mg kg^{-1} ketanserin inhibited it significantly and 0.1 mg kg^{-1} ketanserin abolished it. To confirm the involvement of 5-HT_2 receptors in these responses, we studied the effects in vagotomized guinea-pigs of atropine on the bronchoconstriction induced by the 5-HT_2 agonist, α-methyl-5-HT, infused at rates of 40 and 80 ng kg^{-1} s^{-1}. At both rates, atropine significantly reduced the bronchoconstrictor responses to α-methyl-5-HT.

4 The above results indicate that 5-HT-induced bronchoconstriction is indeed partly mediated by parasympathetic activation within the airways. This activation is mediated by stimulation of 5-HT_2 receptors which are probably located on the postganglionic parasympathetic nerve endings.

Keywords: 5-HT-induced bronchoconstriction; respiratory system; ketanserin; ICS 205-930; α-methyl-5-HT; parasympathetic system

Introduction

5-Hydroxytryptamine (5-HT) is a bronchoconstrictor agent which has been widely used to study bronchial reactivity in several species. 5-HT-induced bronchoconstriction is attributed to the direct action of 5-HT on airway smooth muscle and to a central vagal reflex resulting from the stimulation of irritant receptors in the airways. Parasympathetic nerve activation within the airways may also contribute to the bronchoconstrictor effects of 5-HT, independently of central reflex pathways. 5-HT has been shown to facilitate ganglionic transmission (Wallis & Woodward, 1975; Round & Wallis, 1986) and has been demonstrated in isolated tracheal smooth muscle preparations to induce the release of acetylcholine by interacting with presynaptic neuronal receptors (Aas, 1983).

In vitro results suggest that in the guinea-pig, 5-HT-induced bronchoconstriction could indeed result from parasympathetic system activation in the airways. Thus, in an innervated preparation of guinea-pig trachea, McCaig (1986) reported that atropine partially blocked the increase in intraluminal pressure induced by 5-HT. In guinea-pig tracheal strips, Baumgartner *et al.* (1990) showed that atropine significantly reduced the contractile response to 5-HT. In guinea-pig isolated perfused lungs, Bhattacharya (1955) demonstrated that atropine prevented 5-HT-induced bronchoconstriction. However, the existence of local parasympathetic activation within the airways in the bronchoconstriction induced by 5-HT *in vivo* has not been established.

The aims of our study were therefore: (1) to determine whether the bronchoconstrictor response to 5-HT in the guinea-pig is in part attributable to parasympathetic activation within the airways and (2) to characterize the subtype of 5-HT receptors involved in the interaction between the parasympathetic system and 5-HT, using selective 5-HT receptor agonists and antagonists.

Methods

Animals and general procedures

All experiments were performed on male Hartley strain guinea-pigs weighing 250–300 g (Charles River, France), housed in a temperature-controlled room (21°C) with food and water freely available. Guinea-pigs were anaesthetized i.p. with 40 mg kg^{-1} pentobarbitone sodium. A repeat dose of 10 mg kg^{-1} was given i.p. every 45 min throughout the experiments. These doses were selected so that the animals were surgically anaesthetized during the experiments. The trachea was cannulated, and polyethylene catheters were placed in the external jugular veins for drug administration and in the left common carotid artery for monitoring of blood pressure and heart rate via an indwelling cannula connected to a pressure transducer (Gould). The animals were mechanically ventilated

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and were paralysed by i.v. injection of 0.1 mg kg^{-1} vecuronium bromide repeated every 30 min throughout the experiments. Respiratory frequency was set at 60 cycles min^{-1} and tidal volume at 6 ml kg^{-1}. Body temperature was kept constant at 38°C by placing the animals on a thermostatically controlled heated blanket (Animal Blanket Unit, Harvard) to avoid nonspecific changes in bronchial reactivity (Macquin-Mavier *et al.*, 1989). Before the experiments began, the guinea-pigs were allowed 20 min to recover from the preparation procedure.

Measurement of respiratory mechanics

Airway responses to 5-HT were assessed by measuring respiratory system conductance and compliance. Both parameters were monitored continuously and determined on line from the total respiratory pressure, inspiratory flow and tidal volume signals, as described previously (Lorino *et al.*, 1988). Tracheal pressure was measured with a Validyne MP45 ± 56 cmH_2O differential pressure transducer. Inspiratory flow was measured with a heated Fleisch ≠000 pneumotachograph connected to a Validyne MP45 ± 2.5 cmH_2O differential pressure transducer. Airflow was integrated for measurement of tidal volume. Pressure and flow signals were digitized at a sample rate of 16 Hz and fed into an Apple IIE microcomputer. Respiratory conductance and compliance were calculated for each breath and their averages displayed every 15 s. A large inflation was performed manually every 5 min by occlusion of the expiratory valve for three respiratory cycles to prevent alveolar atelectasis.

Experimental protocol

The following procedure was used for all series of experiments: after the 20 min stabilization period, the guinea-pigs were injected i.v. with a bolus of 1 mg kg^{-1} propranolol to minimize potential changes in bronchoconstrictor responses resulting from changes in levels of circulating catecholamines. Ten minutes later, they were given three sequential 5-HT challenges with increasing doses of 5-HT (40, 60 and 80 ng kg^{-1} s^{-1} respectively) at 15 min intervals. During each challenge, 5-HT was infused i.v. for 5 min until a plateau response was reached. Ten minutes elapsed between each challenge to allow respiratory parameters to return to within 5% of baseline values.

Three studies were performed. In the first, we explored the bronchoconstrictor responses to i.v. administered 5-HT, to see if they included a central vagal reflex and/or a significant peripheral cholinergic component. We reasoned that, if the local effects of 5-HT on cholinergic nerves in the airways were important in the mediation of 5-HT-induced bronchoconstriction, then in vagotomized guinea-pigs, treatment with atropine should reduce the airway responses to 5-HT. Accordingly, three groups of eight guinea-pigs each were pretreated i.v. with saline, bilateral cervical vagotomy or vagotomy plus 3 mg kg^{-1} atropine respectively, 10 min before 5-HT was injected and the dose-response curve recorded.

In the second study, we attempted to establish whether the peripheral cholinergic component of the airway responses to 5-HT may represent a stimulatory action of 5-HT on the efferent parasympathetic ganglia and/or at a site distal to the ganglia. For this purpose, we evaluated the airway responses to 5-HT in two groups of seven vagotomized guinea-pigs each, treated i.v. with either 2 mg kg^{-1} of the ganglion blocking agent hexamethonium or 2 mg kg^{-1} hexamethonium plus 3 mg kg^{-1} atropine 10 min before 5-HT injection and dose-response curve recording.

In the third study, we aimed to characterize the subtype of 5-HT receptors involved in the interaction between 5-HT and the peripheral cholinergic system. We evaluated the airway responses to 5-HT in four groups of vagotomized guinea-pigs treated i.v. with saline ($n = 6$), the 5-HT_3 antagonist ICS 205-930 (1 mg kg^{-1}) ($n = 6$) or one of two doses of the 5-HT_2 antagonist ketanserin (0.01 or 0.1 mg kg^{-1}) ($n = 5$ and 3 respectively) 10 min before 5-HT injection and dose-response curve recording. Since we found that ketanserin abolished 5-HT-induced bronchoconstriction, we studied two additional groups of six vagotomized guinea-pigs each, to confirm the involvement of 5-HT_2 receptors in the peripheral cholinergic activation by 5-HT. These guinea-pigs were treated i.v. with saline or 3 mg kg^{-1} atropine before two consecutive challenges with the 5-HT_2 agonist, α-methyl-5-hydroxytryptamine maleate (α-methyl-5-HT), administered as 5 min infusions at rates of 40 and 80 ng kg^{-1} s^{-1} respectively.

Drugs

5-Hydroxytryptamine hydrochloride, hexamethonium bromide and atropine sulphate were purchased from Sigma and dissolved in physiological saline. Ketanserin was a gift from Janssen and ICS 205-930 (3-tropanyl-indole-3-carboxylate), from Sandoz Ltd. α-Methyl-5-hydroxytryptamine was purchased from Research Biochemicals Incorporated. Ketanserin, ICS 205-930, and α-methyl-5-hydroxytryptamine were dissolved in distilled water. All drugs were injected in a final volume of 1 ml kg^{-1}. All doses are expressed in terms of their corresponding base.

Analysis of data

Data are expressed as means ± s.e. Dose-response curves for 5-HT were analysed by repeated-measures analysis of variance (BMDP statistical software). If there was an interaction between the dose of 5-HT and the treatment, the analysis of variance was followed by the Mann-Whitney U test. P values of less than 0.05 were considered statistically significant.

Results

Effects of vagotomy and of vagotomy plus atropine on 5-hydroxytryptamine-induced bronchoconstriction

5-HT (40–80 ng kg^{-1} s^{-1}) caused dose-related bronchoconstriction. As shown in Figure 1, bilateral cervical vagotomy had no significant effect on 5-HT-induced bronchoconstriction. In contrast, this bronchoconstriction was significantly reduced when the vagotomized guinea-pigs were pretreated with atropine, whatever the degree of bronchoconstriction. Baseline respiratory conductance and compliance were not significantly different in guinea-pigs which underwent bilateral cervical vagotomy or vagotomy plus atropine compared to control levels (Table 1).

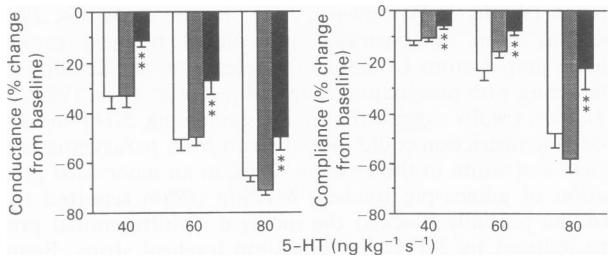

Figure 1 Effects of bilateral cervical vagotomy (stippled columns) or bilateral cervical vagotomy plus atropine (solid columns) on the decreases in respiratory system conductance and compliance induced by 5-hydroxytryptamine (5-HT) infused at rates of 40, 60 and 80 ng kg^{-1} s^{-1}; controls, open columns. Each group included 8 guinea-pigs. Vagotomy did not affect the bronchoconstrictor response to 5-HT, whereas vagotomy plus atropine significantly reduced it, whatever the degree of bronchoconstriction. (** $P < 0.01$ versus controls).

Table 1 Baseline respiratory system conductance and compliance of control guinea-pigs and guinea-pigs treated by vagotomy or vagotomy plus atropine

Treatment	Conductance ($ml\,kPa^{-1}\,s^{-1}$)	Compliance ($ml\,kPa^{-1}$)
Controls	28.5 ± 0.7	3.7 ± 0.1
Vagotomy	27.7 ± 0.5	3.8 ± 0.2
Vagotomy + atropine	27.1 ± 0.9	3.4 ± 0.1

Values are means ± s.e. Eight guinea-pigs were studied in each group treated.

Effects of atropine on 5-hydroxytryptamine-induced bronchoconstriction in vagotomized guinea-pigs treated with hexamethonium

In vagotomized guinea-pigs pretreated with $2\,mg\,kg^{-1}$ hexamethonium, bronchoconstrictor responses to 5-HT were significantly enhanced when 5-HT was infused at rates of 40 and $60\,ng\,kg^{-1}\,s^{-1}$ ($P < 0.05$). As shown in Figure 2, in vagotomized guinea-pigs pretreated with hexamethonium, atropine significantly reduced the bronchoconstrictor responses to 5-HT, whatever the degree of bronchoconstriction.

Effects of ICS 205-930 and ketanserin on 5-hydroxytryptamine-induced bronchoconstriction in vagotomized guinea-pigs

Treatment with $1\,mg\,kg^{-1}$ ICS 205-930 i.v. slightly but significantly enhanced the decrease in respiratory conductance induced by 5-HT; it also tended to enhance the decrease in respiratory compliance induced by 5-HT, although not significantly ($P = 0.056$). Treatment with $0.01\,mg\,kg^{-1}$ ketanserin i.v. significantly reduced 5-HT-induced bronchoconstriction, and treatment with $0.1\,mg\,kg^{-1}$ i.v. ketanserin completely abolished it (Figure 3).

Effects of atropine on the bronchoconstriction induced by α-methyl-5-HT in vagotomized guinea-pigs

α-Methyl-5-HT (40–$80\,ng\,kg^{-1}\,s^{-1}$) induced dose-dependent bronchoconstriction, which was significantly reduced by atropine (Figure 4).

Discussion

In this study, we attempted to determine whether parasympathetic activation within the airways is involved in the bronchoconstrictor responses to 5-HT in the guinea-pig. We conducted all our experiments in guinea-pigs pretreated with propranolol, and some of them in vagotomized guinea-pigs, to eliminate any possible modulation of airway contraction by activation of the sympathetic nervous system and/or the non adrenergic inhibitory system (Clerici *et al.*, 1989).

We found that atropine plus bilateral cervical vagotomy attenuated 5-HT-induced bronchoconstriction, whereas bilateral vagotomy alone had no effect. While vagotomy blocks only the central reflexes, atropine antagonizes the actions of acetylcholine resulting from either centrally or locally induced activation of the cholinergic system. While it is theoretically possible that atropine inhibits 5-HT-induced bronchoconstriction by a direct action at 5-HT receptors, there is no evidence that it exhibits such non-specific activity. Furthermore, Advenier *et al.* (1984) have previously shown that in guinea-pig trachea *in vitro*, atropine is of the order of 10,000 times more potent against acetylcholine than against 5-HT. Our results strongly suggest therefore that, in addition to exerting direct action on airway smooth muscle, 5-HT caused bronchoconstriction which was partly mediated by cholinergic activation within the airways, independently of central reflex pathways. In contrast to the results of previous studies in the dog and cat (Hahn *et al.*, 1978; Parratt *et al.*, 1982) demonstrating marked reduction of the bronchoconstrictor responses to 5-HT after bilateral vagotomy, we did not find a vagally mediated reflex component in the bronchoconstriction

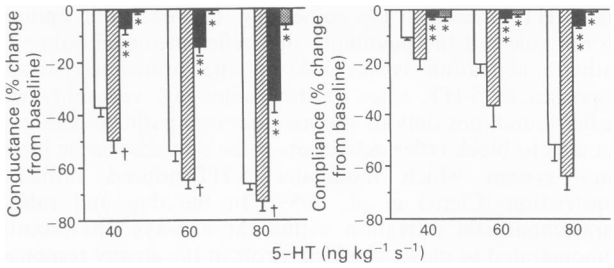

Figure 3 Effects of $1\,mg\,kg^{-1}$ ICS 205-930 (hatched columns) and of 0.01 (solid columns) or $0.1\,mg\,kg^{-1}$ (stippled columns) ketanserin on the decreases in respiratory system conductance and compliance induced by 5-hydroxytryptamine (5-HT) infused at rates of 40, 60 and $80\,ng\,kg^{-1}\,s^{-1}$ in vagotomized guinea-pigs: saline controls (open columns). Six guinea-pigs were included in each of the two groups treated with saline and ICS 205-930 respectively; 5 and 3 guinea-pigs were included in the two groups treated with 0.01 and $0.1\,mg\,kg^{-1}$ ketanserin respectively. ICS 205-930 significantly enhanced the decrease in respiratory conductance induced by 5-HT († $P < 0.05$ versus saline-treated guinea-pigs). Ketanserin significantly reduced 5-HT-induced bronchoconstriction (** $P < 0.01$, * $P < 0.05$ versus saline-treated guinea-pigs)

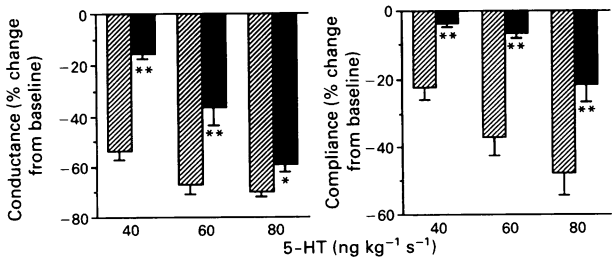

Figure 2 Effects of atropine on the decreases in respiratory system conductance and compliance induced by 5-hydroxytryptamine (5-HT), infused at rates of 40, 60 and $80\,ng\,kg^{-1}\,s^{-1}$ in vagotomized guinea-pigs pretreated with hexamethonium: hexamethonium alone (hatched columns); hexamethonium plus atropine (solid columns). Each group included 7 guinea-pigs. Atropine significantly reduced 5-HT-induced bronchoconstriction, whatever its degree. (** $P < 0.01$, * $P < 0.05$ versus guinea-pigs treated with hexamethonium alone).

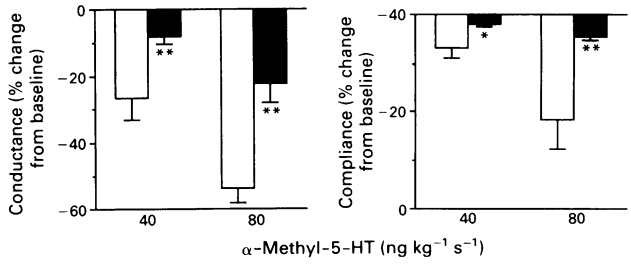

Figure 4 Effects of atropine (solid columns) on the decreases in respiratory system conductance and compliance induced by the $5\text{-}HT_2$ agonist, α-methyl-5-HT, infused at rates of 40 and $80\,ng\,kg^{-1}\,s^{-1}$ in vagotomized guinea-pigs; saline controls (open columns). Each group included 6 guinea-pigs. Atropine significantly reduced the bronchoconstrictor responses to α-methyl-5-HT. (** $P < 0.01$, * $P < 0.05$ versus saline-treated guinea-pigs)

that 5-HT induced in the guinea pig. However, our findings do not rule out the possibility that reflex-mediated parasympathetic activation is involved in the bronchoconstrictor responses to 5-HT, since in the guinea-pig, vagotomy was earlier found, not only to suppress parasympathetic pathways but also to block reflex activation of the non adrenergic inhibitory system which modulates 5-HT-induced bronchoconstriction (Clerici et al., 1989). In the dog and rabbit, parasympathetic activation within the airways was recently demonstrated to play a significant role in the airway responses to other bronchoconstrictor agents, e.g. histamine, platelet activating factor (PAF) and substance P (Shore et al., 1985; Leff et al., 1987; Grunstein et al., 1984). As regards PAF and substance P, parasympathetic activation within the airways appears to be the sole contribution of the parasympathetic system to their bronchoconstrictor effects. Our results demonstrate that this mechanism also contributes to 5-HT-induced bronchoconstriction in the guinea-pig.

Because parasympathetic ganglia lie within the airway wall (Skoogh, 1988), the parasympathetic nerve structures which might be activated within the airways by 5-HT after vagotomy include not only postganglionic pathways but also the parasympathetic ganglia. 5-HT has been shown to activate preganglionic parasympathetic nerve terminals in guinea-pig tracheal strips (Baumgartner et al., 1990), and may also stimulate presynaptic receptors on cholinergic nerve terminals, as demonstrated in the isolated bronchial smooth muscle of the rat (Aas, 1983). To determine the site of the peripheral cholinergic action of 5-HT, we compared the bronchoconstrictor responses to 5-HT by vagotomized guinea-pigs after ganglionic blockade with hexamethonium and after ganglionic plus muscarinic blockade with hexamethonium plus atropine. The observation that hexamethonium alone did not inhibit bronchoconstrictor responses to 5-HT, but that additional atropine treatment did cause a significant reduction suggests that the predominant site of the cholinergic action of 5-HT is located distal to the airway parasympathetic ganglia, i.e. on the parasympathetic nerve terminals. The additional observation that hexamethonium alone appeared actually to enhance 5-HT-induced responses was unexpected, and of uncertain significance.

To characterize the 5-HT receptor subtype involved in the interaction between 5-HT and the parasympathetic system, we studied the effects of the selective 5-HT_3 antagonist, ICS 205-930 (Richardson et al., 1985) and of the selective 5-HT_2 antagonist, ketanserin on the bronchoconstrictor responses to 5-HT in vagotomized guinea-pigs. The release of acetylcholine by 5-HT may result from the excitation of the 5-HT_3 receptors on parasympathetic nerve terminals, as reported for rat bronchi and rabbit heart (Wallis, 1989). However, in our experiments, ICS 205-930 did not reduce but rather enhanced 5-HT-induced bronchoconstriction. In contrast, ketanserin inhibited it. Similar inhibition by ketanserin of the bronchoconstrictor responses to the 5-HT was previously reported in guinea-pig isolated perfused lung by Selig et al. (1989) and in cats by Ball et al. (1983). It is well established that the 5-HT receptors mediating tracheal smooth muscle contraction are of the 5-HT_2 subtype (Cohen et al., 1985; Lemoine & Kauman, 1986). Since there is no evidence that ketanserin interacts with muscarinic receptors (Awouters et al., 1982), the most likely explanation for our results is that ketanserin prevented acetylcholine release. If true, this would suggest that the 5-HT receptors located on the parasympathetic nerve terminals are of the 5-HT_2 subtype. To confirm such involvement of 5-HT_2 receptors in the interaction between 5-HT and the parasympathetic system within the airways, we induced bronchoconstriction by infusing vagotomized guinea-pigs with the selective 5-HT_2 agonist, α-methyl-5-HT (Richardson et al., 1985), and found that atropine significantly reduced α-methyl-5-HT-induced bronchoconstriction.

In conclusion, our data suggest that the bronchoconstriction induced by infusion of 5-HT is elicited by two mechanisms: direct constriction of bronchial smooth muscle and indirect constriction of this muscle by postganglionic stimulation of the parasympathetic nerves. Both mechanisms are mediated by the stimulation of 5-HT_2 receptors.

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(Received July 17, 1990
Revised December 11, 1990
Accepted December 17, 1990)

British Journal of Pharmacology

VOLUME 102 (4) APRIL 1991

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The *British Journal of Pharmacology* is published monthly by the Scientific & Medical Division, Macmillan Press Ltd.

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ISSN 0007–1188

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BRITISH JOURNAL OF PHARMACOLOGY VOLUME 102 (4) 1991

Filmsetting by Santype International Limited, Salisbury, Wiltshire
and printed in Great Britain by Unwin Brothers Ltd., The Gresham Press, Old Woking, Surrey