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Peristalsis evoked by 5-HT and renzapride: evidence for putative 5-HT_4 receptor activation

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The effect of 5-hydroxytryptamine and renzapride was studied on the peristaltic reflex elicited *in vitro* in the guinea-pig ileum. Both agents evoked the reflex at subthreshold intraluminal pressures (50% of threshold) and were blocked by ICS 205-930 (3 μM), but not ondansetron (5 μM). This novel finding suggests strongly that the prokinetic action of renzapride and gut motility stimulating action of 5-hydroxytryptamine are mediated via agonism at the putative 5-HT_4 receptor.

Introduction The mechanism by which certain substituted benzamides (e.g., renzapride, cisapride, and zacopride) enhance gastrointestinal motility cannot be accounted for by their 5-HT_3 receptor antagonistic properties (King & Sanger, 1988). Recently, however, these compounds have been shown to activate a novel 5-hydroxytryptamine (5-HT) receptor (putative 5-HT_4 receptor) mediating neuronally driven longitudinal smooth muscle contraction in guinea-pig ileum (Craig & Clarke, 1990). This raises the possibility that agonism at the putative 5-HT_4 receptor underlies their motility stimulating action. The present investigation was undertaken to determine whether renzapride and 5-HT function to facilitate the peristaltic reflex via agonism at the putative 5-HT_4 receptor.

Methods Male albino guinea-pigs (500–600 g) were killed by asphyxiation with CO_2. Segments of whole ileum, 5 cm in length and proximal to the ileocecal junction, were cannulated at the oral and aboral ends and secured horizontally in a 50 ml, water-jacketed petri dish containing Krebs solution, the composition of which has been detailed previously (Craig & Clarke, 1990). In all experiments, ondansetron (5 μM) was present in the Krebs solution to block 5-HT_3 receptors. The oral end of the segment was connected to a reservoir containing Krebs solution. The aboral end was connected to a vertical column which was open to the air. Intraluminal filling pressure (oral end), controlled by adjusting the elevation of the reservoir, and peristaltic pressure (aboral end) were recorded.

The threshold pressure for peristalsis was determined by raising the reservoir at increments of 0.1 mmHg at 1 min time intervals until peristalsis occurred. A constant threshold pressure, obtained on three consecutive trials, was taken as the control value. The ability of 5-HT (0.1 μM) or renzapride (1.0 μM) to induce peristalsis was investigated by adding these agents to the bathing solution, either in the absence or presence of 3α-tropanyl-1H-3-carboxylic acid ester (ICS 205-930, 3 μM), 1 min after raising pressure to 50% of threshold. The ability of 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP, 10 μM) to elicit peristalsis was tested in some experiments in which the effects of 5-HT or renzapride were blocked by ICS 205-930. The chosen agonist concentrations produce maximum or near-maximum responses at ileal nicotinic cholinoceptors (DMPP) or putative 5-HT_4 receptors (Craig & Clarke, 1990). Control experiments were run to ensure that peristalsis did not develop spontaneously during the agonist test period.

The drugs, dissolved in distilled water, and their sources have been described (Craig & Clarke, 1990).

Results The mean (range) threshold pressures (mmHg) for peristalsis were 0.4 (0.3 to 0.6) (Krebs solution + ondansetron; 17 experiments from 17 guinea-pigs) and 0.4 (0.3 to 0.6) (Krebs + ondansetron + ICS 205-930; 16 experiments from 16 guinea-pigs). Furthermore, in 4 out of 4 experiments, ICS 205-930 (3 μM) added directly to the bathing fluid failed to alter the threshold for peristalsis.

In no instance did peristalsis occur spontaneously in time control experiments (50% threshold pressure). 5-HT (0.1 μM) initiated peristalsis in 4 experiments from 4 different guinea-pigs (Figure 1). The response was slow to develop (ca. 1 min to onset), and in some experiments the rhythmicity of peristaltic waves was interrupted by contractures of circular muscle. In 3 out of the 4 experiments, peristalsis continued intermittently over a subsequent 5 min observation period, but in each case peristaltic activity resulting from 5-HT ceased within 1 min of applying ICS 205-930 (3 μM).

Renzapride (1 μM) evoked peristalsis in 8 experiments from 8 different guinea-pigs. As with 5-HT, the responses were slow to develop and, in some instances, exhibited a tendency to produce a contracture of the ileum (Figure 2). In 6 experiments, peristalsis was monitored for 5 min after the addition of renzapride. In 5 of these experiments intermittent episodes of peristalsis were observed.

5-HT (in 4 out of 4 experiments) and renzapride (in 8 out of 8 experiments) failed to evoke peristalsis in the presence of ICS 205-930 (3 μM). However, ICS 205-930 did not materially

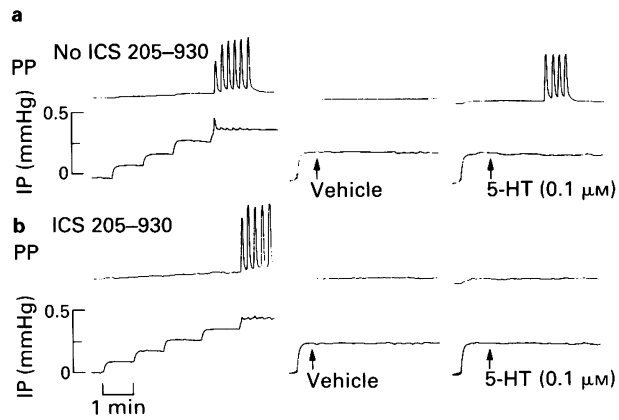

Figure 1 Recordings of intraluminal pressure (IP) and peristaltic pressure (PP) from paired segments of ileum from a single guinea-pig in Krebs solution containing ondansetron (5 μM) to block 5-HT_3 receptors. (a) Left, IP was raised at 1 min intervals to determine the threshold pressure for peristalsis. Centre, IP was maintained at half threshold pressure and vehicle (H_2O, 50 μl), added to the bathing solution, failed to elicit peristalsis. Right, IP was maintained at half threshold pressure, and 5-hydroxytryptamine (5-HT, 0.1 μM), added to the bathing solution, elicited peristalsis after a brief delay. (b) The same as (a) except the bathing fluid contained ICS 205-930 to block putative 5-HT_4 receptors. In the presence of ICS 205-930 (3 μM), 5-HT failed to evoke peristalsis.

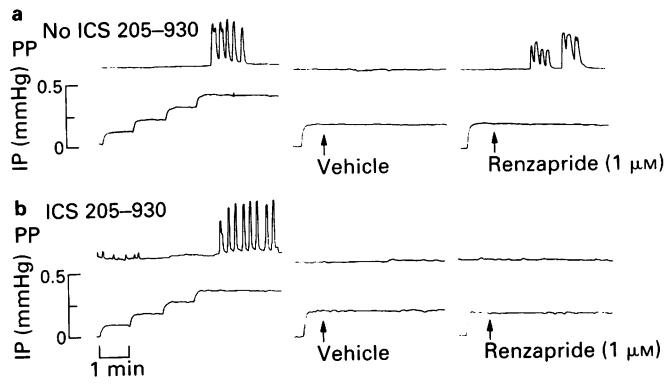

Figure 2 Recordings of intraluminal pressure (IP) and peristaltic pressure (PP) from paired segments of ileum from a single guinea-pig as described in Figure 1. The figure shows that the facilitatory effect of renzapride on the peristaltic reflex (a) is blocked by ICS 205-930 (b).

alter the ability of DMPP (10 μM) to evoke peristalsis (2 out of 3 experiments with ICS 205-930 + 5-HT and 5 out of 5 experiments with ICS 205-930 + renzapride).

Discussion The present study demonstrates, for the first time, that agonism at the putative 5-HT_4 receptor in guinea-pig ileum functions to facilitate the peristaltic reflex. Thus, renzapride and 5-HT, in concentrations consistent with agonism at the putative 5-HT_4 receptor, initiated peristalsis at subthreshold intraluminal pressures. This action was blocked by ICS 205-930, but not by ondansetron, clearly implicating the putative 5-HT_4 receptor over an action at 5-HT_3 receptors (Craig & Clarke, 1990; Craig *et al.*, 1990). Complete blockade by ICS 205-930 suggests an action independent of ileal 5-HT_{1A}, '5-HT_1-like', 5-HT_2 and putative 5-HT_{1P} receptors, all of which are present in the guinea-pig ileum (Craig & Clarke, 1990) and all of which are resistant to inhibition by ICS 205-930 (Bradley *et al.*, 1986; Mawe *et al.*, 1986). Furthermore, ICS 205-930 failed to block peristalsis evoked by DMPP, clearly demonstrating a selectivity of action. The failure of ICS 205-930, itself, to affect the peristaltic reflex suggests that the putative 5-HT_4 receptor may play a secondary or modulatory function in the reflex, rather than a key role in an essential neural circuit.

The prokinetic activity of substituted benzamides in animals and man is well documented, but a firm mechanistic base for enhancing gut motility has proved elusive (Schuurkes, 1987; King & Sanger, 1988; Stanniforth & Pennick, 1990). At 50% threshold pressure, enhanced acetylcholine release by 5-HT and renzapride, via agonism at the putative 5-HT_4 receptor (Craig & Clarke, 1990), may serve to facilitate the reflex, and thus represent the target site for prokinetic activity. Similarly, after years of inconclusive study, a rational explanation is now advanced for the documented (Bülbring & Crema, 1958) facilitatory role of 5-HT on the peristaltic reflex.

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References

BRADLEY, P.B., ENGEL, G., FOZARD, J.R., HUMPHREY, P.P.A., MIDDLEMISS, D.N., MYELCHARANE, E.J., RICHARDSON, B.P. & SAXENA, P.R. (1986). Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. *Neuropharmacol.*, **25**, 563–576.

BÜLBRING, E. & CREMA, A. (1958). Observations concerning the action of 5-hydroxytryptamine on the peristaltic reflex. *Br. J. Pharmacol. Chemother.*, **13**, 444–457.

CRAIG, D.A. & CLARKE, D.E. (1990). Pharmacological characterization of a neuronal receptor for 5-hydroxytryptamine in guinea-pig ileum with properties similar to the 5-hydroxytryptamine$_4$ receptor. *J. Pharmacol. Exp. Ther.*, **252**, 1378–1386.

CRAIG, D.A., EGLEN, R.M., WALSH, L.K.M., PERKINS, L.A., WHITING, R.L. & CLARKE, D.E. (1990). 5-Methoxytryptamine and 2-methyl-5-hydroxytryptamine-induced desensitization as a discriminative tool for the 5-HT_3 and putative 5-HT_4 receptors in guinea-pig ileum. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **342**, 9–16.

KING, F.D. & SANGER, G.J. (1988). Gastrointestinal motility enhancing agents. *Annu. Rep. Med. Chem.*, **23**, 201–210.

MAWE, G.M., BRANCHEK, T.A. & GERSHON, M.D. (1986). Peripheral neural serotonin receptors: Identification and characterization with specific antagoists and agonists. *Proc. Natl. Acad. Sci., U.S.A.*, **83**, 9799–9803.

STANNIFORTH, D.H. & PENNICK, M. (1990). Human pharmacology of renzapride: a new gastrokinetic benzamide without dopamine antagonist properties. *Eur. J. Clin. Pharmacol.*, **38**, 161–164.

SCHUURKES, J.A.J., MEGENS, A.A.H.P., NIEMEGEERS, C.J.E., LEYSEN, J.E. & VAN NUETEN, J.M. (1987). A comparative study of the cholinergic vs the anti-dopaminergic properties of benzamides with gastrointestinal prokinetic activity. In *Cellular Physiology and Clinical Studies of Gastrointestinal Smooth Muscle.* ed. Szurszewski, J.H. pp. 231–247. New York: Elsevier.

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Coronary vasoconstriction in the conscious rabbit following intravenous infusion of L-N^G-nitro-arginine

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Intravenous infusion of L-N^G-nitro-arginine, an inhibitor of endothelial nitric oxide (NO) synthesis, produced vasoconstriction in the coronary, cerebral, renal and duodenal vascular beds of the conscious rabbit. In this study, using radiolabelled microspheres, we provide *in vivo* evidence for a basal NO-dependent vasodilator tone in the coronary vascular bed.

Introduction Intravenous (i.v.) infusion of L-N^G-monomethyl-arginine (L-NMMA), an inhibitor of endothelial nitric oxide (NO) synthesis, increases blood pressure (BP) in anaesthetized rabbits and produces vasoconstriction in a number of vascular beds in the conscious rat (Rees *et al.*, 1989; Gardiner *et al.*, 1990), implying a fundamental role for NO in the maintenance of normal resting vascular tone *in vivo*. Results from *in vitro* experiments suggest a similar role for NO in the coronary circulation (Amezcua *et al.*, 1989). In the present study, by use of a radiolabelled microsphere technique in conscious rabbits, we now show that i.v. infusion of the NO-synthesis inhibitor L-N^G-nitro-arginine (L-NOARG, Moore *et al.*, 1990) produces coronary vasoconstriction *in vivo*.

Methods Male New Zealand White rabbits (2.5–3.5 kg, Froxfield) were anaesthetized with 0.9% alphaxalone/0.3% alphadalone (0.25 ml kg^{-1}, i.v. plus supplements, Saffan, Pitman-Moore) and cannulae were implanted for BP measurement and arterial blood reference sampling (right femoral artery), drug infusion (left jugular vein) and microsphere administration (left ventricle (LV) via left carotid artery). After a 24 h recovery period rabbits were placed in canvas restraining bags for the experimental procedure. BP and heart rate (HR) were recorded continuously and, following a 40 min stabilization period, baseline values were noted and the first suspension of microspheres (^{141}Ce) administered.

Throughout the 75 s microsphere administration cycle a reference blood sample was withdrawn from the femoral artery at 2 ml min^{-1}. Immediately prior to administration, approximately 300,000 microspheres (15 μm diameter, New England Nuclear) were suspended in 1 ml 0.02% Tween 80 by sonication and vortex mixing for 2 min. The microsphere suspension, followed by 1 ml heparinized saline (100 u ml^{-1}), was then steadily administered into the LV cannula over a period of 10 s starting 15 s into the cycle. The reference blood sample, empty aliquot vial and administration line were retained for counting.

The second microsphere suspension (^{103}Ru) was administered in the same manner after a 20 min i.v. infusion (0.2 ml min^{-1}) of either vehicle (saline) or L- or D-NOARG (0.5 mg kg^{-1} min^{-1}, Chemical Dynamics). Each animal received only one infusion and, following the second microsphere administration, was killed with an overdose of pentobarbitone. Tissues of interest were then removed and the left ventricular wall was sectioned into sub-epicardial, mid-myocardial and sub-endocardial layers (Hof, 1983). The initial microsphere suspensions and all samples were counted in a Packard 5530 gamma counter. Tissue blood flows (TBF) and cardiac output (CO), normalized for tissue and body weights, were derived by the reference flow principle (Heymann *et al.*, 1977) following background and crosstalk corrections (Packard Compusphere). Tissue vascular and total peripheral resistances (TVR and TPR) were calculated as BP/TBF and BP/CO respectively. Between group comparisons of baseline values were made by analysis of variance (ANOVA) and the significance of drug-induced changes was tested by Student's paired *t* test. A P value < 0.05 was taken to indicate a significant difference.

Results No between group differences in absolute pre-infusion values (Table 1) were observed with the exception of higher TVR (ileum and skin) and TPR in the saline group. The latter difference was only significant when compared to the D-NOARG group. Infusion of D-NOARG (0.5 mg kg^{-1} min^{-1}) produced a slight fall in CO but, in common with saline infusion, no change in BP, HR, or TPR and no vasoconstriction in any vascular bed studied. However, the same dose of L-NOARG produced significant increases in TVR in the vascular beds of the heart, kidneys, brain and duodenum. These effects, which were accompanied by increases in TPR and BP (31 and 10%) and falls in HR and CO (16 and 16%), are summarized in Figure 1. Coronary vasoconstriction occurred throughout the left ventricular wall and was statistically significant in the sub-endocardial and mid-myocardial layers. D-NOARG produced significant vasodilatation in the vascular bed supplying the back muscle.

Discussion Our results confirm previous observations of renal, mesenteric and cerebral vasoconstriction following i.v.

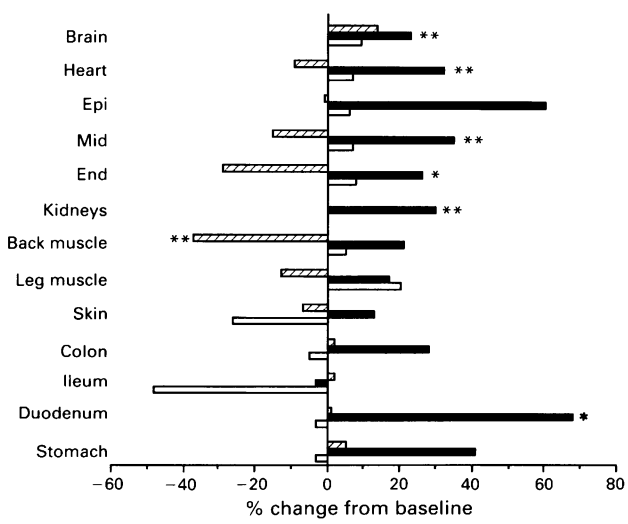

Figure 1 Effect of a 20 min i.v. infusion (0.2 ml min^{-1}) of vehicle (saline, open columns, $n = 5$), L-N^G-nitro-arginine (L-NOARG, 0.5 mg kg^{-1} min^{-1}, solid columns, $n = 6$) or D-NOARG (0.5 mg kg^{-1} min^{-1}, hatched columns, $n = 7$) on tissue vascular resistance in conscious rabbits. Abbreviations as in Table 1. Columns show percentage changes from pre-infusion values calculated from mean data presented in Table 1. * $P < 0.05$ and ** $P < 0.01$ (paired Student's *t* test applied to absolute changes shown in Table 1).

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Table 1 Baseline systemic haemodynamic variables and tissue vascular resistances in conscious rabbits and changes (Δ) produced by i.v. infusion of saline, L-N^G-nitro-arginine (L-NOARG) or D-NOARG

Systemic variable	*Saline* ($n = 5$)		L-NOARG ($n = 6$)		D-NOARG ($n = 7$)	
	Baseline	Δ	*Baseline*	Δ	*Baseline*	Δ
BP	98 ± 4	0 ± 3	98 ± 6	11 ± 3*	93 ± 4	−3 ± 3
HR	258 ± 16	−2 ± 3	261 ± 12	−42 ± 9**	264 ± 20	−5 ± 6
TPR	592 ± 52	41 ± 37	532 ± 41	166 ± 24**	451 ± 22	35 ± 15
CO	173 ± 21	−12 ± 16	189 ± 16	−30 ± 8*	210 ± 14	−22 ± 6*
Organ	*Tissue vascular resistance* (mmHg ml^{-1} min^{-1} g^{-1})					
Brain	152 ± 12	13 ± 7	138 ± 15	32 ± 7**	124 ± 9	17 ± 12
Heart	41 ± 7	3 ± 4	42 ± 5	13 ± 3**	53 ± 4	−5 ± 5
Epi	35 ± 7	2 ± 3	32 ± 4	19 ± 8	40 ± 5	−1 ± 5
Mid	29 ± 5	2 ± 3	30 ± 3	11 ± 2**	40 ± 5	−6 ± 3
End	28 ± 5	2 ± 3	33 ± 4	8 ± 3*	48 ± 8	−14 ± 8
Kidneys	26 ± 2	0 ± 1	19 ± 1	6 ± 1**	21 ± 3	1 ± 3
Back muscle	2089 ± 674	99 ± 71	1906 ± 309	402 ± 611	1857 ± 277	−681 ± 168**
Leg muscle	1761 ± 517	346 ± 243	1488 ± 279	246 ± 566	1382 ± 167	−181 ± 245
Skin	2382 ± 629	−621 ± 435	957 ± 107	126 ± 89	945 ± 126	−61 ± 178
Colon	518 ± 25	−26 ± 27	463 ± 91	129 ± 59	365 ± 70	7 ± 56
Ileum	326 ± 59	−156 ± 59	184 ± 28	−6 ± 29	141 ± 27	3 ± 32
Duodenum	172 ± 47	−5 ± 29	127 ± 17	87 ± 31*	111 ± 22	1 ± 15
Stomach	205 ± 54	−6 ± 29	164 ± 44	68 ± 30	120 ± 25	6 ± 8

Abbreviations and units: BP: blood pressure (mmHg); HR: heart rate (beats min^{-1}); TPR: total peripheral resistance (mmHg ml^{-1} min^{-1} g^{-1}); CO: cardiac output (ml min^{-1} kg^{-1}). Epi, Mid, End: subepicardial, midmyocardial and subendocardial layers of left ventricular wall. Values are means ± s.e.mean from the number of experiments shown in parentheses. * $P < 0.05$ and ** $P < 0.01$ (paired Student's t test).

administration of an inhibitor of NO synthesis (Gardiner *et al.*, 1990). More importantly, the present study demonstrates that acute i.v. infusion of such an agent results in coronary vasoconstriction in a conscious animal.

Inhibition of NO-dependent responses by arginine analogues is L-enantiomer specific and the *in vitro* effects of L-NOARG are reversed by L- but not D-arginine (Moore *et al.*, 1990). This specificity is an established criterion for invoking inhibition of NO production as being responsible for the effects of arginine analogues. In the present study this requirement was met by the demonstration that only L-NOARG produced vasoconstriction.

D-NOARG produced significant vasodilatation in the vasculature of the back muscle. Our results can give no indication of the mechanism for this effect but a positive interaction between NO and D-NMMA has recently been demonstrated *in vitro* (Boulanger *et al.*, 1990).

Our study indicates that there is a basal NO-dependent vasodilator tone in a number of vascular beds, including the coronary circulation. This is likely to be of particular importance in the heart since oxygen extraction is already near maximum at rest and coronary vascular resistance is a major determinant of adequate oxygen delivery to the myocardium. The demonstration that infusion of an inhibitor of endothelial NO synthesis results in coronary vasoconstriction *in vivo* supports the proposal (Vanhoutte & Houston, 1985) that pathophysiological loss of normal endothelial function may predispose to coronary vasospasm and ischaemia.

Note added in proof

Since submission of this manuscript our attention has been drawn to the work of Chu and coworkers (1990) who demonstrated coronary vasoconstriction in conscious dogs following infusion of L-NMMA directly into the left atrium of the heart.

References

AMEZCUA, J.L., PALMER, R.M.J., DE SOUZA, B.M. & MONCADA, S. (1989). Nitric oxide synthesized from L-arginine regulates vascular tone in the coronary circulation of the rabbit. *Br. J. Pharmacol.*, **97**, 1119–1124.

BOULANGER, C., SCHINI, V.B., MONCADA, S. & VANHOUTTE, P.M. (1990). Stimulation of cyclic GMP production in cultured endothelial cells of the pig by bradykinin, adenosine diphosphate, calcium ionophore A23187 and nitric oxide. *Br. J. Pharmacol.*, **101**, 152–156.

CHU, A., CHAMBERS, D.E., LIN, C.C., KUEHL, W.D. & COBB, F.R. (1990). Nitric oxide modulates epicardial coronary basal vasomotor tone in awake dogs. *Am. J. Physiol.*, **258**, H1250–H1254.

GARDINER, S.M., COMPTON, A.M., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1990). Control of regional blood flow by endothelium-derived nitric oxide. *Hypertension*, **15**, 486–492.

HEYMANN, M.A., PAYNE, B.D., HOFFMAN, J.I.E. & RUDOLPH, A.M. (1977). Blood flow measurements with radionuclide-labelled particles. *Prog. Cardiovasc. Dis.*, **20**, 55–79.

HOF, R.P. (1983). Calcium antagonists and the peripheral circulation: differences and similarities between PY 108-068, nicardipine, verapamil and diltiazem. *Br. J. Pharmacol.*, **78**, 375–394.

MOORE, P.K., AL-SWAYEH, O.H., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitro-arginine (L-NOARG), a novel, L-arginine reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.*, **99**, 408–412.

REES, D.D., PALMER, R.M.J. & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3375–3378.

VANHOUTTE, P.M. & HOUSTON, D.S. (1985). Platelets, endothelium, and vasospasm. *Circulation*, **72**, 728–734.

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Relaxation of sheep cerebral arteries by vasoactive intestinal polypeptide and neurogenic stimulation: inhibition by L-N^G-monomethyl arginine in endothelium-denuded vessels

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1 Perivascular nerves of the sheep middle cerebral artery show immunoreactivity for both vasoactive intestinal polypeptide (VIP) and calcitonin gene-related peptide (CGRP).

2 Rings of endothelium-denuded sheep middle cerebral artery precontracted with 5-hydroxytryptamine were relaxed by CGRP (maximum relaxation = 87.8 ± 8.1%, pD_2 = 7.81 ± 0.12, n = 12) and by VIP (maximum relaxation = 55.1 ± 4.1%, pD_2 = 7.65 ± 0.04, n = 18). Rings of endothelium-denuded cat middle cerebral artery precontracted with U46619 were also relaxed by vasoactive intestinal polypeptide (maximum relaxation = 53.1 ± 6.1%, pD_2 = 7.82 ± 0.11, n = 6).

3 Haemolysate (1 $\mu l\,ml^{-1}$) inhibited VIP-induced relaxation in endothelium-denuded sheep and cat middle cerebral artery (n = 6) but had no effect on the CGRP-induced relaxation of the sheep middle cerebral artery (n = 6).

4 The relaxant response to VIP in endothelium-denuded sheep middle cerebral artery was inhibited by methylene blue (10 μM) and augmented by either M&B 22948 (10 μM) or superoxide dismutase (150 units ml^{-1}). Indomethacin (1 μM) had no effect.

5 The addition of L-N^G-monomethyl arginine (100 μM) inhibited both neurogenic and VIP-induced relaxation of endothelium-denuded sheep MCA by 56 ± 6% and 60 ± 6% (n = 5) respectively. The CGRP-induced relaxation was unaffected.

6 It is concluded that neurally mediated vasodilatation in the sheep middle cerebral artery is mediated largely by VIP through a direct action on smooth muscle through a cyclic-GMP-mediated mechanism that appears to involve synthesis of nitric oxide from L-arginine. Vasodilatation by CGRP, which is also contained in perivascular nerves, does not utilize this pathway.

Introduction

Neuronal stimulation of the sheep middle cerebral artery results in a complex mechanical response (Duckles, 1979), part of which is mediated by a relaxant factor that is sensitive to inhibition by haemolysate (Gaw *et al.*, 1990).

Immunohistochemical studies have shown the presence of both vasoactive intestinal polypeptide (VIP)-like and calcitonin gene-related peptide (CGRP)-like immunoreactivity in nerve fibres within the cerebral arteries of several species including man (Larsson *et al.*, 1976; Edvinsson *et al.*, 1980; 1987; Kobayashi *et al.*, 1983; Matsuyama *et al.*, 1983; Edvinsson & Ekman, 1984; Itakura *et al.*, 1984; Gibbins *et al.*, 1984; 1985; Hanko *et al.*, 1985; Saito *et al.*, 1985). The relaxant effects of VIP and CGRP have been shown to be caused by a direct action on the cerebrovascular smooth muscle (Lee & Saito, 1984; Edvinsson *et al.*, 1985; Hongo *et al.*, 1989; Saito *et al.*, 1989). Although the mechanisms of action are not clear, both peptides appear to cause an increase in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels within the muscle cells of cat cerebral arteries (Edvinsson *et al.*, 1985) and they have been shown to hyperpolarize the smooth muscle cells of both cat and rabbit middle cerebral arteries (Saito *et al.*, 1989; Standen *et al.*, 1989).

The objective of the present study was to determine whether the perivascular nerves of the sheep middle cerebral artery contained either VIP or CGRP, or both, to compare the pharmacological properties of these vasodilator peptides with the neurogenic mediator of relaxation, and to investigate whether or not the L-arginine/nitric oxide pathway is involved.

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Methods

Preparation of specimens

Sheep heads were obtained daily from an abattoir. The brain was rapidly excised and transferred to a beaker of oxygenated physiological salt solution (composition, mM: NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.1). The solution was bubbled with a 95% O_2/5% CO_2 gas mixture (BOC Medical Gases). The middle cerebral artery was dissected free of the brain with a short portion of the circle of Willis attached. The middle cerebral arteries from the cat were removed from the cat brain on the evening prior to the experiment and stored overnight (14–16 h) in physiological salt solution at 4°C for use the following morning.

Immunohistochemical labelling

Sheep middle cerebral arteries were cut into 4 mm ring segments, slit open longitudinally and pinned flat onto pieces of Sylgard. They were fixed overnight in Zamboni's fixative (2% formaldehyde, 0.2% v/v of a 15% saturated solution of picric acid in 0.1 M phosphate buffer, pH 7.0–7.4) and then washed 3 times in 80% ethanol and 3 times in 0.1 M phosphate buffered saline, pH = 7.2. The tissue was then incubated overnight at room temperature with primary antiserum against either vasoactive intestinal polypeptide (1:400) or calcitonin gene-related peptide (1:400). After 3 washes with 1% Triton X-100 in phosphate buffered saline, all tissues were incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin diluted 1:50, counterstained with pontamine sky blue to reduce background fluorescence (Cowen *et*

al., 1985) and transferred onto clean slides. The vessels were dried, mounted with 75% (v/v) glycerol (Citifluor) in phosphate buffered saline and viewed under a Zeiss III RS fluorescence microscope fitted with the appropriate FITC filter system (excitation filter = BP 450–490, beam splitter = FT 510, barrier filter = LP 520, short pass filter = KP 560).

Antibody specificity

The specificity of immunoreactivity was determined on trial pieces of artery by preabsorbing the antibody with the appropriate antigen and then using the absorbed antibody as the 'primary antiserum' in the technique described above. No immunoreactivity was seen for either peptide after preabsorption with a 1 μM solution of the appropriate antigen.

Pharmacological studies

In these studies all middle cerebral arteries were denuded of endothelium by intraluminal perfusion with the non-ionic detergent Triton X-100 which results in denudation of the endothelium without causing any damage of the cerebrovascular smooth muscle (Connor & Feniuk, 1987). A stainless steel cannula was inserted via the circle of Willis into the lumen of the middle cerebral artery and the tissue was perfused intraluminally with 0.1% Triton X-100 solution for 1 min at a flow rate of 0.5 ml min^{-1}. The tissue was then perfused with physiological salt solution for 1 min at a flow rate of 0.5 ml min^{-1} before removing the cannula. The section of artery which had been in contact with the cannula was discarded. Rings prepared from sheep middle cerebral arteries that had not been perfused with Triton X-100 contained endothelium as demonstrated by relaxation with substance P (100 nM) following precontraction with 10 μM 5-hydroxytryptamine (5-HT) (relaxation 58 ± 5% of 5-HT induced tone n = 12). Triton X-100 perfusion caused complete destruction of endothelium since no relaxation occurred in any of 12 preparations on addition of substance P (100 nM) following precontraction with 5-HT.

Arterial ring segments of 3–4 mm in length were cut from each middle cerebral artery. Each ring was suspended on two intraluminal parallel wires of 100 μm diameter in a manner similar to that of Nielsen & Owman (1971). The rings were then placed under 0.2 g of tension for 1 h before application of the optimal preloads for each artery, determined in preliminary experiments as 1.0 g for the sheep middle cerebral artery and 0.5 g for the cat middle cerebral artery. The tissues were then left for a 30 min equilibration period following which the middle cerebral arteries were precontracted with a maximal concentration of 5-hydroxytryptamine (5-HT, 10 μM). Absence of functional endothelium was confirmed by lack of relaxation of the precontracted artery on addition of substance P (100 nM). The tissues were washed at 0, 2, 4, 6, 8 and 10 min and then left for 30 min to equilibrate. The tissues were then precontracted with a submaximal concentration of 5-HT (1 μM, giving approximately 80% of 5-HT maximum contraction) and left for 5 min to attain a constant tension. Either CGRP or VIP were then added to the tissue in a cumulative fashion (1 nM–300 nM) and any changes in tone were noted. The tissues were washed at 0, 2, 4, 6, 8 and 10 min and left for 30 min before the addition of either haemolysate solution (1 μl ml^{-1}), L-N^G monomethyl arginine (L-NMMA; 100 μM) or an equivalent volume of drug vehicle as a control. The tissues were left for a further 30 min equilibration period.

The tissues were then precontracted with 5-HT (1 μM) and the addition of either CGRP or VIP was repeated. The changes in tone induced by these agents were expressed as percentages of the resting tone induced by 5-HT (1 μM), and concentration-effect curves were plotted for each compound in the absence and presence of haemolysate or L-NMMA. In each arterial ring the potency of the agonist was calculated as the negative logarithm of the molar concentration necessary to give 50% of the maximum relaxation (pD_2 value). From these individual pD_2 values were calculated the mean and s.e.mean for each series of experiments. Fleming *et al.* (1972) showed that, generally pD_2 values are normally distributed. Moreover, the statistical package that was used in the present study includes a test for normality, which confirmed a normal distribution. A recovery time of 60 min between concentration-effect curves was chosen to avoid problems of tachyphylaxis. The effect of haemolysate on the VIP-induced relaxation of the cat middle cerebral artery was investigated using a similar experimental protocol as that described above. Because of the variability of 5-HT responses in the cat middle cerebral artery, U46619 (100 nM) was used to precontract the arteries.

The effects of other agents on the VIP-induced relaxation of the sheep middle cerebral artery were examined. An identical protocol to that described above was used except that addition of the haemolysate was replaced by the addition of either indomethacin (10 μM), M&B 22948 (10 μM), methylene blue (10 μM) or superoxide dismutase (150 units ml^{-1}).

The effect of L-NMMA on neurogenic relaxation in the sheep middle cerebral artery was also investigated. The arteries were precontracted with 1 μM 5-HT and left for 5 min to attain a constant tension. Electrical field stimulation was carried out with parallel platinum electrodes, one on either side of the arterial ring, and stimulation parameters were chosen which produced supramaximal nerve stimulation (15 s trains of stimuli, 20 V, 8 Hz, 0.2 ms pulse width, 10 min cycle). After obtaining 3 reproducible responses to electrical stimulation, L-NMMA (100 μM) was added and stimulation repeated until a further 3 reproducible responses were obtained. All responses elicited with electrical stimulation were abolished by tetrodotoxin (300 nM).

Drugs

The following compounds were used in the course of these experiments: calcitonin gene-related peptide (human, Sigma Chemical Co., Poole, Dorset), calcitonin gene-related peptide antibody (Immunodiagnostics Ltd, Tyne & Wear), Citifluor (City University, London), goat anti-rabbit immunoglobulin conjugated to fluorescein isothiocyanate (Nordic, Maidenhead), 5-hydroxytryptamine creatinine sulphate (Sigma), indomethacin (Sigma), M&B 22948 (2-*O*-propoxyphenyl- 8-azapurin-6-one; May & Baker, Dagenham), methylene blue (Sigma), L-N^G-monomethyl arginine (William Harvey Institute, London), Sylgard (ICI), substance P (Sigma), superoxide dismutase (bovine, Sigma), Triton X-100 (Sigma), U46619 (11α,9α-epoxymethano prostaglandin H_2, Upjohn, Kalamazoo, MI, U.S.A.), vasoactive intestinal polypeptide (porcine, Sigma), vasoactive intestinal polypeptide antibody (Immunodiagnostics Ltd).

The drugs were dissolved in distilled water to form a stock solution of 10 mM. Exceptions to this were indomethacin and U46619 which were dissolved in 10% ethanol to give 1 mM stock solutions and M&B 22948 which was dissolved in 1 M sodium hydroxide to give a 10 mM stock solution. All subsequent dilutions were carried out with 0.9% NaCl.

Haemolysate solution

The haemolysate solution was prepared by lysis of feline erythrocytes in a hypotonic phosphate buffer, followed by centrifugation and dialysis to produce a final concentration equivalent to 5% whole blood by the method of Bowman & Gillespie (1982). The haemoglobin concentration of the haemolysate was determined by spectrophotometric analysis and it was found to be 8 ± 4 g l^{-1} (n = 3) which is equivalent to a molar concentration of 120 ± 60 μM. The haemolysate was used at a final bath concentration of 1 μl ml^{-1} resulting in a haemoglobin concentration of 120 ± 60 nM.

Statistical analysis

The data are expressed as the mean ± s.e.mean of a group of observations; n refers to the number of heads used. Statistical comparisons between groups were made by Student's t test for paired and unpaired data. $P < 0.05$ was considered to be statistically significant.

Results

Immunohistochemistry

Both VIP-like and CGRP-like immunoreactivity was seen in the perivascular nerves of the sheep middle cerebral artery (Figure 1). A dense plexus of nerves containing VIP-like immunoreactivity covered the vessel wall, mostly running longitudinally down the vessel as single varicose fibres or as small fibre bundles. The pattern formed by the nerves containing CGRP-like immunoreactivity was quite different, consisting of a complex honeycomb of fine fibres and small nerve bundles, overlayed by thick fibre bundles that were probably paravascular nerves traversing the blood vessel. Comparison of the two types of nerve plexi shows that perivascular VIP-immunoreactive nerves are more dense than CGRP-immunoreactive nerves.

Vascular relaxation

In the endothelium-denuded sheep middle cerebral artery, the contractile response elicited with 5-HT (1 μM) was not affected by haemolysate or any of the other agents investigated, therefore any subsequent changes in the dilator responses were not caused by changes in initial tone (Table 1). The potencies of CGRP and VIP in causing relaxation of the sheep middle cerebral artery were similar ($pD_2 = 7.81 \pm 0.12$ for CGRP, $n = 12$ and 7.65 ± 0.04 for VIP, $n = 18$) however, CGRP caused a greater maximal relaxation of the 5-HT (1 μM)-induced tone than VIP (CGRP = 87.8 ± 8.1%, VIP = 55.11 ± 4.1% relaxation, $n = 6$).

The addition of haemolysate did not inhibit the relaxation observed with CGRP (Figure 2), however the relaxation induced by VIP was inhibited by haemolysate and also by methylene blue, a soluble guanylate cyclase inhibitor (Figure 3). VIP also caused a relaxation of U46619-induced tone in the cat middle cerebral artery which was sensitive to inhibition by haemolysate (Figure 4). VIP had a similar potency ($pD_2 = 7.82 \pm 0.11$, $n = 6$) and caused a similar degree of relaxation in the cat (53.1 ± 6.1%, $n = 6$) as in the sheep middle cerebral artery.

The cyclic GMP phosphodiesterase inhibitor M&B augmented the relaxant effects of VIP (Figure 5). The relaxant effect of VIP was also augmented by superoxide dismutase; however, the cyclo-oxygenase inhibitor indomethacin had no effect (Figure 5).

The addition of L-NMMA did not affect either the 5-HT-induced tone or the CGRP-induced relaxant response (Figure 6). However, VIP-induced relaxation was inhibited by L-NMMA (100 μM), which caused depression of the concentration-relaxation curve (Figure 6) and inhibition, by 60 ± 6%, at the highest concentration of VIP.

Nerve stimulation caused relaxation to 38 ± 3% of 5-HT-induced tone. This neurogenic relaxant response was significantly inhibited by L-NMMA (100 μM) by 55.9 ± 5.5% ($n = 5$) and significantly increased by superoxide dismutase (150 units ml^{-1}) to 160 ± 12% ($n = 4$).

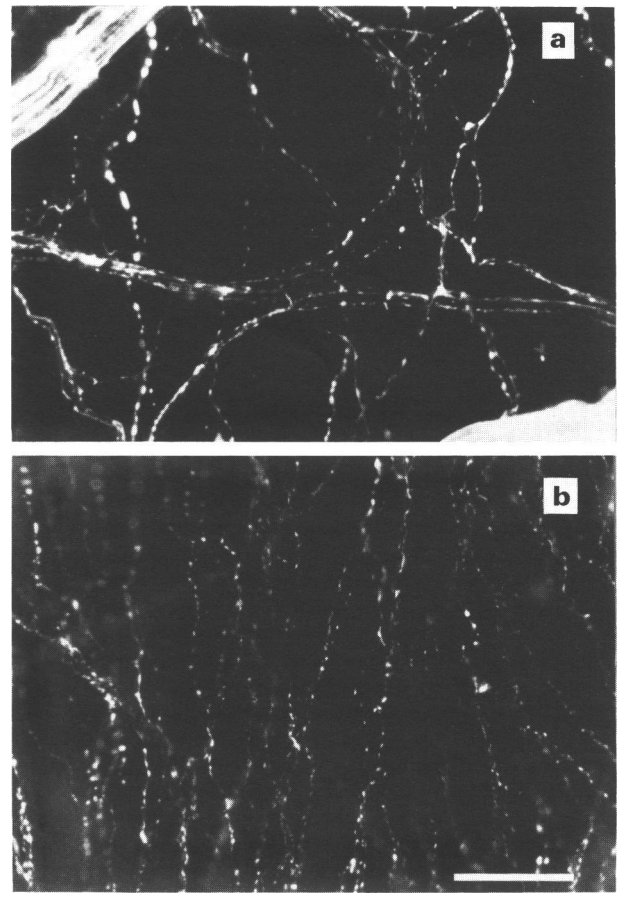

Figure 1 Fluorescence micrographs of calcitonin gene related peptide (a) and vasoactive intestinal polypeptide (b) immunoreactive nerve fibres in stretch preparations of sheep middle cerebral artery. The micrographs illustrate the differences in pattern and relative density of each nerve fibre type. The scale bar represents 50 μm for both plates.

Discussion

Both VIP and CGRP appear to be present within the perivascular nerves of the sheep middle cerebral artery and the

Table 1 Initial contractile response obtained to 5-hydroxytryptamine (5-HT, 1 μM) before addition of either calcitonin-gene related peptide (CGRP) or vasoactive intestinal polypeptide (VIP) in the sheep middle cerebral artery

		Contractile response with 5-HT (1 μM)		
	Agonist treatment	*Before treatment*	*After treatment*	n
CGRP	None	0.98 ± 0.12 g	1.09 ± 0.09 g	6
	Haemolysate	0.92 ± 0.12 g	0.96 ± 0.10 g	6
VIP	None	1.25 ± 0.20 g	1.23 ± 0.11 g	12
	Haemolysate	1.35 ± 0.21 g	1.30 ± 0.25 g	6
	Methylene blue	1.02 ± 0.19 g	1.04 ± 0.20 g	6
	M&B22948	0.89 ± 0.15 g	0.86 ± 0.15 g	6
	Superoxide dismutase	1.03 ± 0.17 g	0.94 ± 0.13 g	6
	L-NMMA	1.04 ± 0.18 g	1.17 ± 0.28 g	5

Results are expressed as the mean ± s.e.mean and n represents the number of preparations. L-NMMA = L-N^G-monomethyl arginine.

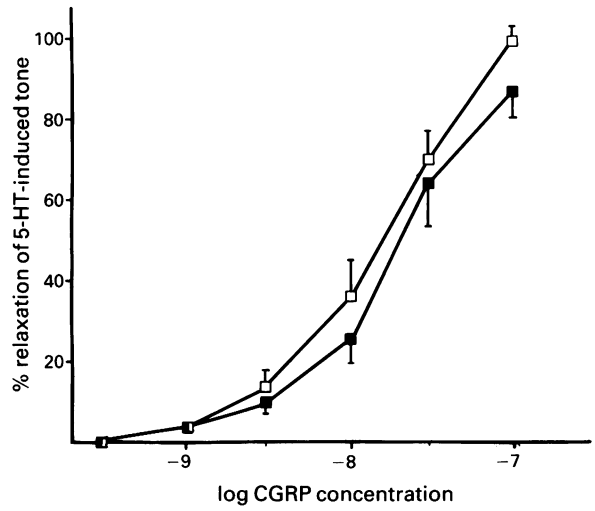

Figure 2 Concentration-effect curves showing the relaxant effects of calcitonin gene-related peptide (CGRP) on tone induced by 5-hydroxytryptamine (5-HT, 1 μM) in the sheep middle cerebral artery denuded of endothelium. The curves show the relaxation elicited by CGRP in the absence (■) or presence (□) of haemolysate (1 μl ml^{-1}). The responses were calculated as a percentage of the 5-HT-induced tone and are expressed as the mean of 6 experiments with s.e.mean shown by vertical bars. No significant differences between responses at any given concentration were observed.

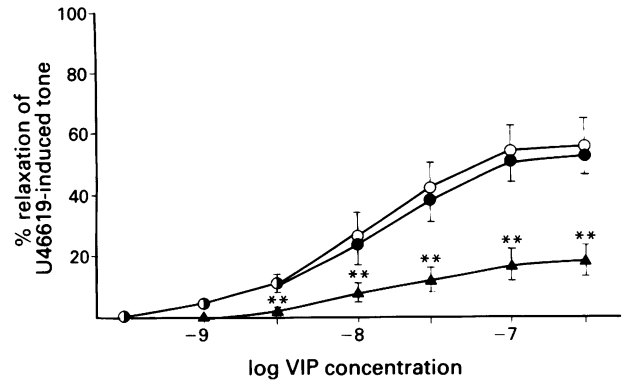

Figure 4 Concentration-effect curves showing the relaxant effects of vasoactive intestinal polypeptide (VIP) on tone induced by U46619 (100 nM) in the cat middle cerebral artery denuded of endothelium. The curves show the relaxation elicited by VIP either alone (●), in the presence of drug vehicle (○) or in the presence of haemolysate 1 μl ml^{-1} (▲). The responses were calculated as a percentage of the U46619-induced tone and are expressed as the mean of 6 experiments; s.e.mean shown by vertical bars. Significant differences from the vehicle responses were depicted by ** $P < 0.01$.

results from the pharmacological studies have also shown that both of these agents are effective relaxant agents in the sheep middle cerebral artery. Both of these agents may therefore be regarded as potential mediators of neurogenic relaxation in the sheep cerebral vasculature. Similar results have been obtained in other species (Lee & Saito, 1984; Edvinsson *et al.*, 1985; Saito *et al.*, 1989).

The VIP-induced relaxation of the sheep middle cerebral artery, like the neurogenic mediator of relaxation (Gaw *et al.*, 1990), was susceptible to inhibition by a haemolysate solution from feline erythrocytes. This inhibitory action of haemolysate appeared to be selective since the relaxation induced by CGRP, another vasodilator peptide, was unaffected. Methylene blue also inhibited VIP-induced relaxation. Since both haemoglobin and methylene blue inhibit the activation of soluble guanylate cyclase by nitric oxide, endothelium-derived relaxing factor (EDRF) and nitrovasodilators (Murad *et al.*, 1978; Gruetter *et al.*, 1981; Martin *et al.*, 1985) it would appear that the relaxant action of VIP was mediated through a similar system to that of these agents. The inhibition of the VIP-induced relaxation of the cat middle cerebral artery by haemolysate also suggests that this mechanism is not confined to the sheep. Intracellular cyclic GMP causes relaxation by activation of protein kinases leading to dephosphorylation of the myosin light chain in the contractile apparatus and also phosphorylation of other cellular proteins (Rapoport *et al.*, 1983). The ability of M&B 22948, a cyclic GMP phosphodiesterase inhibitor, to augment the VIP-induced relaxation further supports the possibility that VIP may cause relaxation by an increase in cyclic GMP levels. Superoxide dismutase is known to prevent the destruction of EDRF, a free radical, by removal of the superoxide anion (Rubanyi & Vanhoutte, 1988). The augmentation of the relaxant effect of VIP observed with superoxide dismutase suggests that VIP also acts through an intermediate which is sensitive to destruction by the superoxide anion. The relaxant response of VIP was

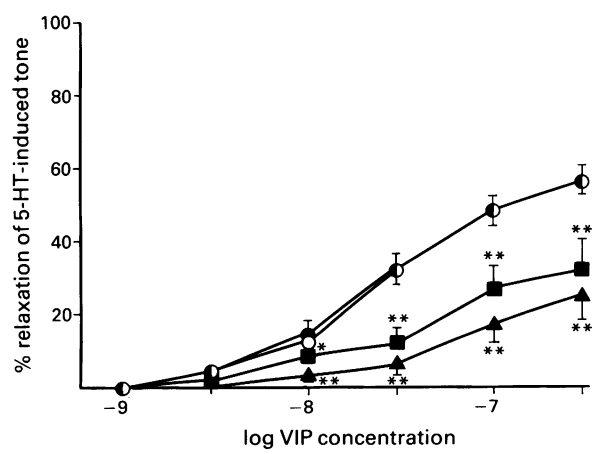

Figure 3 Concentration-effect curves showing the relaxant effects of vasoactive intestinal polypeptide (VIP) on tone induced by 5-hydroxytryptamine (5-HT, 1 μM) in the sheep middle cerebral artery denuded of endothelium. The curves show the relaxation elicited by VIP either alone (●) or in the presence of the drug vehicle (○), haemolysate 1 μl ml^{-1} (▲) or methylene blue 10 μM (■). The responses were calculated as a percentage of the 5-HT induced tone and are expressed as the mean of 6 experiments; s.e.mean shown by vertical bars. Significant differences from the vehicle responses are depicted by ** $P < 0.01$.

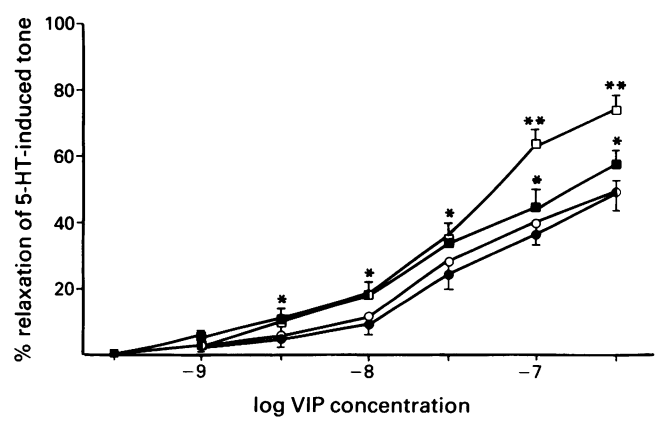

Figure 5 Concentration-effect curves showing the relaxant effects of vasoactive intestinal polypeptide (VIP) on 5-hydroxytryptamine (5-HT, 1 μM)-induced tone in the sheep middle cerebral artery denuded of endothelium. The curves show the relaxation elicited by VIP either alone (●) or in the presence of indomethacin 1 μM (○), M&B22948 10 μM (□) or superoxide dismutase 150 units ml^{-1} (■). The responses were calculated as a percentage of the 5-HT-induced tone and are expressed as the mean of 6 experiments; s.e.mean shown by vertical bars. Significant differences from the vehicle responses are depicted by * $P < 0.05$ and ** $P < 0.01$.

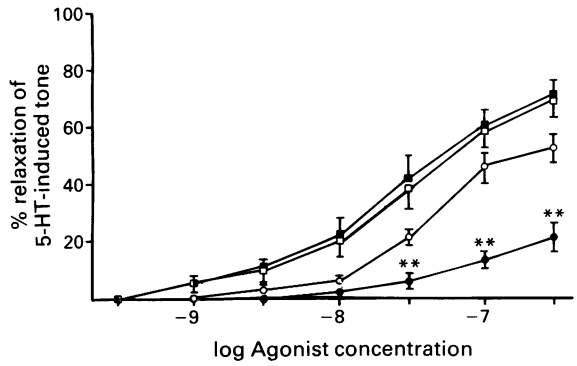

Figure 6 Concentration-effect curves showing the relaxant effects of calcitonin gene-related peptide (□■) or vasoactive intestinal polypeptide (○●) on tone induced by 5-hydroxytryptamine (5-HT, 1 μM) in the sheep middle cerebral artery. The curves show the relaxation elicited by each agonist in the absence (open symbols) or presence (closed symbols) of L-N^G-monomethyl arginine (L-NMMA) (100 μM) in endothelium-denuded preparations. The responses are calculated as a percentage of the 5-HT-induced tone and are expressed as the mean of 5 experiments; s.e.mean shown by vertical bars. Significant differences in the presence of L-NMMA are denoted by ** $P < 0.01$.

not mediated through EDRF since in all of these experiments the tissues had no functional endothelium. The lack of effect of indomethacin indicates that cyclo-oxygenase products were not involved in the VIP-induced relaxation and also shows that the inhibitory effect of haemolysate was not caused by inhibition of vasodilator prostaglandin synthesis as has been suggested by other authors (Toda, 1988).

In these experiments VIP appears to have caused relaxation of the sheep cerebral artery by the production of free radicals which then activate guanylate cyclase to increase the intracellular cyclic GMP concentration resulting in smooth muscle relaxation. It is not clear from these experiments whether the VIP acts to stimulate the formation of a free radical within the arterial smooth muscle cells, to cause the release of a free radical from another cell type within the artery wall, such as macrophages, or if the VIP itself is processed to form free radicals. In the present experiments where endothelium was absent, both the VIP-induced and neurogenic relaxation of the sheep middle cerebral artery were susceptible to inhibition by L-NMMA, an inhibitor of the pathway which produces nitric oxide from L-arginine in endothelial cells and macrophages (Hibbs *et al.*, 1987; Palmer *et al.*, 1988). The presence of VIP apparently stimulates the conversion of L-arginine to nitric oxide via a system which is independent of the endothelium. Recent studies in the rat anococcygeus muscle have also demonstrated a similar mechanism where the neurogenic relaxation was selectively inhibited by L-NMMA (Gillespie *et al.*, 1989). A recent study has reported that L-NMMA causes non-selective inhibition of vasodilators in the rat aorta by an endothelium-dependent mechanism (Thomas *et al.*, 1989). It is unlikely that the actions of L-NMMA described here can be ascribed to nonselective action since there was no functional endothelium in any of the tissues examined and the relaxant responses to CGRP were unaffected by L-NMMA.

Immunohistochemical studies have shown a dense network of nerves containing VIP-like immunoreactivity in the sheep middle cerebral artery. It is not possible to state conclusively that VIP is the neurogenic mediator of relaxation from these studies; however, it does seem plausible since VIP is present in perivascular nerves and both VIP and the neurogenic mediator appear to act through a similar mechanism which is sensitive to both haemolysate (Gaw *et al.*, 1990) and L-NMMA. It is not possible to rule out a minor contribution by CGRP to the neurogenic relaxant response, but any such contribution would have to be very small since CGRP is not affected by haemolysate and the CGRP innervation of the sheep middle cerebral artery is sparser than the VIP innervation. Haemolysate has been shown to inhibit the neurogenic relaxation observed in cat middle cerebral artery (Lee *et al.*, 1984) and other studies have also shown a reduction in the neurogenic dilator response of cat middle cerebral artery using VIP antiserum (Bevan *et al.*, 1986). However, it has been reported that VIP-induced relaxation of the cat middle cerebral artery was not affected by the addition of haemolysate (Lee *et al.*, 1987). In the present experiments, the addition of haemolysate does inhibit the ability of VIP to reduce U46619-induced tone in cat middle cerebral artery, therefore it is feasible that VIP may be the neurogenic mediator of relaxation in both the cat and sheep middle cerebral artery. If VIP acts in the human cerebral vasculature via a mechanism similar to that in the sheep then the inhibitory effects of haemolysate released from a subarachnoid blood clot could inhibit the actions of VIP. The resulting loss in vasodilator control could therefore lead to an enhancement of responses to constrictor agents resulting in vasospasm.

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References

BOWMAN, A. & GILLESPIE, J.S. (1982). Block of some non-adrenergic inhibitory responses of smooth muscle by a substance from haemolysed erythrocytes. *J. Physiol.*, **328**, 11–25.

BEVAN, J.A., BUGA, G.M., MOSKOWITZ, M.A. & SAID, S.I. (1986). *In vitro* evidence that vasoactive intestinal polypeptide is a transmitter of neuro-vasodilation in the head of the cat. *Neuroscience*, **19**, 597–604.

CONNOR, H.E. & FENIUK, W. (1987). Role of the endothelium in haemoglobin-induced contraction of dog basilar artery. *Eur. J. Pharmacol.*, **140**, 105–108.

COWEN, T., HAVEN, A.J. & BURNSTOCK, G. (1985). Pontamine sky blue: a counterstain for background autofluorescence in fluorescence and immunofluorescence histochemistry. *Histochemistry*, **82**, 205–208.

DUCKLES, S.P. (1979). Neurogenic dilator and constrictor responses of mice arteries *in vitro*: differences between dogs and sheep. *Circ. Res.*, **44**, 482–490.

EDVINSSON, L. & EKMAN, R. (1984). Distribution and dilatory effect of vasoactive intestinal polypeptide (VIP) in human cerebral arteries. *Peptides*, **5**, 329–331.

EDVINSSON, L., FAHRENKRUG, J., HANKO, J., OWMAN, C., SUNDLER, F. & UDDMAN, R. (1980). VIP (vasoactive intestinal polypeptide)-containing nerves of intracranial arteries in mammals. *Cell Tiss. Res.*, **208**, 135–142.

EDVINSSON, L., FREDHOLM, B., HAMEL, E., JANSEN, I. & VERRECCHIA, C. (1985). Perivascular peptides relax cerebral arteries concomitant with stimulation of cyclic adenosine monophosphate accumulation or release of an endothelium-derived relaxing factor in the cat. *Neurosci. Lett.*, **58**, 213–217.

EDVINSSON, L., EKMAN, R., JANSEN, I., MCCULLOCH, J. & UDDMAN, R. (1987). Calcitonin gene-related peptide and cerebral blood vessels: Distribution and vasomotor effects. *J. Cereb. Blood Flow Metab.*, **7**, 720–728.

FLEMING, W.W., WESTFALL, D.P., DE LA LANDE, I.S. & JELLET, L.B. (1972). Log-normal distribution of equieffective doses of norepinephrine and acetylcholine in several tissues. *J. Pharmacol. Exp. Ther.*, **181**, 339–345.

GAW, A.J., WADSWORTH, R.M. & HUMPHREY, P.P.A. (1990). Neurotransmission in the sheep middle cerebral artery: modulation of responses by 5-HT and haemolysate. *J. Cereb. Blood Flow Metab.*, **10**, 409–416.

GIBBINS, I.L., BRAYDEN, J.E. & BEVAN, J.A. (1984). Perivascular nerves with immunoreactivity to vasoactive intestinal polypeptide in cephalic arteries of the cat: distribution, possible origins and functional implications. *Neuroscience*, **13**, 1327–1346.

GIBBINS, I.L., FURNESS, J.B., COSTA, M., MACINTYRE, I., HILLYARD, C.J. & GIRGIS, S. (1985). Colocalisation of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous,

vascular and visceral sensory neurones of guinea pigs. *Neurosci. Lett.*, **57**, 91–95.

GILLESPIE, J.S., LIU, X. & MARTIN, W. (1989). The effects of L-arginine and N^G-monomethyl L-arginine on the response of the rat anococcygeus muscle to NANC nerve stimulation. *Br. J. Pharmacol.*, **98**, 1080–1082.

GRUETTER, C.A., KADOWITZ, P.J. & IGNARRO, L.J. (1981). Methylene blue inhibits coronary arterial relaxation and guanylate cylcase activation by nitroglycerin, sodium nitrite and amyl nitrite. *Can. J. Physiol. Pharmacol.*, **59**, 150–156.

HANKO, J., HARDEBO, J.E., KAHRSTROM, J., OWMAN, C. & SUNDLER, F. (1985). Calcitonin gene-related peptide is present in mammalian cerebrovascular nerve fibres and dilates pial and peripheral arteries. *Neurosci. Lett.*, **57**, 91–95.

HIBBS, J.B., TAINTOR, R.R. & VAVRIN, Z. (1987). Macrophage toxicity: role for arginine deaminase and imino nitrogen oxidation to nitrite. *Science*, **235**, 473–476.

HONGO, K., TSUKAHARA, T., KASSEL, N.F. & OGAWA, H. (1989). Effect of subarachnoid haemorrhage on calcitonin gene-related peptide-induced relaxation in rabbit basilar artery. *Stroke*, **20**, 100–104.

ITAKURA, T., OKUNO, T., NAKAKITA, K., KAMEI, I., NAKA, Y., NAKAI, K., IMAI, H., KOMAI, N., KIMURA, Y. & MAEDA, T. (1984). A light and electron microscopic immunohistochemical study of vasoactive intestinal polypeptide- and substance P-containing nerve fibres along the cerebral blood vessels: Comparison with aminergic and cholinergic nerve fibres. *J. Cereb. Blood Flow Metab.*, **4**, 407–414.

KOBAYASHI, S., KYOSHIMA, K., OLSCHOWKA, J.A. & JACOBOWITZ, D.M. (1983). Vasoactive intestinal polypeptide immunoreactive and cholinergic nerves in the whole mount preparation of the major cerebral arteries of the rat. *Histochemistry*, **79**, 377–381.

LARSSON, L.I., EDVINSSON, L., FAHRENKRUG, J., HAKANSON, R., OWMAN, C., SCHAFFALITZSKY, D.E., MUCKADELL, O.S. & SUNDLER, F. (1976). Immunohistochemical localisation of a vasodilatory polypeptide (VIP) in cerebrovascular nerves. *Brain Res.*, **113**, 410–444.

LEE, T.J.-F., LINNIK, M.D., SHIRASAKI, Y., IMAI, H., McILHANY, M.P. & YU, J.G. (1987). Neurogenic vasodilatation in large cerebral arteries of the cat. In *Vasodilatation*, ed. Vanhoutte, P.M., pp. 101–109. New York: Raven Press.

LEE, T.J.-F., McILHANY, M.P. & SARWINSKI, S. (1984). Erythrocyte extracts enhance neurogenic vasoconstriction of dog cerebral arteries. *J. Cereb. Blood Flow. Metab.*, **4**, 474–476.

LEE, T.J.-F. & SAITO, A. (1984). Vasoactive intestinal polypeptide-like substance: The potential transmitter for cerebral vasodilation. *Science*, **226**, 898–901.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232**, 708–716.

MATSUYAMA, T., SHIOSAKA, S., MATSUMOTO, M., YONEDA, S., KIMURA, K., ABE, H., HAYAKAWA, T., INONE, H. & TOHYAMA, M. (1983). Overall distribution of vasoactive intestinal polypeptide-containing nerves in the walls of cerebral arteries: an immunohistochemical study using whole mount preparations. *Neuroscience*, **10**, 89–96.

MURAD, F., MITTAL, C., ARNOLD, W.P., KATSUKI, S. & KIMURA, H. (1978). Guanylate cyclase: Activation by azide, nitro compounds, nitric oxide and hydroxyl radical and inhibition by hemoglobin and myoglobin. *Adv. Cyclic Nucleotide Res.*, **9**, 145–158.

NIELSEN, K.C. & OWMAN, C. (1971). Contractile response and amine receptor mechanism in isolated middle cerebral artery of the cat. *Brain Res.*, **27**, 33–42.

PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988). L-Arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Res. Commun.*, **153**, 1251–1256.

RAPOPORT, R.M., DRAZNIN, M.B. & MURAD, F. (1983). Endothelium-dependent relaxation in rat thoracic aorta may be mediated through cyclic GMP-dependent protein phosphorylation. *Nature*, **306**, 174–176.

RUBANYI, G.M. & VANHOUTTE, P.M. (1988). Modulation of the release and biological activity of endothelium-derived relaxing factor by oxygen-derived free radicals. In *Endothelium Derived Relaxing and Contracting Factors*, ed. Vanhoutte, P.M., pp. 91–105. London, New York: Humana Press.

SAITO, A., WU, J.Y. & LEE, T.J.-F. (1985). Evidence for the presence of cholinergic nerves in cerebral arteries: an immunohistochemical demonstration of choline acetyltransferase. *J. Cereb. Blood Flow Metab.*, **5**, 327–334.

SAITO, A., MASAKI, T., UCHIYAMA, Y., LEE, T.J.-F. & GOTO, K. (1989). Calcitonin gene-related peptide and vasodilator nerves in large cerebral arteries of cats. *J. Pharmacol. Exp. Ther.*, **248**, 455–462.

STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarising vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **245**, 177–180.

TODA, N. (1988). Haemolysate inhibits cerebral artery relaxation. *J. Cereb. Blood Flow Metab.*, **8**, 46–53.

THOMAS, G., COLE, E.A. & RAMWELL, P.W. (1989). N^G-monomethyl L-arginine is a non-specific inhibitor of vascular relaxation. *Eur. J. Pharmacol.*, **170**, 123–124.

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Prejunctional prostanoid receptors on cardiac adrenergic terminals belong to the EP_3 subtype

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1 The effects of prostaglandin E_2 (PGE_2) and of several synthetic prostanoids on the cardiac response to sympathetic nerve stimulation in guinea-pig atria have been evaluated.

2 PGE_2 (0.01–100 nM), sulprostone (0.01–100 nM) and misoprostol (0.1–100 nM), but not butaprost (0.1–100 nM), dose-dependently reduced the increase in cardiac contractility induced by electrical field stimulation of sympathetic terminals.

3 The EP_1 antagonist AH6809 (1 μM) did not modify the inhibition of cardiac response induced by PGE_2, sulprostone and misoprostol.

4 In preparations preloaded with [^{3}H]-noradrenaline, tritium overflow induced by electrical field stimulation was greatly and significantly reduced by 100 nM PGE_2 and by 100 nM sulprostone.

5 These results indicate that PGE_2 and other synthetic prostanoids reduce noradrenaline release from cardiac adrenergic nerve terminals acting on prejunctional inhibitory receptors belonging to the EP_3 subtype.

Introduction

Prostaglandins have a widespread role in modulating adrenergic neurotransmission in several peripheral preparations (for reviews see Hedqvist, 1977; Malik, 1978). In rabbit, rat and cat isolated hearts, prostaglandins of the E and I series reduce noradrenaline release induced by sympathetic nerve stimulation (Hedqvist *et al.*, 1970; Khan & Malik, 1980; 1982; Marques *et al.*, 1983), as well as the increase in heart rate due to adrenergic nerve stimulation (Hedqvist *et al.*, 1970). We have recently demonstrated that prostaglandin E_2 (PGE_2) is able to reduce the response to adrenergic nerve stimulation in guinea-pig isolated atria (Mantelli *et al.*, 1990).

Several receptors for prostaglandins have been recently described (Coleman *et al.*, 1984; Coleman, 1988), and the existence of three subtypes of receptors for PGE_2 (EP receptors) has been proposed (Coleman *et al.*, 1987a,b).

In this work we have attempted to characterize the EP receptors involved in the inhibitory effect of PGE_2 on adrenergic neurotransmission in guinea-pig atria, by using agonists and antagonists selective for the various EP receptor subtypes.

Methods

Male guinea pigs, weighing 200–300 g, were killed by a blow on the head. The hearts were rapidly removed, the atria were isolated and mounted vertically in a 15 ml glass chamber, containing Tyrode solution of the following composition (mM): NaCl 115, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 10. The solution was aerated with a gas mixture of 95% O_2 and 5% CO_2 and had a pH of 7.4. The temperature was kept at 30°C in order to reduce the metabolic demand of the preparations. The preparations were stimulated at a constant rate (4 Hz) slightly higher than the spontaneous rate, by square wave pulses of 0.5 ms duration and twice the threshold voltage intensity through two platinum punctate electrodes connected with a Tektronix pulse generator. The isometric contraction was recorded by an isometric transducer on a pen recorder (Battaglia Rangoni KV 135) and on a dual beam oscilloscope (Tektronix D 13). Experiments were performed after an equilibration period of at least 1 h.

Sympathetic nerve terminals were stimulated by using the method of field stimulation during the functional refractory period, as previously described (Ledda & Mantelli, 1984; Mantelli *et al.*, 1990). Trains of field pulses (75 mA, 1 ms), consisting of one pulse for each of 10 consecutive contractions, were applied at 15 min intervals through two platinum plates parallel to the preparations, connected with a second pulse generator (MARB 82/2/200); a control circuit allowed timing of the field pulses to begin 10–20 ms after each driving pulse, during the absolute refractory period. Since the response to each train of field stimulation remained reproducible for many consecutive tests it was possible to obtain concentration-response curves for the agonists in the same preparation by adding cumulative concentrations 10 min before field stimulation. The experiments with the antagonist were done in different preparations, and the antagonist was added to the bathing solution before starting field stimulation. The control responses induced by field stimulation were taken as 100% response. All the experiments were carried out in the presence of atropine 1 μM in order to eliminate the parasympathetic component of the response to field stimulation.

For the experiments on noradrenaline overflow detection, guinea-pig atria were incubated with 5 μCi of [^{3}H]-noradrenaline (specific activity 13.7 Ci m mol^{-1}) for 40 min in 2 ml Tyrode solution oxygenated and maintained at 30°C. After this period the preparations were placed in 50 ml Tyrode solution containing ascorbic acid 300 μM, atropine 1 μM and phenoxybenzamine 1 μM; radioactivity was washed out for 30 min by replacing the solution 4 times. Then atria were mounted vertically in an organ bath similar to that used for functional studies but containing 2.5 ml Tyrode solution. As previously described the preparations were stimulated at a constant rate (4 Hz); trains of 100 field pulses were applied during the absolute refractory period, in order to obtain detectable [^{3}H]-noradrenaline release, at 15 min intervals. The incubation medium was collected every 5 min. After two collections of bathing solution (for measurement of basal tritium overflow) the first train was applied at the 13th min (S_1), and Tyrode solution was again sampled at the 15th min. Five consecutive trains of field pulses were repeated. When the effect of drugs was tested they were added to Tyrode solution 5 min after S_2 in order to evaluate the effect on basal and stimulus-induced overflow. Insta-gel (15 ml) was added to each sample and radioactivity was measured on a Packard 1500 Tri-carb liquid scintillation analyzer.

The drugs used were: atropine sulphate (BDH), prostaglandin E_2 (PGE_2, Sigma), sulprostone (Shering), misoprostol (Schiapparelli-Searle), butaprost (Bayer), AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid, Glaxo), nor-

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adrenaline hydrochloride (Sigma), phenoxybenzamine hydrochloride (SKF), [^{3}H]-noradrenaline (Amersham). Stock solutions (10 mM) of PGE_2 and butaprost were prepared in ethanol; misoprostol was dissolved in dimethylsulphoxide (DMSO) and AH6809 in 1% bicarbonate solution; further dilutions were made in Tyrode solution.

Results

Effect of prostaglandin E_2 on the sympathetic response to field stimulation

Guinea-pig atria developed a transient increase in contractility in response to electrical field stimulation: tension increased from 421 ± 19 to 624 ± 24 mg (n = 23) in about 10 s and then declined, returning to baseline in about 1 min.

PGE_2 (0.01–100 nM) concentration-dependently and significantly reduced the cardiac response to field stimulation. Records taken from a typical experiment, showing the response to field stimulation and the effect of increasing concentrations of PGE_2, are shown in Figure 1. Figure 2 shows the concentration-response curve for PGE_2: the maximum inhibitory effect of PGE_2 consisted of about 80% reduction of the control response to field stimulation; the IC_{50} value was 0.59 nM. The inhibitory effect of PGE_2 was due to a prejunctional effect since 10 nM PGE_2 did not modify either basal cardiac contractility or the positive inotropic effect induced by exogenous noradrenaline (not shown). Moreover, in guinea-pig atria preloaded with [^{3}H]-noradrenaline, 100 nM PGE_2 greatly reduced the stimulus-evoked tritium overflow, while leaving the basal tritium outflow unaffected (Figure 3). The ratio between the tritium overflow evoked by electrical stimulation during the second period of stimulation and that of the first period (S_2/S_1), and that between S_3 (in the presence of PGE_2) and S_2 are shown in Table 1.

Effect of synthetic prostanoids on the sympathetic response to field stimulation

Since PGE_2 is able to stimulate all the EP receptor subtypes, we also tested several synthetic prostanoids with more specific agonist activity for the various EP receptor subtypes, such as misoprostol (an EP_2, EP_3 mixed agonist), sulprostone (an EP_1, EP_3 mixed agonist), and butaprost (an EP_2 selective agonist). The concentration-inhibitory effect curves for all these agonists are shown in Figure 2 in comparison with the concentration-effect curve for PGE_2. Sulprostone was the more active agonist, since it reduced the cardiac response to adrenergic nerve stimulation in the same concentration-range as PGE_2 (0.01–100 nM); the agonist also showed a maximum inhibitory effect similar to that of PGE_2 (a near 80% reduction in the response to adrenergic nerve stimulation), and an IC_{50} slightly lower than that of PGE_2 (0.41 nM). Misoprostol was active at concentrations between 0.1 and 100 nM; it showed a maximum inhibitory effect similar to that of PGE_2 and sulprostone and a slightly higher IC_{50} value (2.5 nM). Finally butaprost (0.1–100 nM) was ineffective in modifying the response to field stimulation (Figure 2). Moreover the drug, at the highest concentration tested (100 nM), left the concentration-effect curve for PGE_2 completely unaffected (n = 3; not shown), thus showing that it was devoid of any antagonist activity on PGE_2 receptors. None of these drugs was able to affect basal cardiac contractility or the positive inotropic effect induced by exogenous noradrenaline (not shown).

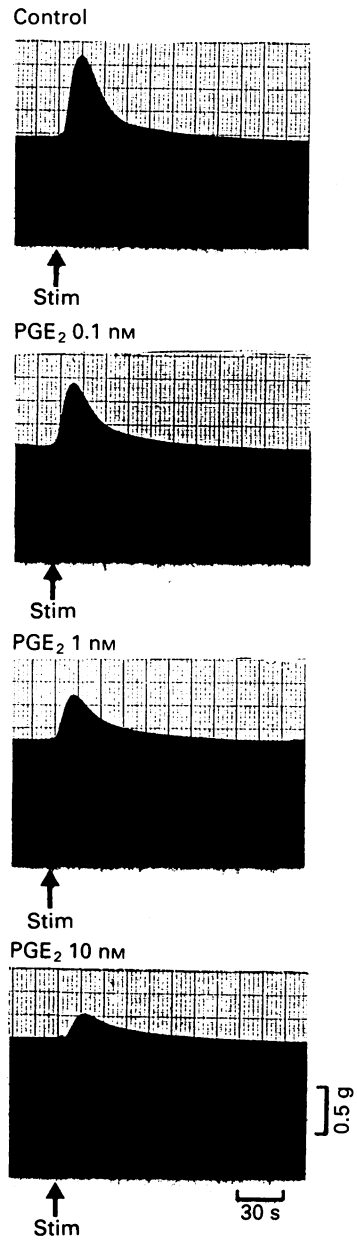

Figure 1 Records from a typical experiment showing the effect of increasing concentrations of prostaglandin E_2 (PGE_2) on the response induced by electrical field stimulation in guinea-pig isolated atria. The arrows indicate the beginning of field stimulation.

The inhibitory effect of PGE_2 and sulprostone was unaffected by a concentration of the EP_1 receptor antagonist AH6809 (1 μM), which has been reported to block EP_1 receptors in other peripheral preparations (Coleman *et al.*, 1985) (Figure 4); the effect of misoprostol was also not modified by AH6809 (not shown). Moreover 100 nM sulprostone, alone and in the presence of 1 μM AH6809, reduced the tritium overflow induced by field stimulation in preparations preloaded

Table 1 Ratios between the increase in the tritium overflow induced by two consecutive trains of field stimulation in guinea-pig atria preloaded with [^{3}H]-noradrenaline

Pretreatment	S_2/S_1	*Treatment*	S_3/S_2	
—	0.91 ± 0.03	PGE_2	0.13 ± 0.03	(n = 4)
—	0.97 ± 0.05	Sulprostone	0.09 ± 0.03	(n = 3)
AH6809	1.11 ± 0.24	Sulprostone	0.09 ± 0.01	(n = 3)

All S_3/S_2 ratio values were significantly different from the respective S_2/S_1 ratio values according to paired Student's t test ($P < 0.01$).

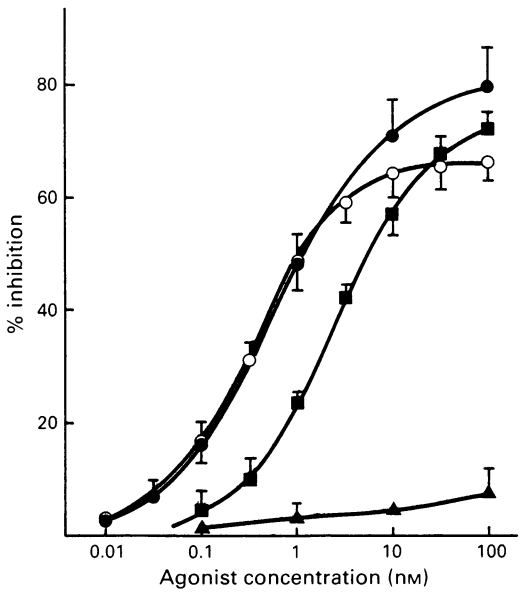

Figure 2 Effects of prostaglandin E_2 (PGE_2) and synthetic prostanoids on the cardiac response to field stimulation in guinea-pig isolated atria. The control response was taken as 100%: (●) PGE_2 ($n = 5$); (○) sulprostone ($n = 4$); (■) misoprostol ($n = 6$); (▲) butaprost ($n = 4$).

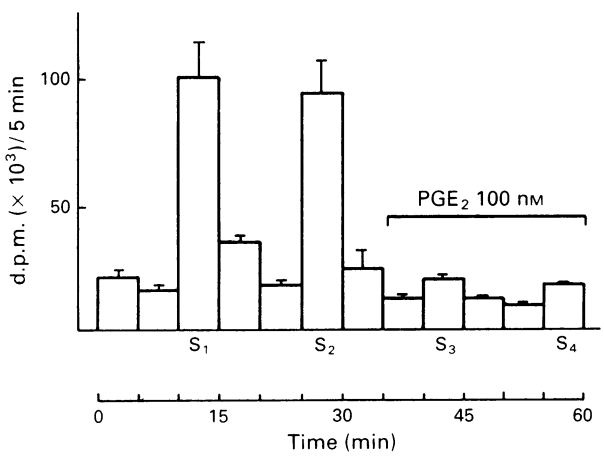

Figure 3 Basal and stimulation-evoked tritium overflow in guinea-pig isolated atria preloaded with [^{3}H]-noradrenaline in the absence and in the presence of prostaglandin E_2 (PGE_2) ($n = 4$).

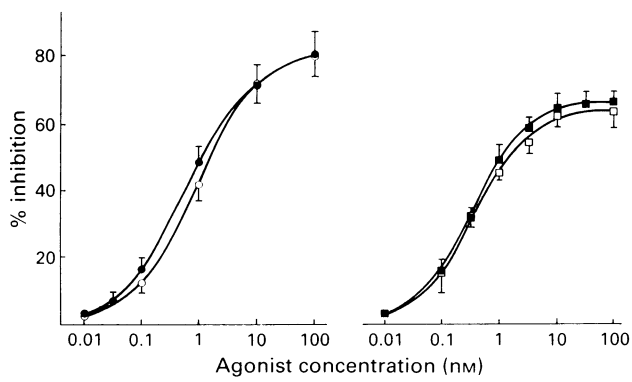

Figure 4 Effect of prostaglandin E_2 (PGE_2) and sulprostone, alone and in the presence of AH6809, on the cardiac response to field stimulation in guinea-pig isolated atria: (●) PGE_2 alone ($n = 5$); (○) PGE_2 in the presence of 1 μM AH6809 ($n = 4$); (■) sulprostone alone ($n = 4$); (□) sulprostone in the presence of 1 μM AH6809 ($n = 4$).

with [^{3}H]-noradrenaline; the S_2/S_1 and S_3/S_2 ratios for sulprostone are given in Table 1.

Discussion

The present study has confirmed our previous observation that PGE_2 is able to reduce the response to sympathetic nerve stimulation in guinea-pig atria (Mantelli *et al.*, 1990). It has also been shown in this paper that the reduction in the cardiac response to adrenergic stimulation is readily attributable to an inhibition of noradrenaline release, since PGE_2 greatly reduced the tritium overflow induced by electrical field stimulation in preparations preloaded with [^{3}H]-noradrenaline, while leaving unaffected the response to exogenous noradrenaline. A similar effect of prostaglandins has been observed in the heart of other animal species (i.e., rabbit, rat, dog; Hedqvist *et al.*, 1970; Wennmalm, 1971; Fuder & Muscholl, 1974; Khan & Malik, 1982; Marques *et al.*, 1983).

It was the aim of the present study to characterize the subtype of receptors responsible for the inhibitory effect of PGE_2. The receptors for PGE_2 (EP receptors) have been identified in many peripheral preparations (for a review see Rao, 1988); the most recent classification of prostanoid receptors indicates that the effects of PGE_2 are mediated by three different subtypes of EP receptors in different preparations (Coleman *et al.*, 1987a; 1987b; Coleman, 1988). They have been indicated as EP_1, EP_2, EP_3, and are preferentially activated by different synthetic prostanoids. The analysis of our results, obtained in guinea-pig isolated atria, suggests that the prostaglandin receptors present on adrenergic nerve endings and responsible for the inhibition of adrenergic neurotransmission belong to the EP_3 subtype according to Coleman's classification. Both sulprostone and misoprostol, which share the ability to stimulate EP_3 receptors, displayed an inhibitory effect superimposable on that of PGE_2. The selective EP_2 agonist, butaprost, was ineffective in our experimental model, thus excluding the presence of EP_2 receptors. On the other hand, the inhibitory effect of the EP agonists did not seem to be mediated by EP_1 receptors since AH6809, an antagonist able to block the effects mediated by EP_1 receptors (Coleman *et al.*, 1985; McKenniff *et al.*, 1988), did not modify the inhibitory effect of PGE_2 or sulprostone. It is interesting to note that our results are in agreement with those obtained in guinea-pig vas deferens, where an inhibitory role on neurotransmitter release for prostanoids has been reported (Ambache & Zar, 1970; Hedqvist & Von Euler, 1972). Recent observations show that the contractions elicited by trains of field pulses in such preparations were reduced by PGE_2 and sulprostone in a similar way, and that this effect was not antagonized by an EP_1 antagonist (Coleman *et al.*, 1987b), thus suggesting the presence of EP_3 receptors. Results similar to those described above have also been obtained in the rat gastric mucosa, where the well known inhibitory effect of prostaglandins on acid secretion was attributed to an action mediated by EP_3 receptors (Reeves *et al.*, 1988). Therefore, available data seem to suggest that EP_3 receptors are present in those tissues where prostaglandins mediate an inhibitory effect on secretory mechanisms.

In conclusion, the results of the present study demonstrate that PGE_2 and synthetic prostanoid agonists inhibit the cardiac response to adrenergic nerve stimulation by reducing noradrenaline release, and suggest that the prejunctional prostanoid receptors responsible for this effect belong to the EP_3 subtype.

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References

AMBACHE, N. & ZAR, M.A. (1970). An inhibitory effect of prostaglandin E_2 on neuromuscular transmission in the guinea-pig vas deferens. *J. Physiol.*, **208**, 30–32P.

COLEMAN, R.A. (1988). Prostaglandin receptors, *Prostaglandin Perspect.*, **4**, 33–35.

COLEMAN, R.A., HUMPHREY, P.P.A., KENNEDY, I. & LUMLEY, P. (1984). Prostanoid receptors – the development of a working classification. *Trends Pharmacol. Sci.*, **5**, 303–306.

COLEMAN, R.A., KENNEDY, I. & SHELDRICK, R.L.G. (1985). AH6809, a prostanoid EP_1 receptor blocking drug. *Br. J. Pharmacol.*, **85**, 273P.

COLEMAN, R.A., KENNEDY, I. & SHELDRICK, R.L.G. (1987a). Evidence for the existence of three subtypes of PGE_2 sensitive (EP) receptors in smooth muscle. *Br. J. Pharmacol.*, **91**, 323P

COLEMAN, R.A., KENNEDY, I., SHELDRICK, R.L.G. & TOLOWINSKA, I.Y. (1987b). Further evidence for the existence of three subtypes of PGE_2-sensitive (EP-) receptors. *Br. J. Pharmacol.*, **91**, 407P.

FUDER, H. & MUSCHOLL, E. (1974). The effect of methacoline on noradrenaline release from the rabbit heart perfused with indometacin. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **285**, 127–132.

HEDQVIST, P. (1977). Basic mechanisms of prostaglandin action on autonomic neurotransmission. *Ann. Rev. Pharmacol. Toxicol.*, **17**, 259–279.

HEDQVIST, P., STJARNE, L. & WENNMALM, A. (1970). Inhibition by prostaglandin E_2 of sympathetic neurotransmission in the rabbit heart. *Acta Physiol. Scand.*, **79**, 139–141.

HEDQVIST, P. & VON EULER, U.S. (1972). Prostaglandin-induced neurotransmission failure in the field-stimulated isolated vas deferens. *Neuropharmacology*, **11**, 177–187.

KHAN, M.T. & MALIK, K.U. (1980). Sympathetic nerve stimulation of the isolated rat heart: release of a prostaglandin-like substance and the inhibitory effect of prostacyclin on the output of ^{3}H-norepinephrine. *Adv. Prostaglandin Thromboxane Res.*, **8**, 1241–1243.

KHAN, M.T. & MALIK, K.U. (1982). Modulation by prostaglandins of the release of ^{3}H noradrenaline evoked by potassium and nerve stimulation in the isolated rat heart. *Eur. J. Pharmacol.*, **78**, 213–218.

LEDDA, F. & MANTELLI, L. (1984). Difference between the prejunctional effects of phenylephrine and clonidine in isolated guinea-pig atria. *Br. J. Pharmacol.*, **81**, 491–499.

MALIK, K.U. (1978). Prostaglandins – modulation of adrenergic nervous system. *Fed. Proc.*, **37**, 203–207.

MANTELLI, L., AMERINI, S. & LEDDA, F. (1990). Different effects of prostaglandins on adrenergic neurotransmission in atrial and ventricular preparations. *Br. J. Pharmacol.*, **99**, 717–720.

MARQUES, M.C.A., LADOSKY, E.M.P. & FONTELES, M.C. (1983). The presynaptic effect of aspirin and prostaglandin E_2 in the isolated cat heart. *Braz. J. Med. Biol. Res.*, **16**, 133–140.

McKENNIFF, M., RODGER, I.W., NORMAN, P. & GARDINER, P.J. (1988). Characterization of receptors mediating the contractile effects of prostanoids in guinea-pig and human airways. *Eur. J. Pharmacol.*, **153**, 149–159.

RAO, C.V. (1988). Receptors for various prostaglandins. In *Prostaglandins: Biology and Chemistry of Prostaglandins and Related Eicosanoids.* ed. Curtis-Prior, P.B. pp. 171–178. Edinburgh: Churchill Livingstone.

REEVES, J.J., BUNCE, K.T., SHELDRICK, R.L.G. & STABLES, R. (1988). Evidence for the PGE receptor subtype mediating inhibition of acid secretion in the rat. *Br. J. Pharmacol.*, **95**, 805P.

WENNMALM, A. (1971). Studies on the mechanisms controlling the secretion of neurotransmitters in the rabbit heart. *Acta Physiol. Scand.*, **83** suppl. 363, 1–36.

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Relationship between lipolysis and cyclic AMP generation mediated by atypical β-adrenoceptors in rat adipocytes

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1 The nature of the β-adrenoceptor(s) mediating adenylyl cyclase activation in rat adipocyte ghosts by (−)-isoprenaline and the lipolytically selective β-adrenoceptor agonist, BRL 37344, was investigated by use of the β_1-selective antagonist, CGP 20712A. The results were compared with lipolysis in adipocytes.

2 While in lipolysis BRL 37344 was a full and 10 times more potent agonist than (−)-isoprenaline, in adenylyl cyclase activation similar pD_2 values for both agonists were found. BRL 37344 was only a partial agonist on rat adipocyte adenylyl cyclase, with an intrinsic activity of 0.62.

3 With CGP 20712A small rightward shifts of the (−)-isoprenaline concentration-response curve (CRC) were observed at concentrations up to 10 μM, while at 100 μM and 1 mM clear rightward shifts occurred. The BRL 37344 CRC was not shifted with antagonist concentrations up to 10 μM. Only at 100 μM and 1mM CGP 20712A were rightward shifts observed.

4 CGP 20712A concentrations of 10 μM and 100 μM depressed the maximum of the (−)-isoprenaline CRC to 89 and 60%, while the BRL 37344 CRCs retained the control maximum effect (62% of (−)-isoprenaline). Only at 1 mM CGP 20712A, was the CRC of BRL 37344 depressed, while the (−)-isoprenaline maximum was diminished further.

5 It was concluded that as with lipolysis, (−)-isoprenaline acts both through typical β_1- and atypical β_3-adrenoceptors for activation of adenylyl cyclase, while BRL 37344 acts solely through atypical β_3-adrenoceptors.

6 The results also demonstrate that the relationship between adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and lipolysis is different for BRL 37344 and (−)-isoprenaline. Although the maximum activation of adenylyl cyclase by BRL 37344 is only 62% of that by (−)-isoprenaline, the distance between the lipolysis and adenylyl cyclase CRCs is much larger in the case of BRL 37344, indicating a larger transduction reserve for this agonist.

Introduction

It is now well established that rat adipocyte lipolysis is predominantly mediated by atypical β-adrenoceptors (Zaagsma & Nahorski, 1990, and references cited therein). An atypical or hybrid β-adrenoceptor was already suggested by Harms *et al.* (1974) and by De Vente *et al.* (1980), who demonstrated low affinity of this adrenoceptor for both β_1- and β_2-selective and non-selective antagonists. In addition, for the adipocyte β-adrenoceptor atypically low stereoselectivity ratios of antagonist enantiomers were found compared to typical β_1- (cardiac) and β_2- (skeletal muscle) adrenoceptors (Harms *et al.*, 1977). The introduction of a novel series of β-adrenoceptor agonists that were lipolytically selective (Arch *et al.*, 1984) strengthened the hypothesis of an atypical β-adrenoceptor mediating lipolysis. Important progress in this field was made by the recent isolation of a human gene coding for a β_3-adrenoceptor with clear atypical β-adrenoceptor properties when expressed in chinese hamster ovary cells (Emorine *et al.*, 1989).

Recently, direct evidence was obtained that rat adipocyte lipolysis is indeed mediated predominantly by atypical β-adrenoceptors, while the typical β_1-adrenoceptors play at most a small, subordinate role (Hollenga & Zaagsma, 1989). It was found that when (−)-isoprenaline-induced lipolysis was antagonized with the highly β_1-selective antagonist, CGP 20712A, small but consistent shifts to the right of the concentration-response curves (CRCs) occurred at antagonist concentrations up to 10 μM, while only at 100 μM CGP 20712A was a clear rightward shift found. In contrast, with the lipolytically selective compound, BRL 37344, which was shown to be about 10 times more potent in lipolysis than (−)-isoprenaline, no rightward shifts of the CRC were observed at CGP 20712A concentrations up to 10 μM. Only at 100 μM CGP 20712A was a clear rightward shift found. It was concluded that the main action of (−)-isoprenaline is through atypical β-adrenoceptors, and a small part through typical β_1-adrenoceptors, while BRL 37344 acts solely through atypical β-adrenoceptors (Hollenga & Zaagsma, 1989).

In the present study the activation of adenylyl cyclase in rat adipocyte ghosts by (−)-isoprenaline and BRL 37344 was investigated. Both agonists were also studied in the presence of increasing concentrations of CGP 20712A, and interesting differences between lipolysis and adenylyl cyclase activation were observed.

Methods

Adipocyte ghosts isolation

Epididymal fat pads from male Wistar rats (210–230 g) were used. Adipocytes were isolated as described (Hollenga & Zaagsma, 1989), except that no adenosine was used in the isolation medium. Immediately after fat cell isolation, adipocyte ghosts were prepared by lysis essentially as described by Bojanic *et al.* (1985). The isolated adipocytes were lysed in 20 volumes of Tris-HCl 2 mM, EGTA 2 mM, pH 7.4, at room temperature for 15 min, followed by centrifugation at 50,000*g* for 20 min at 4°C. Supernatant and congealed fat cake were removed and the pellet was washed with ice-cold Tris-HCl 80 mM, $MgSO_4$ 4 mM, EDTA 0.2 mM, isobutyl methylxanthine (IBMX) 0.4 mM, pH 7.4, and centrifuged again at 50,000*g* for 20 min at 4°C. The final pellet was resuspended in the same buffer solution and stored in liquid nitrogen. Protein concentration was determined according to Lowry *et al.* (1951), with bovine serum albumin used as standard.

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Adenylyl cyclase stimulation

Adenylyl cyclase stimulation experiments were carried out by incubating 20–50 μg protein for 20 min at 37°C in a buffer solution composed of Tris-HCl 120 mM, $MgCl_2$ 3.5 mM, adenosine 5'-triphosphate (ATP) 1 mM, dithiotreitol 1 mM, phosphocreatinine 20 mM, creatine phosphokinase 5 u, guanosine 5'-triphosphate (GTP) 0.1 mM and IBMX 0.5 mM, with the appropriate concentrations of drugs under investigation, in a final volume of 100 μl. When (−)-isoprenaline was used as agonist, $Na_2S_2O_5$ 50 $\mu g\,ml^{-1}$ was added as antioxidant. The incubation was terminated by adding 400 μl Tris-HCl 50 mM, EDTA 4 mM, pH 7.4, and subsequently placing the test tubes in a boiling waterbath. After 10 min the tubes were placed on ice and were centrifuged for 5 min at 4°C at 2000*g*. The supernatant was stored in polyethylene vials at −20°C until the adenosine 3':5'-cyclic monophosphate (cyclic AMP) determination was carried out.

Isolation of cyclic AMP binding protein

Fresh bovine adrenals, obtained from the local slaughterhouse, were transported to the laboratory on ice. Fat and medulla were removed and the adrenal cortices were homogenized with an Ultra-Turrax, in 2 volumes of Littlefields medium, composed of sucrose 250 mM, Tris 50 mM, KCl 25 mM, $MgCl_2$ 5 mM, 2-mercapto-ethanol 6 mM and theophylline 8 mM, pH 7.4. The homogenate was centrifuged for 5 min at 2000*g* at 4°C. The supernatant was centrifuged at 50,000*g* for 20 min at 4°C. The supernatant remaining after this centrifugation step was lyophilized and stored at −20°C.

Determination of cyclic AMP

Determination of cyclic AMP production was carried out essentially according to Brown *et al.* (1971) by incubating the samples with 1.7 mg bovine adrenal binding protein and 10 μCi [^{3}H]-cyclic AMP in a buffer composed of Tris-HCl 50 mM, EDTA 4 mM, pH 7.4 in a final volume of 300 μl for at least 3 h at 4°C to reach equilibrium. When necessary, samples were diluted with incubation buffer. The incubation was terminated by mixing with charcoal suspension (Norit A special; $2.5\,g\,l^{-1}$) at 4°C, mixing, and subsequent centrifugation to remove unbound [^{3}H]-cyclic AMP. The upper layer was put in scintillation vials and 3 ml scintillation fluid was added. The samples were counted for 5 min in a Beckman type LS 1701 liquid scintillation counter with an efficiency of 46%.

Data analysis

Data are presented as means ± s.e.mean. Dose-response curves of cyclic AMP production are expressed relative to (−)-isoprenaline maximum. Schild plots were constructed according to Arunlakshana & Schild (1959). When the slopes did not significantly differ from unity (two-tailed Student's *t* test, $P < 0.05$), pA_2 values were calculated for each concentration of antagonist according to: $pA_2 = -\log \{[\text{antagonist}]/(DR - 1)\}$ (MacKay, 1978).

Materials

Collagenase (type II, $390\,u\,mg^{-1}$), (−)-isoprenaline hydrochloride, dithiotreitol, phosphocreatine, creatinephophokinase, GTP, IBMX, 2-mercapto-ethanol, theophylline and cyclic AMP were purchased from Sigma (St. Louis, U.S.A.). Demineralized bovine serum albumin was from Organon Technika (Oss, The Netherlands). All buffer salts were from Merck. [^{3}H]-cyclic AMP was from The Radiochemical Center (Amersham, UK). Scintillation fluid (Picofluor) was purchased from Packard Instruments B.V. (Groningen, The Netherlands).

BRL 37344 (4-[2-[(2-hydroxy-2-(3-chlorophenyl)ethyl)-amino]propyl]-phenoxyacetic acid) and CGP 20712A (1-[2-((3-carbamoyl-4-hydroxy)-phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)-phenoxy]-propan-2-ol) were kind gifts of Beecham (Epsom, U.K.) and Ciba Geigy (Basle, Switzerland), respectively.

Results

Adenylyl cyclase stimulation of rat white adipocyte ghosts by (−)-isoprenaline and BRL 37344 was linear with time and protein concentration (data not shown). Basal cyclic AMP production was 0.66 ± 0.17, maximal cyclic AMP production by (−)-isoprenaline was $2.89 \pm 0.69\,nmol\,mg^{-1}$ protein $20\,min^{-1}$ ($n = 6$). In Figure 1 the concentration-response curves (CRCs) for both agonists are depicted together with the lipolytic CRCs in rat isolated adipocytes, taken from our previous study (Hollenga & Zaagsma, 1989). In lipolysis, both compounds were full agonists, with pD_2 values ($-\log EC_{50}$) of 7.28 ± 0.11 for (−)-isoprenaline and 8.34 ± 0.07 for BRL 37344, the latter being 10 times more potent. Surprisingly, the pD_2 values for adenylyl cyclase activation were similar for both agonists, being 6.07 ± 0.10 for (−)-isoprenaline and 5.99 ± 0.24 for BRL 37344. Moreover, compared to (−)-isoprenaline, BRL 37344 was a partial agonist on adenylyl cyclase stimulation, with an intrinsic activity of 0.62.

When the CRCs were performed with increasing concentrations of CGP 20712A, differences between (−)-isoprenaline and BRL 37344 were found. From 100 nM to 10 μM CGP 20712A small rightward shifts of the (−)-isoprenaline CRC were observed. At higher antagonist concentrations of 100 μM and 1 mM the rightward shifts enlarged. Furthermore, with these CGP 20712A concentrations a depression of the maximum response of the (−)-isoprenaline CRC was found. The Schild plot constructed from these data was clearly biphasic, the slope of the steep part being 0.80 ± 0.15, not significantly different from unity. From the shifts with 100 μM and 1 mM antagonist, after subtraction of the 'high affinity part' of the Schild plot (Bond & Clarke, 1988), a pA_2 value for CGP 20712A for antagonizing (−)-isoprenaline-induced cyclic AMP production by the higher concentrations of the antagonist of 4.53 ± 0.17 ($n = 8$) was calculated (Figure 2a).

With BRL 37344 different results were found. No significant shift of the BRL 37344 CRC was observed up to 10 μM CGP 20712A. At 100 μM antagonist a small rightward shift was found, which increased at 1 mM CGP 20712A. With BRL 37344, no depression of the CRC occurred up to 100 μM of the antagonist. Only at 1 mM CGP 20712A was a clear depression of the CRC seen. The Schild plot constructed from the data obtained with 100 μM and 1 mM CGP 20712A had a slope of

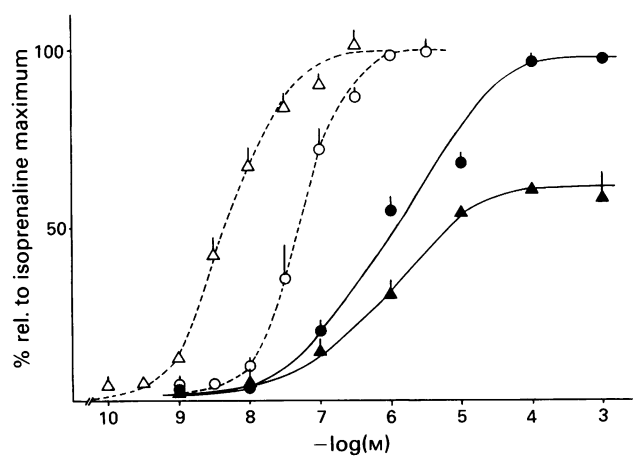

Figure 1 Lipolysis (open symbols) and adenosine 3':5'-cyclic monophosphate (cyclic AMP) production (closed symbols) in Wistar rat adipocytes and adipocyte ghosts induced by (−)-isoprenaline (○, ●) and BRL 37344 (△, ▲). Both lipolysis and cyclic AMP production are expressed as % relative to (−)-isoprenaline maximum.

1.20 ± 0.19, which was also not significantly different from unity. A pA_2 value of 4.60 ± 0.17 ($n = 6$) was calculated for CGP 20712A antagonizing BRL 37344 induced adenylyl cyclase stimulation (Figure 2b).

Discussion

The present study provides information on two steps involved in β-adrenoceptor-mediated lipolysis in rat adipocytes. First, the activation of adenylyl cyclase by (−)-isoprenaline and BRL 37344, and second, the relationship between cyclic AMP generation and lipolysis for both agonists.

One of the most striking observations is that BRL 37344, while being a full and 10 fold more potent agonist than (−)-isoprenaline on lipolysis, is only a partial agonist on cyclic AMP production, with a similar potency to (−)-isoprenaline. For BRL 37344 a 220 fold difference in ED_{50} values for lipolysis and cyclic AMP generation was found, while for (−)-isoprenaline only a 16 fold difference was obtained. As a consequence, maximum lipolysis induced by BRL 37344 is already reached at 20% of the maximum cyclic AMP production, whereas with (−)-isoprenaline maximum lipolysis required 50%.

When adenylyl cyclase stimulation by (−)-isoprenaline and BRL 37344 was antagonized with the β_1-selective antagonist CGP 20712A, a similar pattern was observed to that seen with lipolysis (Hollenga & Zaagsma, 1989). With low antagonist concentrations of 100 nM to 10 μM, small rightward shifts of the (−)-isoprenaline CRC were found, indicating a small contribution of typical β_1-adrenoceptors. The rightward shift increased when higher antagonist concentrations of 100 μM to 1 mM were applied. While with BRL 37344 no significant shift was observed at concentrations up to 10 μM, at 100 μM and 1 mM concentrations of CGP 20712A rightward shifts were found. The pA_2 values calculated for antagonism of cyclic AMP production by the higher CGP 20712A concentrations were 4.53 and 4.60, being similar to the pA_2 values of 4.80 and 4.61 reported for CGP 20712A antagonism of (−)-isoprenaline- and BRL 37344-induced lipolysis, respectively (Hollenga & Zaagsma, 1989). Because of the similarity of the findings in the present study on cyclic AMP generation with those reported for lipolysis (Hollenga & Zaagsma, 1989) it can be concluded that adenylyl cyclase activation is also predominantly mediated by atypical β-adrenoceptors, with a small contribution of the typical β_1-adrenoceptors. BRL 37344 acts solely through the atypical β-adrenoceptors.

In the presence of 10 μM CGP 20712A, i.e. under complete β_1-adrenoceptor blockade, the CRCs for lipolysis (Hollenga & Zaagsma, 1989) and adenylyl cyclase activation (Figure 2) revealed that with (−)-isoprenaline the difference in EC_{50} values increased from 16 fold to 50 fold, whereas with BRL 37344 a 295 fold difference was found, comparable to the value found in the absence of CGP 20712A. Thus, the marked discrepancy between cyclic AMP generation and lipolysis observed with both agonists, but in particular with BRL 37344, was entirely due to occupation of the atypical β-adrenoceptors.

This discrepancy could be explained by functional compartmentalization of cyclic AMP, which implies more efficient activation of protein kinase A involved in triglyceride lipase phosphorylation in the case of BRL 37344 (Honnor *et al.*, 1985). The existence of functional and non-functional cyclic AMP compartments in fat cells was already indicated by the results of Schimmel (1984). With hamster adipocytes this author found that 1 μM forskolin, a diterpene acting directly on adenylyl cyclase, while producing relatively large amounts of cyclic AMP, hardly stimulated lipolysis. When 0.01 μM isoprenaline was added, which itself did not stimulate cyclic AMP production, the two compounds acted synergistically on lipolysis but not on cyclic AMP generation, and glycerol production dramatically increased. It was concluded that this potentiation could arise if isoprenaline directed the cyclic AMP produced in response to forskolin into the appropriate compartment coupled to lipolysis. Alternatively, the coexistence of cyclic AMP-dependent and cyclic AMP non-dependent mechanisms might be responsible for the observed differences with BRL 37344 and (−)-isoprenaline, BRL 37344 being the most potent in stimulating the cyclic AMP non-dependent mechanism. On the basis of the differential inhibition by the adenosine A_1-receptor agonist N^6-phenylisopropyladenosine of lipolysis and adenylyl cyclase activation induced by isoprenaline as compared to forskolin, Allen & Quesenberry (1988) also considered the possibility of a cyclic AMP-independent mechanism. In addition, a second regulatory factor, acting synergistically with cyclic AMP, might by involved, BRL 37344 again being more potent in activating this factor than (−)-isoprenaline.

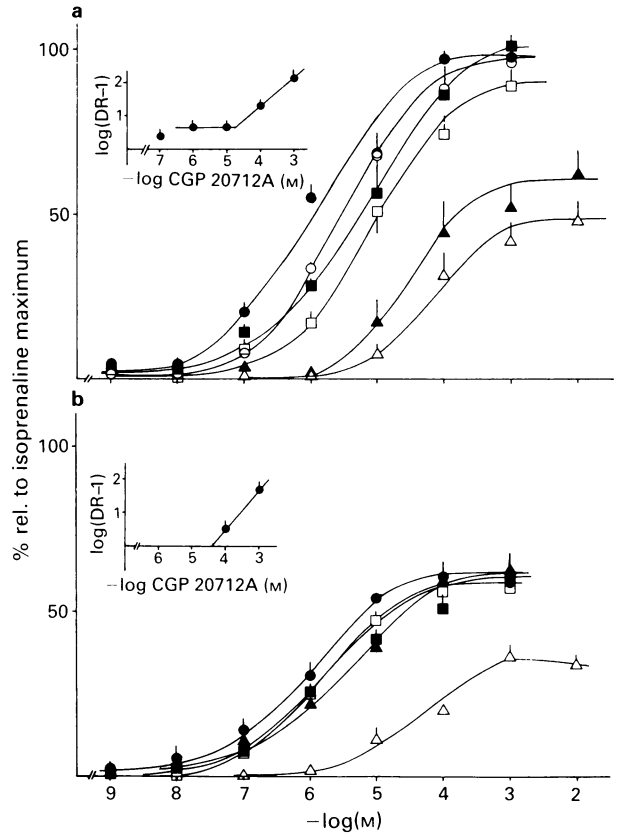

Figure 2 Antagonism of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production in Wistar rat adipocyte ghosts, induced by (−)-isoprenaline (a) and BRL 37344 (b), with CGP 20712A. Control (●), CGP 20712A 100 nM (○), 1 μM (■), 10 μM (□), 100 μM (▲), and 1 mM (△). Cyclic AMP production is expressed as % relative to (−)-isoprenaline maximum. The insets show Schild plots calculated from the individual experiments.

Interestingly, in rat brown fat tissue even larger differences between adenylyl cyclase activation and stimulation of the respiratory rate were observed (Muzzin *et al.*, 1988). From the EC_{50} values reported in that paper, for BRL 37344, being again a partial agonist on adenylyl cyclase, a difference of 5111 fold between cyclic AMP generation and increase in oxygen consumption could be calculated and for (−)-isoprenaline a 1521 fold difference, indicating a tighter coupling of the functional cyclic AMP pool and/or alternative factors with the respiration stimulation of brown adipocytes in the case of BRL 37344.

It is known that competitive antagonists at high concentrations can act non-competitively, resulting in depression of the CRCs, particularly when applied with agonists having a low intrinsic activity (Kenakin, 1984). Bojanic & Nahorski (1984) observed a dose-dependent depression of rat reticulocyte adenylyl cyclase activation by isoprenaline with the photo-

affinity β-adrenoceptor antagonist *para*-aminobenzylcarazolol (*p*ABC). They noticed however, that there was no difference in *p*ABC behaviour with or without N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)-hexanoate (SANAH) and photolysis, indicating that the depression of maximal adenylyl cyclase activity occurred during (slowly?) reversible interaction of the antagonist with the β-adrenoceptor, instead of being a result of irreversible β-adrenoceptor blockade.

In the present study, CGP 20712A at high concentrations caused depression of the CRCs of both agonists. However, (−)-isoprenaline-induced maximum cyclic AMP production already started to decrease at 10 μM CGP 20712A, while the BRL 37344 CRC was found to be depressed only at 1 mM CGP 20712A. Since (−)-isoprenaline activates both the atypical and the typical β_1-adrenoceptor, the total amount of cyclic AMP produced after stimulation with this agonist could be the sum of the production by the individual β-adrenoceptors. The atypical β-adrenoceptor, maximally activated by BRL 37344, contributes 62% to the maximum amount obtained with (−)-isoprenaline. At 100 μM CGP 20712A the β_1-adrenoceptor-mediated contribution by (−)-isoprenaline is totally depressed, while the activation of the atypical β-adrenoceptor, which is known to have a low affinity for the antagonist (Hollenga & Zaagsma, 1989), is still maximal, as can be seen from the experiments with BRL 37344 in the absence and presence of CGP 20712A of 100 μM and lower. The observation that at 100 μM CGP 20712A, (−)-isoprenaline still stimulates adenylyl cyclase to 60% of its own maximum response, comparable to the control maximum BRL 37344 activity, whereas at 1 mM both the (−)-isoprenaline and the BRL 37344 response are depressed, supports this view.

In conclusion, the present study shows that, as already demonstrated for lipolysis, adenylyl cyclase activation in rat adipocytes by BRL 37344 is solely and by (−)-isoprenaline is predominantly mediated by atypical β_3-type adrenoceptors. Although this adrenoceptor apparently is not very efficiently coupled to adenylyl cyclase, given the maximum activity of 62% compared to (−)-isoprenaline, the generated cyclic AMP is activating the triglyceride lipase-sensitive pool of protein kinase A very well. In the case of BRL 37344, this results in a larger distance between the lipolysis and adenylyl cyclase CRCs than in the case of (−)-isoprenaline, implying that the high lipolytic activity of BRL 37344 is due to a larger transduction reserve. It would be most interesting to investigate this relationship in other cell types.

References

ARCH, J.R.S., AINSWORTH, A.T., CAWTHORNE, M.A., PIERCY, V., SENNIT, M.V., THODY, V.E., WILSON, C. & WILSON, S. (1984). Atypical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature*, **309**, 163–165.

ALLEN, D.O. & QUESENBERRY, J.T. (1988). Quantitative differences in the cyclic AMP-lipolysis relationships for isoproterenol and forskolin. *J. Pharmacol. Exp. Ther.*, **244**, 852–858.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BOJANIC, D., JANSEN, J., NAHORSKI, S.R. & ZAAGSMA, J. (1985). Atypical characteristics of the β-adrenoceptor mediating cyclic AMP generation and lipolysis in the rat adipocyte. *Br. J. Pharmacol.*, **84**, 131–137.

BOJANIC, D. & NAHORSKI, S.R. (1984). Irreversible antagonism of beta-adrenoceptors with para-amino-benzyl-carazolol provides further evidence for an atypical rat adipocyte beta-adrenoceptor. *J. Rec. Res.*, **4**, 21–35.

BOND, R.A. & CLARKE, D.E. (1988). Agonist and antagonist characterization of a putative adrenoceptor with distinct pharmacological properties from the α- and β-subtypes. *Br. J. Pharmacol.*, **95**, 723–734.

BROWN, B.L., ALBANO, J.D.M., EKINS, R.P., SGHERZI, A.M. & TAMPION, W. (1971). A simple and sensitive saturation assay method for the measurement of adenosine-3',5'-cyclic monophosphate. *Biochem. J.*, **121**, 561–562.

DE VENTE, J., BAST, A., VAN BREE, L. & ZAAGSMA, J. (1980). β-Adrenoceptor studies. 6. Further investigations on the hybrid nature of the rat adipocyte β-adrenoceptor. *Eur. J. Pharmacol.*, **63**, 73–83.

EMORINE, L.J., MARULLO, S., BRIEND-SUTREN, M.-M., PATEY, G., TATE, K., DELAVIER-KLUTCHKO, C. & STROSBERG, A.D. (1989). Molecular characterization of the human β_3-adrenergic receptor. *Science*, **245**, 1118–1121.

HARMS, H.H., ZAAGSMA, J. & DE VENTE, J. (1977). Differentiation of β-adrenoceptors in right atrium, diaphragm and adipose tissue of the rat using stereoisomers of propranolol, alprenolol, nifnalol and practolol. *Life Sci.*, **21**, 123–128.

HARMS, H.H., ZAAGSMA, J. & VAN DER WAL, B. (1974). β-Adrenoceptor studies. III. On the β-adrenoceptors in rat adipose tissue. *Eur. J. Pharmacol.*, **25**, 87–91.

HOLLENGA, CH. & ZAAGSMA, J. (1989). Direct evidence for the atypical nature of functional β-adrenoceptors in rat adipocytes. *Br. J. Pharmacol.*, **98**, 1420–1424.

HONNOR, R.C., DHILLON, G.S. & LONDOS, C. (1985). cAMP dependent protein kinase and lipolysis in rat adipocytes. I. Cell preparation, manipulation and predictability in behavior. *J. Biol. Chem.*, **260**, 15122–15129.

KENAKIN, T.P. (1984). The classification of drugs and drug receptors in isolated tissues. *Pharmacol. Rev.*, **36**, 165–222.

MUZZIN, P., SEYDOUX, J., GIACOBINO, J.-P., VENTER, J.-G. & FRASER, C. (1988). Discrepancies between the affinities of binding and action of the novel β-adrenergic agonist BRL 37344 in rat brown adipose tissue. *Biochem. Biophys. Res. Commun.*, **156**, 375–382.

LOWRY, O.H., ROSENBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MACKAY, D. (1978). How should values of pA_2 and affinity constants for pharmacological competitive antagonists be estimated? *J. Pharm. Pharmacol.*, **30**, 312–313.

SCHIMMEL, R.J. (1984). Stimulation of cAMP accumulation and lipolysis in hamster adipocytes with forskolin. *Am. J. Physiol.*, **246**, C63–C68.

ZAAGSMA, J. & NAHORSKI, S.R. (1990). Is the adipocyte β-adrenoceptor a prototype for the recently cloned atypical 'β_3-adrenoceptor'? *Trends Pharmacol. Sci.*, **11**, 3–7.

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Selective and competitive antagonism by suramin of ATP-stimulated catecholamine-secretion from PC12 phaeochromocytoma cells

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1 Suramin, a putative P_2-antagonist, (10 to 300 μM) inhibited the adenosine 5'-triphosphate (ATP)-stimulated secretion of [^{3}H]-noradrenaline or endogenous dopamine from phaeochromocytoma PC12 cells in a concentration-dependent manner. Suramin (300 μM) did not affect the dopamine-secretion stimulated by high K^+ or nicotine.

2 Suramin shifted the concentration-response curve for ATP to the right. The antagonism was competitive with a pA_2 value of 4.52.

3 ATP also stimulated an increase in intracellular Ca^{2+} concentration as determined by fura-2 methods. Suramin antagonized this effect over the same concentration range that antagonized the ATP-stimulated catecholamine secretion.

4 These results suggest that suramin can be used as a selective and competitive antagonist of ATP in experiments concerning mechanisms of catecholamine-secretion.

Introduction

Extracellular adenosine and adenine nucleotides have been regarded as modulators in various kinds of cells (Burnstock, 1978; Gordon, 1986; Bean & Friel, 1990). Receptors mediating the effects of adenosine and adenine nucleotides are classified broadly into two types (P_1 and P_2) based mainly upon the order of potency of various agonists (Burnstock & Kennedy, 1985). It was reported recently that suramin blocks the effects of adenosine 5'-triphosphate (ATP) in smooth muscles and it was suggested that suramin acts as a P_2-purinoceptor antagonist (Dunn & Blakeley, 1988; Den Hertog *et al.*, 1989). We have previously reported that suramin inhibits an inward current activated by ATP in PC12 phaeochromocytoma cells (Nakazawa *et al.*, 1990b). However, it was not known whether suramin acts purely as a P_2-purinoceptor antagonist or exerts some other effects on neuronal responses, including catecholamine-secretion.

In the present study, we have measured catecholamine-secretion from PC12 cells and Ca^{2+}-concentrations in these cells to investigate the selectivity and the mechanism of action of suramin.

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 with an atmosphere of 10% CO_2, and then plated into collagen-coated polystyrene dishes (35 mm in diameter) at 1×10^6 cells per dish. After an additional 2 to 3 day-culture, the cells were used for experimentation.

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Dopamine- and noradrenaline-secretion

Secretion of dopamine from PC12 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, nicotine, or high K (155 mM Na in BS was substituted with K) in the absence or presence of suramin. The incubation solution (0.8 ml) 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 contents.

Dopamine contents were 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 a 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 analyzed with a chromatographic data processor (Shimadzu C-R3A).

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

In some experiments, noradrenaline-secretion was measured instead of dopamine-secretion according to Inoue & Kenimer (1988). Briefly, the cells were incubated with [^{3}H]-noradrenaline (43.7 Ci mmol^{-1}, 0.125 μCi per dish) for 1 h at 37°C and

then incubated with BS for 1 h at room temperature. After washing with BS, the cells were used in experiments. The ratio of noradrenaline-secretion was calculated from radioactivity in the incubation solution and that remaining in the cells as in the case of the dopamine-secretion described above.

Intracellular Ca^{2+} concentrations

Intracellular Ca^{2+} concentrations were measured with fura-2, a fluorescent Ca^{2+} indicator (Grynkiewicz *et al.*, 1985). The cultured cells were removed from dishes by pipetting and loaded with fura-2 for 30 min by incubation with the membrane permeable dye, fura-2/AM (acetoxymethyl ester form, 10 μM) dissolved in BS. The cells were then rinsed with BS and were placed in the spectrometer (3×10^6 cells ml^{-1}) for intracellular Ca^{2+} measurements (Japan Spectroscopic, CAF-100). Fluorescence was excited at 340 nm and 380 nm, and intensity of the fluorescence (F340 and F380) at 500 nm was measured from the cells at 37°C. Ca^{2+} concentrations were calculated from the ratio of the fluorescence intensity (R = F340/F380) and a K_d value of 224 nM according to Grynkiewicz *et al.* (1985). R_{max} and R_{min} were obtained after treatment with ionomycin (10 μM) and EGTA (10 mM), respectively. ATP or ATP plus suramin was applied to the cell suspension as a solution containing 100 fold the final concentrations. The final concentrations were readily attained because the cell suspension was continuously stirred during the Ca^{2+} measurement.

Drugs

Drug used were ATP, nicotine (Sigma) and suramin monosodium salt (Bayer). [^{3}H]-noradrenaline was from New England Nuclear, and fura-2/AM (dissolved in DMSO) was from Dojin.

Data analysis

Data analysis was made according to Tallarida & Jacob (1979). In the curve-fitting for concentration-response data, curves were fitted to the data using an equation, $E/(E_{max} - E) = ([ATP]/EC_{50})^n$, where E is an effect induced by each concentration of ATP. In Schild analysis, the concentration of ATP (A) which induced half-maximal response without suramin and the concentration (A') which induced the same response with suramin of each concentration (B) were obtained from the concentration-response curves, and log (A'/A − 1) was plotted against −log B.

Statistical analyses were made by Student's *t* test. Comparison was made within same batches (i.e., the data in a series of experiments obtained in a day). Significant difference ($P < 0.05$) was indicated by an asterisk in figures.

Results

Effect of suramin on secretion of noradrenaline and dopamine

We previously characterized ATP-stimulated catecholamine-secretion by loading PC12 cells with [^{3}H]-noradrenaline (Inoue *et al.*, 1989; 1990). The receptors mediating the noradrenaline secretion can be classified as the P_2-type (Burnstock & Kennedy, 1985), judging from the order of agonist potency (Inoue *et al.*, 1990). Suramin (10 to 300 μM) inhibited the [^{3}H]-noradrenaline-secretion in a concentration-dependent manner (Figure 1a), suggesting that suramin antagonized the P_2-purinoceptor-mediated response in these cells. Figure 1b shows the effects of suramin on ATP-stimulated dopamine-secretion from PC12 cells as determined with h.p.l.c.-e.c.d. (see Methods). Suramin blocked the ATP-stimulated dopamine-secretion with a concentration-dependency similar to that seen for blockade of the ATP-stimulated noradrenaline secretion (Figure 1a). We characterized the effects of suramin further by measuring its effect on stimulated dopamine-secretion.

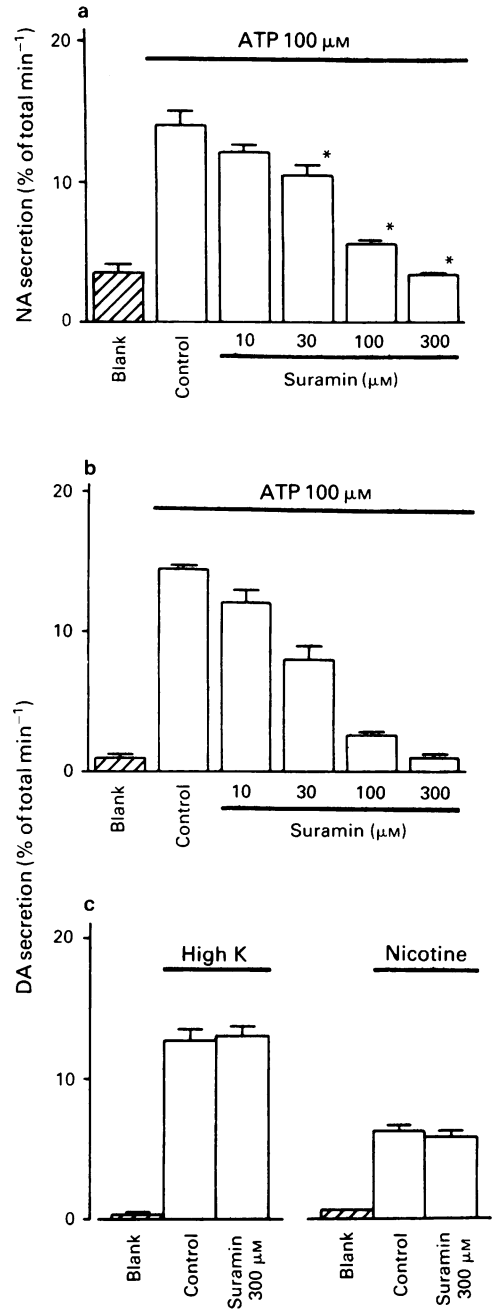

Figure 1 Effects of suramin on noradrenaline (NA) (a) and dopamine (DA)-secretion (b, c) from PC12 cells. (a, b) Concentration-dependent inhibition by suramin (10 to 300 μM) of extracellular ATP (100 μM)-stimulated noradrenaline (a) or dopamine-secretion (b). (c) Lack of effects of suramin (300 μM) on dopamine-secretion induced by high-K (155 mM) or nicotine (10 μM). Each column represents the mean from three dishes of the same batch. Bars are s.e.mean. Data shown were representative of three (a, b) or two (c) experiments. The blank was obtained without stimulation.

Suramin (300 μM) did not affect the dopamine-secretion stimulated by high-K (155 mM) or nicotine (10 μM) (Figure 1c). These results suggest that the blockade is selective for ATP-stimulated secretion. Figure 2 shows the concentration-response curves for the ATP-stimulated dopamine-secretion. Suramin (30 and 100 μM) shifted the curve to the right in a concentration-dependent manner (Figure 2). The maximal response was not altered in the presence of 30 μM suramin (Figure 2), suggesting that the antagonism is competitive.

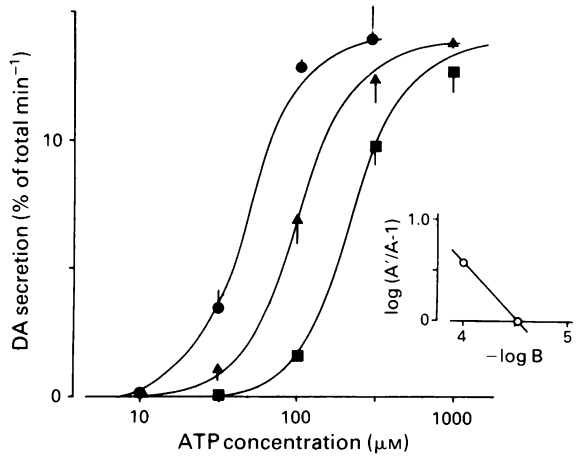

Figure 2 Concentration-response curves for ATP-stimulated dopamine (DA)-secretion in the absence (●) and presence of 30 (▲) or 100 μM (■) suramin. Each symbol represents the mean from three dishes. Vertical lines are s.e.mean. Smooth curves were fitted to the data using the values: $n = 2.5$, and $EC_{50} = 50$ (control), 102 (30 μM suramin) or 234 μM (100 μM suramin). Inset: Schild plot for the antagonism by suramin.

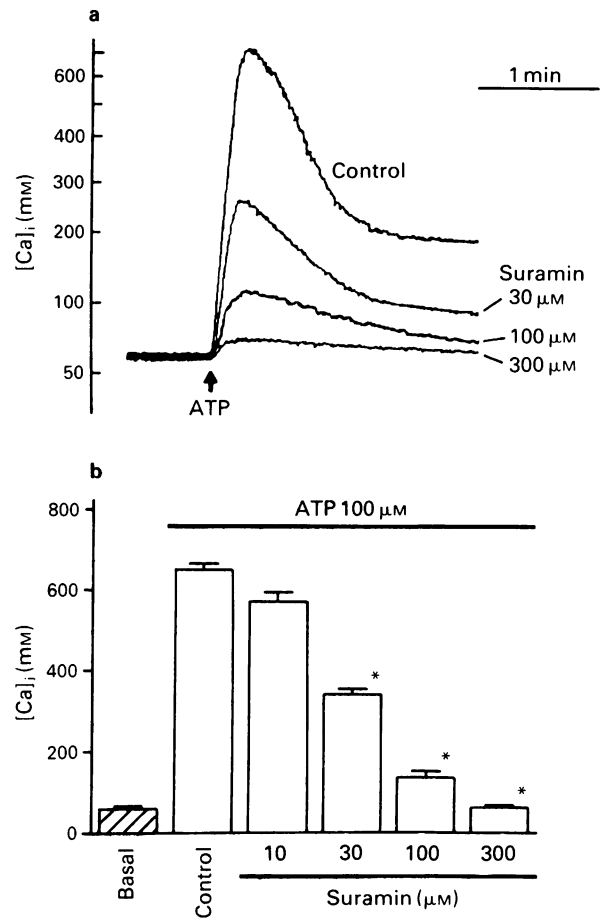

Figure 3 Increase in intracellular free Ca^{2+} concentrations ($[Ca]_i$) induced by ATP and its suppression by suramin. (a) Time course of changes in Ca^{2+} concentrations obtained from cell suspension (3×10^6 cells ml^{-1}). ATP (100 μM) with or without suramin was applied at the time indicated by an arrow, and continuously present in cell suspension. (b) Concentration-dependent antagonism by suramin of ATP-induced increase in Ca^{2+} concentrations. Each column represents the mean of peak Ca^{2+} concentrations after administration of 100 μM ATP with or without suramin. Data were obtained with cells from three dishes of the same batch. Bars are s.e.mean. The data shown were representative of three experiments. The basal Ca^{2+} concentration was measured just before application of drugs.

Competitive antagonism was also indicated by the slope obtained from a Schild plot (Figure 2, inset): the calculated slope (1.08) was close to the value (1.00) predicted from an ideal competitive antagonism (Tallarida & Jacob, 1979). The pA_2 value for suramin determined from the Schild plot was 4.52.

Effect of suramin on the ATP-stimulated increase of intracellular Ca^{2+}

Figure 3a shows a typical recording of intracellular Ca^{2+} concentrations in PC12 cells obtained with fura-2. The intracellular Ca^{2+} concentration was increased from a basal value of about 60 nM to about 700 nM by extracellular ATP (100 μM). Simultaneous administration of suramin suppressed the increment of Ca^{2+} concentration increase without affecting the time-course of the increase (Figure 3a). Suramin antagonized the intracellular Ca response to ATP in a concentration-dependent manner (Figure 3a, b).

Discussion

We have studied the effects of suramin on the ATP-stimulated catecholamine-secreted from PC12 cells. Suramin antagonized the effects of ATP on secretion, in a manner similar to its reported antagonism of ATP-induced smooth muscle contraction (Dunn & Blakeley, 1988) or relaxation (Den Hertog *et al.*, 1989). Thus, suramin may generally antagonize responses mediated through P_2-purinoceptors.

Suramin antagonized the ATP-stimulated catecholamine-secretion but not that stimulated by nicotine (Figure 1). Lack of blocking effects on the nicotine-induced secretion is in accordance with our previous result showing that suramin blocks an inward current activated by ATP but not that activated by nicotine (Nakazawa *et al.*, 1990b). Suramin thus discriminates P_2-purinoceptors from nicotinic receptors as well as from muscarinic receptors (Dunn & Blakeley, 1988; Nakazawa *et al.*, 1990b) and α_1-adrenoceptors (Den Hertog *et al.*, 1989).

The slope of the concentration-response curve for ATP increases in the presence of suramin in the smooth muscle cells (Dunn & Blakeley, 1988; Den Hertog *et al.*, 1989) whereas such an increase was not observed in the present study (Figure 2). The discrepancy suggests that the response to ATP is almost completely mediated through suramin-sensitive receptors in PC12 cells but not in smooth muscle cells.

Suramin suppressed the stimulation of intracellular Ca^{2+} concentration by ATP at the same concentrations that inhibited the ATP-stimulated catecholamine-secretion (Figure 3). These results suggest that the inhibition of catecholamine-secretion by suramin is due to a decrease in free Ca^{2+} concentrations. Suramin also blocked the ATP-activated inward current in PC12 cells at these concentrations (Nakazawa *et al.*, 1990b). The ATP-activated channels are Ca-permeable (Nakazawa *et al.*, 1990a) and the Ca-influx through the channels directly causes the catecholamine-secretion (Inoue *et al.*, 1990). Therefore, it is probable that suramin inhibits the ATP-stimulated catecholamine-secretion by blocking the activation of the channels coupled to the receptors (Bean & Friel, 1990) rather than some subsequent process. This may be indirect evidence that suramin does not exert significant effects other than competition with ATP.

In conclusion, suramin selectively and competitively antagonized the ATP-stimulated catecholamine-secretion from PC12 cells. The antagonistic effect of suramin may arise from its competition with ATP at receptors coupled to the ion channels. Suramin, at the concentrations used in the present study, can be regarded as a pure P_2-purinoceptor antagonist in experiments concerning catecholamine-secretion.

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References

BEAN, B.P. & FRIEL, D.D. (1990). ATP-activated channels in excitable cells. In *Ion Channels*, vol. 2, ed. Narahashi, T. pp. 169–203. New York: Plenum Press.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinoceptor. In *Cell Membrane Receptors for Drugs and Hormones: a Multidisciplinary Approach*. ed. Straub, R.W. & Bolis, L. pp. 107–118. New York: Raven Press.

BURNSTOCK, G. & KENNEDY, C. (1985). A dual function for adenosine 5'-triphosphate in the regulation of vascular tone; excitatory cotransmitter with noradrenaline from perivascular nerves and locally released inhibitory intravascular agent. *Circ. Res.*, **58**, 319–330.

DEN HERTOG, A., NELEMANS, A. & DEN AKKER, J.V. (1989). The inhibitory action of suramin on the P_2-purinoceptor response in smooth muscle cells of guinea-pig taenia caeci. *Eur. J. Pharmacol.*, **166**, 531–534.

DUNN, P.M. & BLAKELEY, A.G.H. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluoresence properties. *J. Biol. Chem.*, **260**, 3440–3450.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

INOUE, K. & KENIMER, J.G. (1988). Muscarinic stimulation of calcium influx and norepinephrine release in PC12 cells. *J. Biol. Chem.*, **263**, 8157–8161.

INOUE, K., NAKAZAWA, K., FUJIMORI, K., OBAMA, T., SAITO, I. & TAKANAKA, A. (1990). Extracellular ATP-evoked norepinephrine release coupled with receptor-operated Ca permeable channels in PC12 cells. *Jpn. J. Pharmacol.*, **52**, (suppl. I), 100P.

INOUE, K., NAKAZAWA, K., FUJIMORI, K. & TAKANAKA, A. (1989). Extracellular adenosine 5'-triphosphate-evoked norepinephrine secretion not relating to voltage-gated Ca channels in pheochromocytoma PC12 cells. *Neurosci. Lett.*, **106**, 294–299.

NAKAZAWA, K., FUJIMORI, K., TAKANAKA, A. & INOUE, K. (1990a). An ATP-activated conductance in pheochromocytoma cells and its modification by extracellular calcium. *J. Physiol.*, **428**, 257–272.

NAKAZAWA, K., FUJIMORI, K., TAKANAKA, A. & INOUE, K. (1990b). Reversible and selective antagonism by suramin of ATP-activated inward current in PC12 phaeochromocytoma cells. *Br. J. Pharmacol.*, **101**, 224–226.

TALLARIDA, R.J. & JACOB, L.S. (1979). Kinetics of drug-receptor interaction: interpreting dose-response data. In *The Dose-Response Relation in Pharmacology*. pp. 49–84. New York: Springer-Verlag.

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Differences in the K^+-channels opened by cromakalim, acetylcholine and substance P in rat aorta and porcine coronary artery

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1 The effects of acetylcholine and substance P on the efflux of $^{86}Rb^+$ and $^{42}K^+$ from rat aorta and pig coronary artery, respectively, were compared with those of the K^+ channel opening agent, cromakalim.

2 In rat aorta preloaded with $^{86}Rb^+$ and/or $^{42}K^+$, acetylcholine produced transient, concentration-dependent increases in the efflux rate coefficients of these tracers (maximum $\approx$ 35%). These effects were abolished by endothelial cell removal.

3 Donor/acceptor experiments with rat aorta suggested that at least some of the efflux of $^{86}Rb^+$ seen in the presence of acetylcholine was not derived from the endothelium, but came from the smooth muscle itself.

4 Acetylcholine (10 μM)-induced $^{86}Rb^+$ efflux was reduced by tetraethylammonium (TEA, 10 mM) to 33% and ouabain (300 μM) to 54% of control. Preincubation with Ba^{2+} (100 μM) did not significantly inhibit acetylcholine-induced efflux.

5 Acetylcholine-induced $^{42}K^+/^{86}Rb^+$ efflux was unaffected by preincubation with glibenclamide (10 μM). In contrast, the $^{42}K^+/^{86}Rb^+$ efflux induced by cromakalim was inhibited by glibenclamide (50 nM) by 50%.

6 Acetylcholine (0.3–10 μM)-induced inhibition of phenylephrine (1 μM)-induced tone was abolished by endothelial cell removal but unaffected by glibenclamide. Cromakalim-induced relaxations were endothelium-independent and were inhibited by glibenclamide in a concentration-dependent manner.

7 L^G-monomethyl L-arginine (L-NMMA, 250 μM) produced a significant (37 $\pm$ 14%) inhibition of acetylcholine-induced $^{86}Rb^+$ efflux whereas D^G-monomethyl L-arginine was without effect. In the tissue bath L-NMMA inhibited relaxations produced by acetylcholine (0.3–10 μM), but was without effect on responses to cromakalim.

8 In the pig coronary artery, substance P induced an endothelium-dependent efflux of $^{86}Rb^+$ and $^{42}K^+$, which was unaffected by preincubation with glibenclamide (10 μM) or L-NMMA (250 μM).

9 The present study shows that acetylcholine and substance P each open K^+-channels in arterial smooth muscle. However, the insensitivity of the stimulated $^{86}Rb^+/^{42}K^+$ efflux to inhibition by glibenclamide suggests that the K^+-channel opened by these agents is different from the K^+-channel opened by cromakalim. In addition, the inability of L-NMMA to inhibit fully the acetylcholine- and substance P-stimulated $^{86}Rb^+$ efflux suggests that in rat aorta and pig coronary artery the endothelium-derived hyperpolarizing factor(s) (EDHF) is different from endothelium-derived relaxing factor (EDRF).

Introduction

The existence of channels which can be modulated by the internal adenosine 5'-triphosphate (ATP) concentration of cells was first described in cardiac muscle (Trube & Hescheler, 1983; Noma, 1983) and has since been demonstrated in skeletal muscle (Spruce *et al.*, 1985; 1987), pancreatic β-cells (Cook & Hales, 1984) and cortical neurones (Ashford *et al.*, 1988). More recently, ATP-sensitive K^+-channels (K_{ATP}) have been identified in arterial smooth muscle, where they have been suggested to be the site of action of the K^+-channel opening drugs (Standen *et al.*, 1989), typified by cromakalim (Hamilton *et al.*, 1986).

In inside-out patches derived from rabbit mesenteric arteries, cromakalim opened K^+-channels which had been closed by applying a high concentration of ATP to the cytoplasmic side of the membrane (Standen *et al.*, 1989). The effects of cromakalim were reversed by the sulphonylurea glibenclamide, a potent and selective blocker of K_{ATP} in pancreatic β-cells and cardiac cells (Sturgess *et al.*, 1985; Schmid-Antomarchi *et al.*, 1987). Thus, the effects of cromakalim and other K^+-channel openers may be exerted via the opening of a K^+-channel similar to K_{ATP} in pancreatic β-cells and cardiac muscle. This is supported by a number of reports in which glibenclamide has been shown to inhibit the effects of cromakalim, both *in vitro* and *in vivo* (Buckingham *et al.*, 1989; Cavero *et al.*, 1989; Quast & Cook, 1989a). However, considerable evidence also suggests that other channel types such as large conductance Ca^{2+}-activated (Gelband *et al.*, 1988; Hu *et al.*, 1990) and delayed outward rectifier K^+-channels (Beech & Bolton, 1989) may be involved in the response to cromakalim.

Stimulation of the endothelium by acetylcholine and substance P leads to the release of factors which relax (EDRF) and hyperpolarize (EDHF) vascular smooth muscle (Furchgott & Zawadski, 1980; Zawadski *et al.*, 1983; Taylor *et al.*, 1988). In rat aorta, acetylcholine-induced hyperpolarization was accompanied by an increase in $^{86}Rb^+$ efflux from the tissue, reflecting an increase in the permeability of the cell membrane to K^+ (Taylor *et al.*, 1988). In the rabbit middle cerebral artery the hyperpolarizing effects of acetylcholine were reversed by glibenclamide, suggesting that the effects of acetylcholine in these arterial vessels were mediated through ATP-dependent K^+-channels (Standen *et al.*, 1989; Brayden, 1990).

The aim of the present study was to investigate the type of K^+-channel associated with the effects of two endothelium-dependent vasodilators, acetylcholine and substance P and that associated with the K^+-channel opening drug, cromakalim. The glibenclamide sensitivity of each response and the

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effects of the inhibitor of EDRF synthesis, N^G-monomethyl L-arginine (L-NMMA) (Rees *et al.*, 1989) were assessed and compared. Preliminary accounts of some of the data have already been presented (Quast & Baumlin, 1990; Quast & Bray, 1990).

Methods

Preparation of rat aorta

Male rats (Sprague-Dawley $\approx$ 150 g) were anaesthetized with ether and exsanguinated. Sections of the descending thoracic aorta ($\approx$15 mm long) were removed, carefully placed onto stainless steel tissue holders and bathed in a HEPES-buffered physiological salt solution (PSS) at 37°C, pH 7.4. In some experiments the endothelium was removed by rubbing the intimal surface of the aorta with a small wooden stick. Small rings of aorta (2–3 mm) were then cut from some of the preparations to examine in separate tissue bath experiments, the contractile and relaxant properties of the tissue following this rubbing procedure.

Measurement of $^{86}Rb^+$ and $^{42}K^+$ efflux

For the measurement of tracer efflux the tissues were preincubated for 120 min in PSS to which had been added $^{86}Rb^+$ (5 μCi ml^{-1}) or $^{42}K^+$ (5 μCi ml^{-1}) or both. The tissues were then transferred to thermostatic perfusion chambers containing PSS (perfused at 3 ml min^{-1}). The perfusate was collected at 1 min intervals and counted for radioactivity in the Čerenkov mode at an efficiency of 50% ($^{86}Rb^+$) or 65% ($^{42}K^+$). The radioactivity remaining at the end of the experiment was determined by dissolving the tissue in Lumasolve (at 50°C) overnight. The sample was then supplemented with 500 μl of 1 N HCL and 10 ml scintillant (OPTIFLUOR, Packard) and counted. The efflux data were expressed in terms of the efflux rate coefficient of $^{86}Rb^+$ or $^{42}K^+$ efflux (k, 10^{-2} min^{-1}).

In general, drug effects were expressed as the area under the curve (AUC) of the k vs time plots. In some experiments the peak drug effect was also calculated as percentage change (ΔK%) of the basal value of k, determined during the 10 min period prior to drug application. Results obtained from dual isotope experiments were evaluated as previously described (Quast & Baumlin, 1988).

Inhibition of efflux was generally studied by a double pulse protocol. After a period of equilibration the tissues were exposed to the test substance for 5–20 min (see respective Figures) followed by a period of washout. The blocker (inhibitor) was then added for 20 min before re-exposure to the test substance in the continuing presence of this agent. AUCs were calculated for each exposure period and the results expressed as a ratio of the second to the first peak (AUC_2/AUC_1). In experiments using L-NMMA or D-NMMA, a single pulse protocol was used. Tissues were exposed to L-NMMA (250 μM) or D-NMMA (250 μM) during the $^{86}Rb^+$ loading period and were washed for 25 min with PSS containing L-NMMA/D-NMMA. The tissues were then exposed to acetylcholine (10 μM) for 5 min. Direct comparison of AUCs was made between tissues exposed to L-NMMA/D-NMMA and those exposed only to vehicle.

Donor/acceptor experiments

Rings of rat aorta with the endothelium intact or rubbed (control experiments) were placed on tissue holders. After a period of equilibration, a piece of aorta of similar size, from a randomized group of rings which had been pre-labelled with $^{86}Rb^+$ and rubbed to remove the endothelium (acceptor), was placed next to (and above) the unlabelled (donor) ring. The tissues were then perfused with PSS from the bottom of the chamber upwards and exposed to relaxants following a double pulse protocol, being first exposed to acetylcholine (10 μM) followed by cromakalim (1 μM).

Tissue bath studies

Aortic rings were mounted in conventional tissue baths containing PSS, bubbled with 95% O_2, 5% CO_2 and maintained at 37°C, pH 7.4. Tissues were placed under an initial tension of 1 g. After a period of equilibration the rings were precontracted with phenylephrine (1 μM) and once the response was maintained, were exposed to a single concentration of acetylcholine (0.3–10 μM) or cromakalim (1–10 μM). Following washout, glibenclamide (1 or 10 μM) or L-NMMA (250 μM) was added for 40 min before exposure to phenylephrine and relaxant drug in the continuing presence of glibenclamide or L-NMMA. The relaxation to acetylcholine/cromakalim after exposure to glibenclamide or L-NMMA was expressed as a fraction of that obtained before exposure to these antagonists.

Pig coronary arteries

Pig hearts were obtained from the local slaughter house within 30 min of death and placed in PSS at room temperature. The left and right circumflex coronary arteries were carefully dissected and divided into sections 15 mm (for efflux studies) and 2–3 mm in length (for tissue bath studies).

The rings were mounted for isometric tension recordings under an initial tension of 4 g. After a period of equilibration (30 min) the tissues were retensioned and left for a further hour. After this time the PSS was replaced and the rings retensioned every 30 min for a further 60–90 min. Tissues were then precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10 μM) until a maintained contraction was achieved, followed by addition of a single contraction of susbtance P (30 nM) or cromakalim (0.1–10 μM). Following washout, the tissue was exposed to vehicle (dimethylsulphoxide (DMSO)), glibenclamide (1 or 10 μM) or L-NMMA (250 μM) for 40 min before re-exposure to $PGF_{2\alpha}$ and substance P/cromakalim in the continuing presence of test substance.

For efflux studies the protocol used for pig coronary arteries was identical to that employed in rat aorta experiments. In these experiments a 5 min exposure period to substance P was used.

Drugs and solutions

The following drugs were used: acetylcholine chloride (Fluka), stock solution in twice distilled water; (±)-cromakalim (synthesized at Sandoz), stock solution in DMSO; substance P (Bachem), stock solution in 150 mM acetic acid; (−)-noradrenaline L-hydrogentartrate (Fluka), stock solution in ascorbic acid (1 mM); $PGF_{2\alpha}$ (Upjohn), stock solution in 9% benzylic alcohol; barium chloride, stock solution in twice distilled water; tetraethylammonium (TEA, Janssen), stock solution in PSS; ouabain (Fluka), stock solution in twice distilled water. All dilutions were made in twice distilled water. Final concentrations of solvents did not exceed 0.1% and had no effect on the basal levels of either $^{86}Rb^+$ or $^{42}K^+$ efflux or on basal tension levels. $^{86}Rb^+$ was obtained from Amersham International (U.K.). $^{42}K^+$ was obtained as $^{42}K_2CO_3$ (Eidgenössisches Institut für Reaktorforschung, Würenlingen, Switzerland). The composition of the physiological salt solution was (mM): NaCl 120, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1.2, glucose 11, HEPES 20, pH 7.4 at 37°C, gassed with 95%O_2/5%CO_2.

Statistics

Results are expressed as the mean ± s.e.mean. Difference in tissue responses was assessed by a two-tailed unpaired Student's t test. Inhibitory effects of agents on tracer efflux stimulated by cromakalim, acetylcholine or substance P were assessed by measuring AUCs in the presence and absence of inhibitory substance and by expressing these as a ratio (AUC_2/AUC_1).

Results

Studies in rat aorta

Effects of acetylcholine Figure 1a shows the effects of exposure to acetylcholine (10 μM) followed by cromakalim (1 μM) on the $^{86}Rb^+$ efflux from unrubbed (endothelium intact) and rubbed (endothelium-denuded) aortic rings. Clearly, the effects of acetylcholine were fully dependent on the presence of an intact endothelium. Although in endothelium-denuded preparations, the time taken to reach maximum cromakalim-induced $^{86}Rb^+$ efflux was increased, the peak change in efflux was not significantly different in the two experimental groups ($P > 0.05$, $n = 6$–8). In intact tissues, preloaded with $^{86}Rb^+$, acetylcholine (0.3–30 μM) produced a concentration-dependent increase in the efflux of $^{86}Rb^+$ (Figure 1b) which reached a peak after 3 min and thereafter rapidly declined to baseline levels during the 20 min exposure time to acetylcholine (Figure 1a). In contrast, in the presence of cromakalim (1 μM) peak changes in k were observed only after 10 min (unrubbed tissues) and 15 min (rubbed tissues) (Figure 1a). Although there was some decline in the efflux coefficient after this time, the response to cromakalim was significantly maintained above control levels during the 20 min exposure period (Figure 1a).

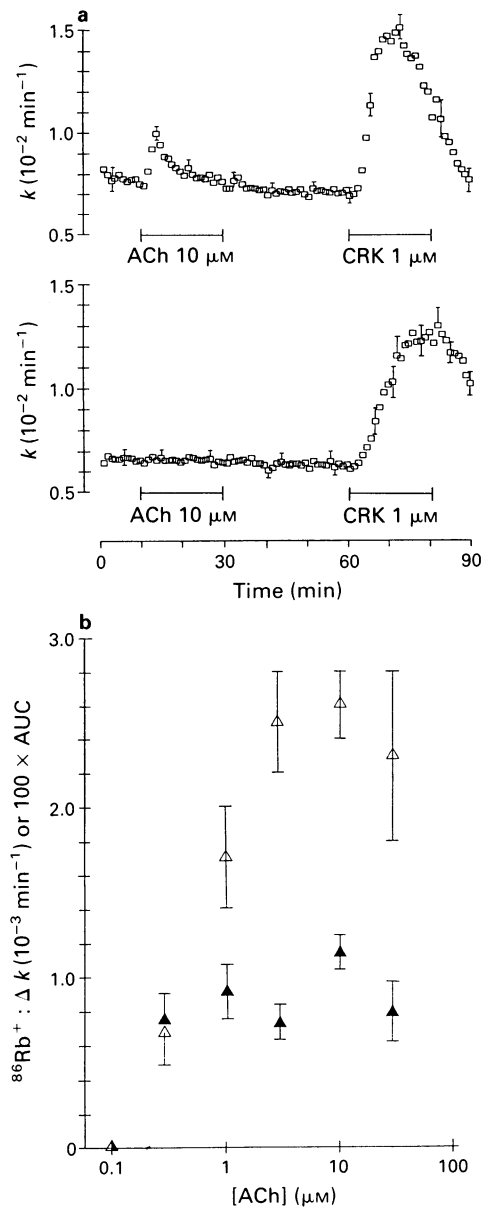

Figure 1 Effects of acetylcholine (ACh) and cromakalim (CRK) on the rate constant, k, of $^{86}Rb^+$ efflux from rat aorta. (a) Effects of ACh (10 μM) and CRK (1 μM) in; Upper trace, endothelium-intact preparations; Lower trace, rubbed tissues. At times indicated, ACh and CRK were each perfused for 20 min. (b) Dependence of $^{86}Rb^+$ efflux on ACh concentration as assessed by the peak increase in k (Δ) and the area under curve of the k vs time plot (AUC, ▲). Values are mean of 6–8 observations; vertical bars show s.e.mean. Some error bars have been omitted for clarity.

The effects of acetylcholine and cromakalim in the above flux experiments correlated with data obtained in tissue bath studies. In these experiments both acetylcholine (0.3 and 10 μM) and cromakalim (1 and 10 μM) inhibited phenylephrine (1 μM)-induced tone (Figure 2, Table 1). However, relaxations to acetylcholine were transient in nature, whereas cromakalim-induced responses were maintained. Of the two, only acetylcholine-induced relaxation was inhibited by endothelial cell removal (data not shown).

Donor/acceptor experiments Figure 3 shows the efflux of $^{86}Rb^+$ from 'acceptor' rings of rat aorta which were denuded of endothelium. In the presence of a 'donor' tissue with an intact endothelium, acetylcholine produced a small increase in the efflux of $^{86}Rb^+$ from the 'acceptor' tissue (Figure 3a). The efflux observed was 25 ± 5% of that observed for acetylcholine under the conditions described for Figure 1. In contrast, if the endothelium was removed from the 'donor' no efflux of $^{86}Rb^+$ from the acceptor tissue was observed (Figure 3b). In control and test conditions the responses to cromakalim were of similar magnitude (Figure 3a,b).

Inhibition studies

Glibenclamide: Figure 4a shows the effects of acetylcholine (10 μM) on the tracer efflux from intact aortic rings labelled, in separate experiments, with $^{86}Rb^+$ or $^{42}K^+$. Acetylcholine (10 μM) induced a transient increase in the efflux of both $^{86}Rb^+$ and $^{42}K^+$ (Figure 4a). Following a washout period of 30 min a second exposure to acetylcholine produced a second increase in flux of both tracers, which was in the case of $^{86}Rb^+$, 87 ± 7% and for $^{42}K^+$, 104 ± 6% of initial control response ($n = 6$). Pretreatment of tissues with glibenclamide (10 μM) alone, had no effect on the basal efflux of either $^{86}Rb^+$ or $^{42}K^+$. In addition, this concentration of glibenclamide was without effect on acetylcholine-stimulated increases in both $^{86}Rb^+$ and $^{42}K^+$ efflux (Figure 4b, Table 2).

In dual-labelling experiments cromakalim (1 μM) induced a relatively maintained efflux of $^{86}Rb^+$ and $^{42}K^+$ from rat aorta (Figure 4c). In contrast to the lack of effect of glibenclamide on acetylcholine-induced efflux, the effects of cromakalim were inhibited by glibenclamide (50 nM–10 μM) in a concentration-dependent manner (Figure 4d, Table 2). In the tissue bath, glibenclamide inhibited cromakalim-induced relaxation in rings precontracted with phenylephrine (1 μM) but was without effect on acetylcholine-induced vasorelaxations (Figure 2, Table 1).

L-NMMA: Preincubation with L-NMMA (250 μM) during the $^{86}Rb^+$-loading period (120 min) produced a significant (37 ± 14%) reduction in the efflux of $^{86}Rb^+$ produced by subsequent exposure to acetylcholine (10 μM), but was without significant effect on cromakalim-induced fluxes (Table 2). In contrast, exposure to D-NMMA (250 μM) was without effect on responses to acetylcholine (Table 2).

In tissue bath experiments, the application of L-NMMA sometimes produced a slowly developing contraction of the aorta and a potentiation of the maximum tension developed to phenylephrine. In the continuing presence of L-NMMA (250 μM) the relaxant effects of acetylcholine (0.3–10 μM) were inhibited (Table 1). This effect could be overcome to some extent by further increasing the concentration of acetylcholine (Table 1).

Tetraethylammonium: Figure 5a shows control responses to acetylcholine when a double pulse protocol was used. Following washout of acetylcholine, pretreatment with TEA (10 mM)

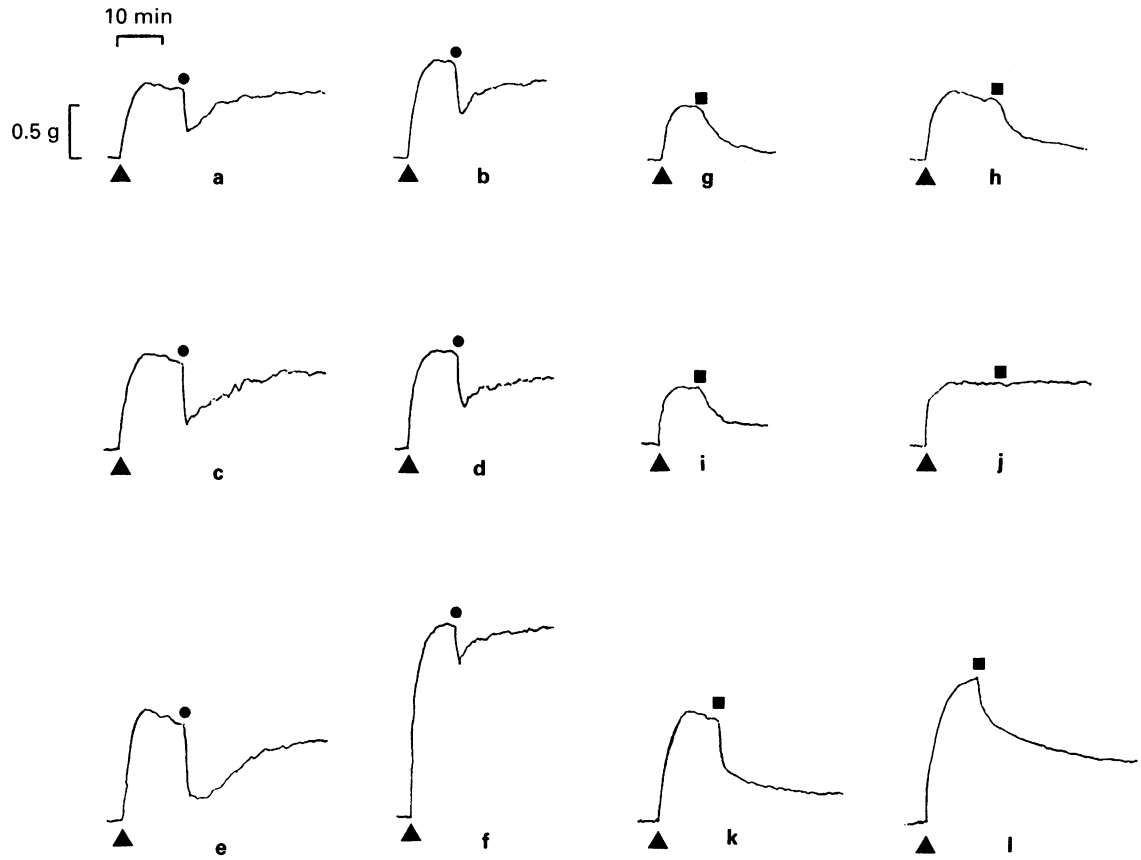

Figure 2 Representative traces showing the effects of glibenclamide (GBC) and L^G-monomethyl L-arginine (L-NMMA) on acetylcholine (ACh, ●, left panel) and cromakalim (CRK)-induced relaxations (■, right panel) in rat aorta. Left panel: (a,c,e) control responses. Tissues were precontracted with phenylephrine (PE, 1 μM) (▲), and then exposed to ACh (0.3 μM, ●). Following washout, vehicle, dimethylsulphoxide (DMSO), GBC (10 μM) or L-NMMA (250 μM) was added for 40 min. Re-exposure to PE (▲) and ACh (●) in presence of (b) DMSO; (d) GBC; (f) L-NMMA. Right panel: (g,i,k) control responses. Tissues were precontracted with PE (1 μM) (▲), and then exposed to CRK (10 μM, ■). Re-exposure to PE (▲) and CRK (■) in presence of (h) vehicle (DMSO); (j) GBC (10 μM); (l) L-NMMA (250 μM).

for 20 min produced a small but significant increase in the basal $^{86}Rb^+$ efflux rate coefficient (Figure 5b). In the continuing presence of TEA (10 mM), the response to acetylcholine (measured as AUC post exposure to TEA/AUC pre-exposure) was reduced to 33% of control values (no TEA) (Table 2). TEA (10 mM) completely inhibited cromakalim (1 μM)-induced efflux (Table 2).

Ba^{2+}: Although a small reduction in acetylcholine-induced $^{86}Rb^+$ efflux was observed in the presence of Ba^{2+} (100 μM) this was not significant (Figure 5c, Table 2). In contrast, the flux-inducing effects of cromakalim were almost completely inhibited by Ba^{2+} (100 μM, Table 2).

Ouabain: With an identical protocol, exposure to ouabain (300 μM) produced a slowly developing but progressively increasing baseline. In the continuing presence of ouabain, both acetylcholine- and cromakalim-induced effluxes were significantly inhibited (Table 2) compared to control responses where ouabain was not included in the perfusing solution (acetylcholine response, Figure 5d).

Studies in pig coronary arteries

The basal rates of efflux of $^{86}Rb^+$ and $^{42}K^+$ (measured between 30–35 min into efflux, $n = 6$ preparations) from pig coronary artery were 0.328 ± 0.003 10^{-2} min^{-1} and 0.751 ± 0.02 10^{-2} min^{-1}, respectively. In the absence of agonist these rates were well maintained, in contrast to the basal efflux in rat aorta which tended to fall slightly throughout the experiment (see Figure 1).

Effects of substance P on tracer efflux In pig coronary artery rings, substance P (30 nM) induced a small, concentration-dependent, transient increase in the efflux of $^{86}Rb^+$ and $^{42}K^+$ (Figure 6a). In a manner similar to that of acetylcholine in rat

Table 1 Vasorelaxant effects of acetylcholine (ACh), substance P and cromakalim (CRK) and their inhibition by glibenclamide (GBC) and L^G-monomethyl L-arginine (L-NMMA)

	Rat aorta[a]		*Pig coronary*[a]	
	ACh	*CRK*	*Substance P*	*CRK*
Substance	0.3 μM/10 μM	0.3 μM/10 μM	30 nM	0.3 μM/10 μM
(Control)	87 ± 7/98 ± 9	90 ± 6/84 ± 6	102 ± 5	98 ± 5/103 ± 4
GBC (1 μM)	96 ± 6/99 ± 7	29 ± 8*/30 ± 3*	97 ± 6	10 ± 7*/85 ± 6*
GBC (10 μM)	97 ± 7/97 ± 5	20 ± 8*/35 ± 4*	99 ± 8	4 ± 1*/52 ± 6*
L-NMMA (250 μM)	34 ± 4*/51 ± 6*	83 ± 14/74 ± 5	98 ± 12	111 ± 14/106 ± 3

[a] Tissues were precontracted with phenylephrine 1 μM (aorta) or prostaglandin $F_{2\alpha}$ 10 μM (pig coronary artery). Results in presence of inhibitor or vehicle are expressed as % of initial relaxant response in absence of inhibitor (see Methods). Values are mean ± s.e.mean of 4–7 observations; * denotes $P < 0.05$ compared to control value.

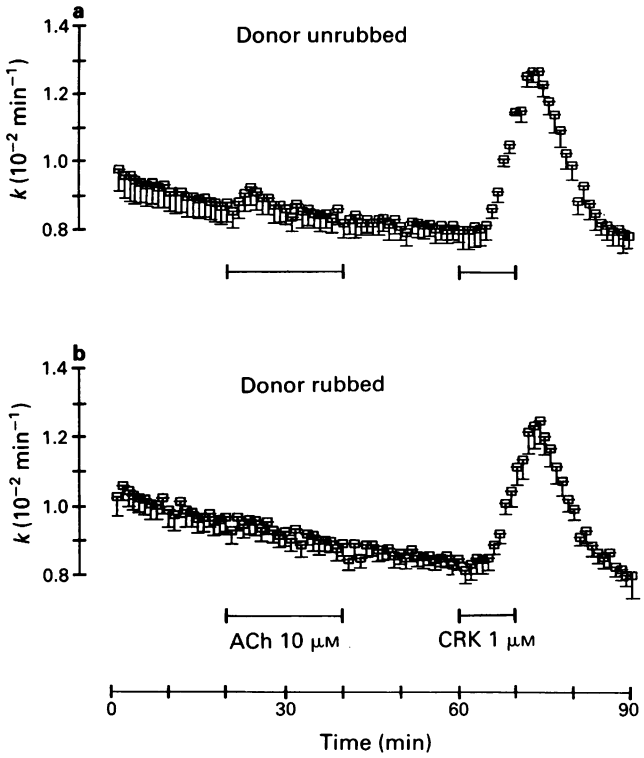

Figure 3 Donor/acceptor experiments in rat aorta, showing the efflux of $^{86}Rb^+$ induced by acetylcholine (ACh, 10 μM) and cromakalim (CRK, 1 μM), from rubbed (endothelium-denuded) rings of rat aorta (a) in the presence of an unrubbed, unlabelled donor tissue; (b) in the presence of a rubbed, unlabelled donor tissue. ACh and CRK were perfused at the times indicated by the horizontal bars. Values are mean for 6 experiments; vertical bars show s.e.mean.

aorta (see Figure 1), this effect of substance P was completely abolished by removal of the endothelium, whereas in the same tissue segments (i.e. rubbed tissues) the response to cromakalim was unaffected (results not shown).

Following washout of substance P, a second increase in efflux of both $^{86}Rb^+$ and $^{42}K^+$ could be produced, although this was reduced by approximately 20% when compared to the initial peak (Figure 6a, Table 3). Preincubation with glibenclamide (10 μM) for 20 min had no effect on the basal efflux of $^{86}Rb^+$ or $^{42}K^+$ and in the continuing presence of glibenclamide, efflux induced by substance P was unaffected (Figure 6b). In contrast, the effects of cromakalim were inhibited by glibenclamide (0.5–10 μM) in a concentration-dependent manner (Figure 7 and Table 3).

In a separate series of experiments, tissues were preincubated with L-NMMA (250 μM) during the $^{86}Rb^+$ loading period and for a further 30 min before exposure to a single concentration of substance P (30 nM). L-NMMA was without effect on either the basal or substance P-induced increase in $^{86}Rb^+$ efflux in this tissue (Table 3).

Tissue bath studies Exposure of coronary artery rings to $PGF_{2\alpha}$ (10 μM), usually produced a slowly developing contraction which reached a maintained level after approximately 30–60 min. In some preparations the contraction developed more rapidly (15 min). However, no differences were observed in the inhibitory effects of substance P, following either type of contraction (Figure 8).

Substance P (30 nM) produced a maximal relaxation of pig coronary artery rings which had been precontracted with $PGF_{2\alpha}$ (10 μM) (Figure 8). Like the inhibitory effects of acetylcholine in rat aorta, this relaxation was transient in nature and inhibited completely by endothelial removal (data not shown). These mechanical changes were completely unaffected by preincubation of the tissues for 40 min with glibenclamide

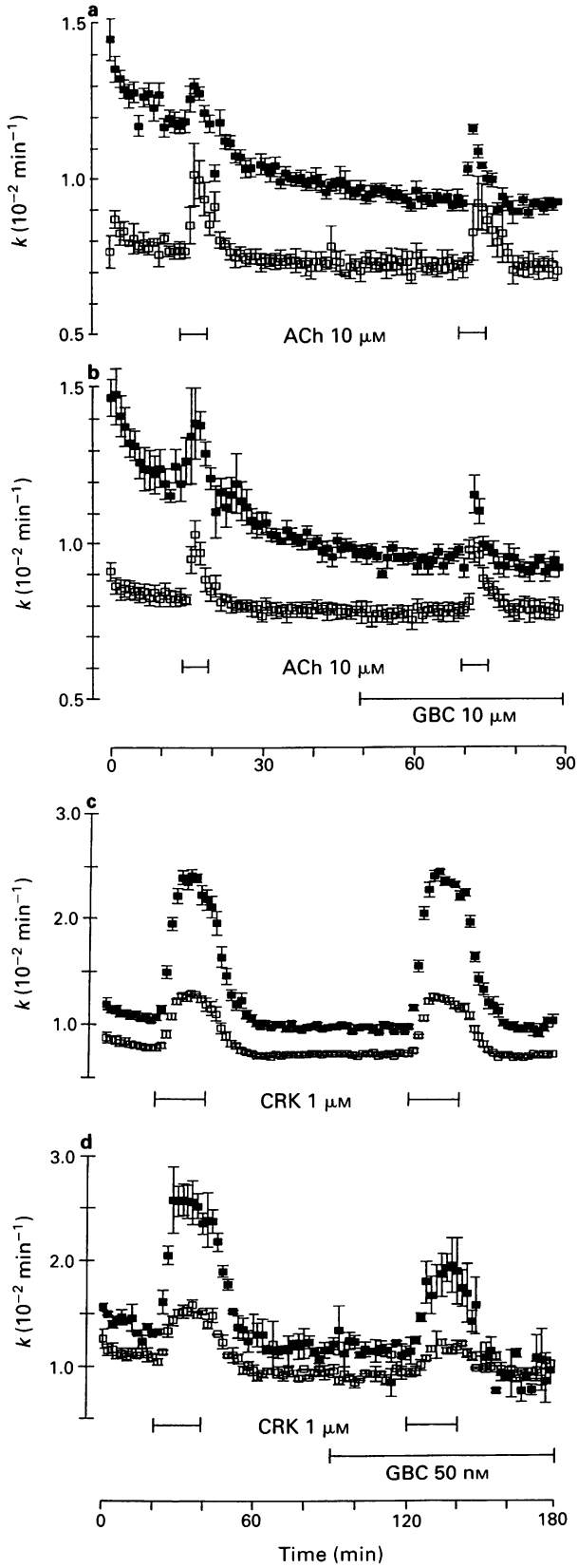

Figure 4 Effects of glibenclamide (GBC) on acetylcholine (ACh) and cromakalim (CRK)-induced efflux of $^{42}K^+$ (■) and $^{86}Rb^+$ (□) from rat aorta. (a) Control: ACh (10 μM) was perfused for 5 min at the times indicated. (b) Effect of GBC on ACh-induced tracer efflux. GBC (10 μM) was added 20 min prior to the second exposure to ACh as indicated. (c) Control: double pulse protocol for CRK (1 μM, perfused for 20 min). (d) Effect of GBC (0.05 μM, added 30 min prior to the second exposure to CRK) on CRK-induced tracer efflux. (a and b) Effluxes of $^{42}K^+$ and $^{86}Rb^+$ were determined in separate experiments; (c and d) double labelling experiments. Values are mean with s.e.mean shown by vertical bars, $n = 6$.

Table 2 Comparison of $^{86}Rb^+$ effluxes induced by acetylcholine (10 μM) or cromakalim (1 μM) in rat aorta[a]

Substance[b]	*Acetylcholine* AUC_2/AUC_1 (%)[c]	*Cromakalim* AUC_2/AUC_1 (%)[c]
(Control)	0.87 ± 0.06 = 100%	1.09 ± 0.07 = 100%
GBC 0.05 μM	/	48 ± 10*
0.5 μM	/	0 ± 0*
10 μM	104 ± 6	0 ± 0*
L-NMMA 250 μM	63 ± 14*	82 ± 9
D-NMMA 250 μM	96 ± 8	/
TEA 10 mM	33 ± 10*	0 ± 0*
Ba^{2+} 100 μM	83 ± 10	20 ± 3*
Ouabain 300 μM	54 ± 6*	73 ± 5*

[a] A double pulse protocol was used, except for L^G-monomethyl L-arginine (L-NMMA) and D-NMMA which were present during the $^{86}Rb^+$ loading period (120 min) and a 10 min wash (see Figure 5 and Methods).

[b] The applied agents were without effect on the basal rate of $^{86}Rb^+$ efflux ($k = 0.65 \pm 0.03\ 10^{-2}\,min^{-1}$) except tetraethylammonium (TEA), Ba^{2+} and ouabain which increased k by 0.07 ± 0.02, 0.08 ± 0.06 and 0.13 ± 0.03 $10^{-2}\,min^{-1}$ after 20 min perfusion time (n = 6–10, mean ± s.e.mean).

[c] Data are expressed as AUC_2/AUC_1 (mean ± s.e.mean, n = 6–10) where AUC_1 was 0.016 ± 0.002 (n = 10) for 20 min stimulation with acetylcholine (10 μM) and 0.164 ± 0.0005 (n = 9) for cromakalim (1 μM) (AUC in dimensionless units, see Quast & Baumlin, 1988). AUC_2/AUC_1 was then normalized to 100%. Statistically significant differences, compared to control values are indicated by * $P < 0.05$. GBC = glibenclamide.

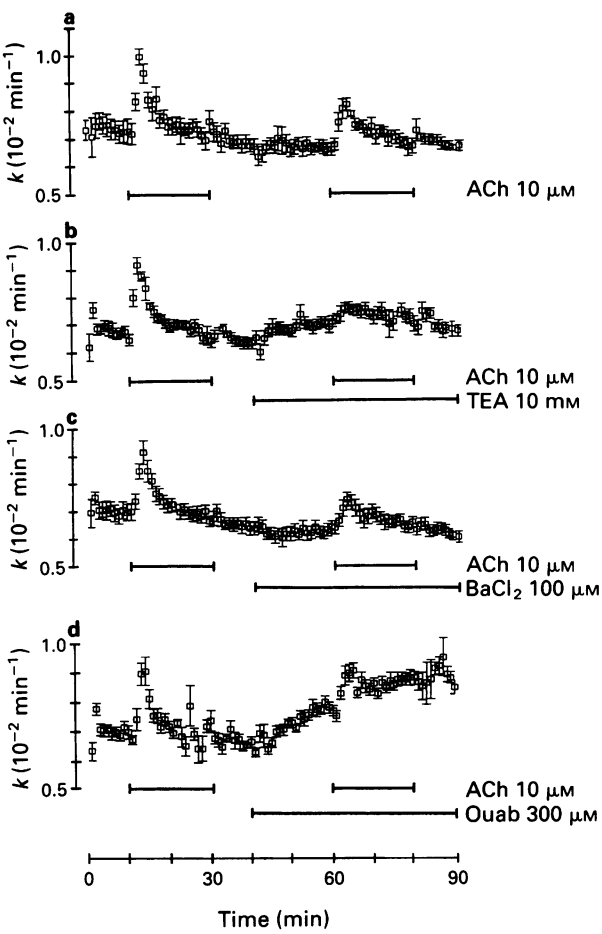

Figure 5 Acetylcholine (ACh)-induced $^{86}Rb^+$ efflux in the presence of tetraethylammonium (TEA), Ba^{2+} and ouabain (Ouab). (a) Control responses to ACh (10 μM), using a double pulse protocol. After first exposure to ACh, tissues were superfused with PSS containing (a) vehicle, (b) TEA, 10 mM, (c) Ba^{2+}, 100 μM or (d) ouabain, 300 μM for 20 min, prior to re-exposure to ACh. Values are mean of 6–8 observations; vertical bars show s.e.mean.

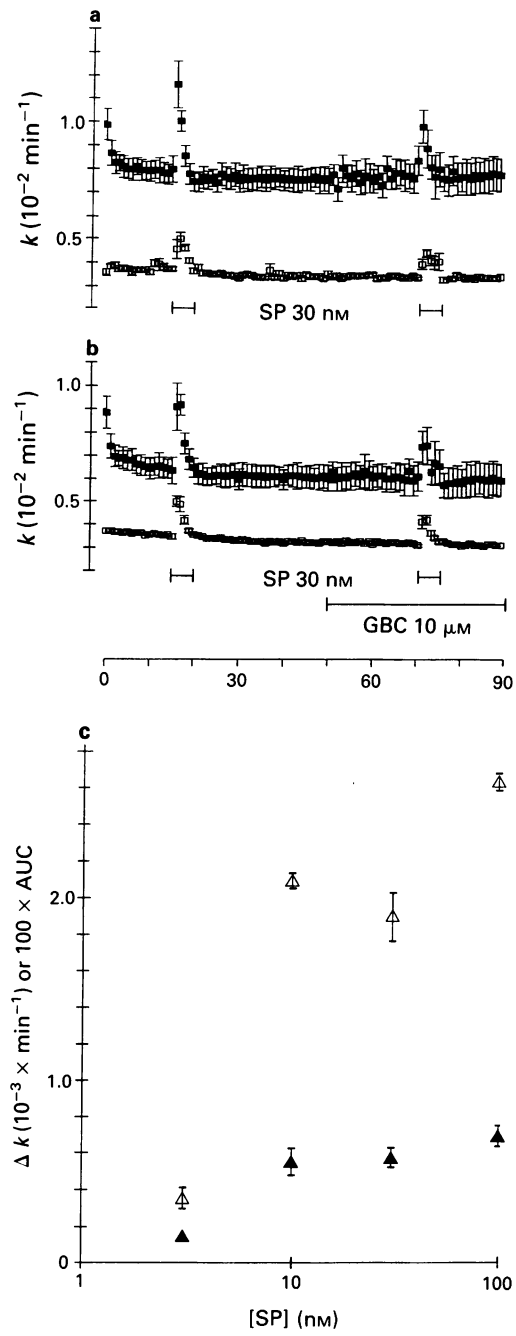

Figure 6 Substance P (SP)-induced efflux of $^{42}K^+$ (■) and $^{86}Rb^+$ (□) from pig coronary artery rings and effect of glibenclamide (GBC). (a) Effect of substance P (30 nM) perfused for 5 min at the times indicated (control). (b) Effect of GBC (10 μM), added 20 min prior to the second exposure to substance P. (c) Plot of Δk (△) and AUC (▲) vs concentration of substance P. Effluxes of $^{42}K^+$ and $^{86}Rb^+$ were determined in separate experiments. Values are mean of 6–8 observations; s.e.mean shown by vertical bars.

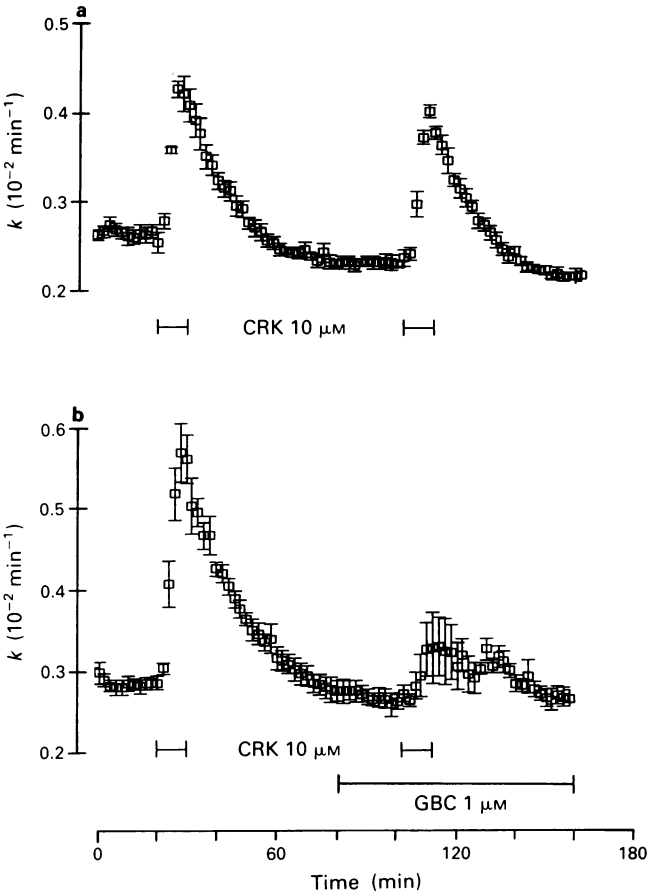

Figure 7 Effect of glibenclamide (GBC) on cromakalim (CRK)-induced $^{86}Rb^+$ efflux from pig coronary artery rings. (a) Control responses to CRK (10 μM) using a double pulse protocol. Tissues were exposed to CRK for 10 min at the times indicated. (b) Effect of GBC (1 μM), added 20 min prior to and during second exposure to CRK. Values are mean of 8 observations; s.e.mean shown by vertical bars.

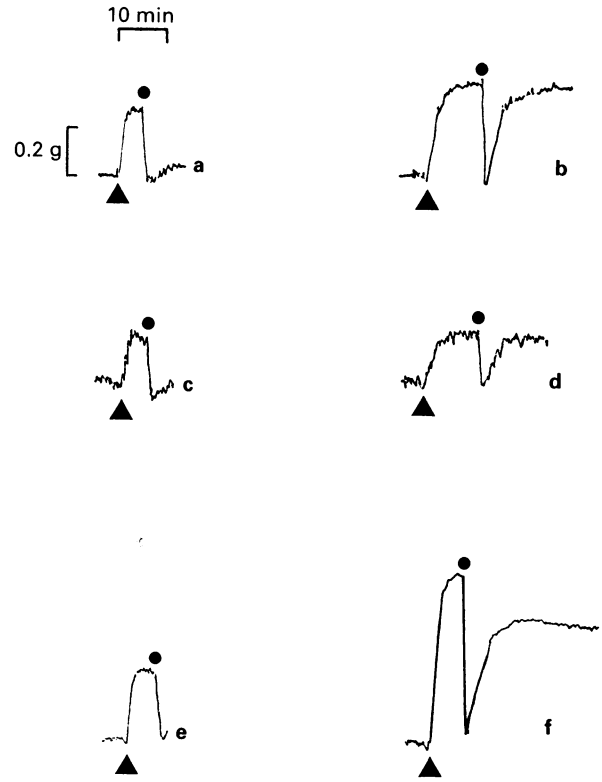

Figure 8 Representative traces showing the effects of glibenclamide (GBC) and L^G-monomethyl L-arginine (L-NMMA) on substance P-induced relaxations in pig coronary artery. (a,c,e) Control responses. Tissues were precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10 μM) (▲), and then exposed to substance P (30 μM, ●). Following washout, vehicle (DMSO), GBC (10 μM) or L-NMMA (250 μM) was added for 40 min. Re-exposure to $PGF_{2\alpha}$ and substance P in presence of (b) DMSO, (d) GBC, (f) L-NMMA.

(10 μM). Although the contractile response to $PGF_{2\alpha}$ was potentiated by preincubation with L-NMMA (see Figure 8f), the inhibitory effects of substance P were unaffected by this pretreatment (Table 1).

Cromakalim (1 and 10 μM) produced a slowly developing inhibition of tone in coronary artery rings, which could be inhibited by pretreatment with glibenclamide (1 and 10 μM). The inhibitory effects of cromakalim were not significantly inhibited by pretreatment with L-NMMA (Table 1).

Table 3 Effects of glibenclamide (GBC) and L^G-monomethyl L-arginine (L-NMMA) on the $^{86}Rb^+$ efflux induced by substance P (30 nM) and cromakalim (1 and 10 μM) in porcine coronary artery

Substance	*Substance P* (30 nM) AUC_2/AUC_1 (%)	*Cromakalim* (1 μM/10 μM) AUC_2/AUC_1 (%)
(Control)	0.82 ± 0.02 = 100%	1.0 ± 6 = 100% (1 μM) 1.0 ± 6 = 100% (10 μM)
GBC 0.1 μM	/	27 ± 1*/55 ± 1*
0.3 μM	/	8 ± 4*/28 ± 3*
3 μM	92 ± 14	/
10 μM	113 ± 17	/
L-NMMA 250 μM	111 ± 13	/

Substance P: AUC_1 = 0.0056 ± 0.0004 (absolute units, n = 14, mean ± s.e.mean)
Cromakalim: AUC_1 (1 μM) = 0.034 ± 0.002 (absolute units, n = 8, mean ± s.e.mean) (10 μM) = 0.062 ± 0.005 (absolute units, n = 8, mean ± s.e.mean)
* $P < 0.05$, compared to control.

Neither substance P nor cromakalim had any effects on the basal tension of coronary artery rings (data not shown).

Discussion

The present study has shown that several major differences exist between the pharmacological profiles of acetylcholine- and substance P-induced $^{86}Rb^+/^{42}K^+$ efflux in comparison to cromakalim-induced effects. These include: (1) the complete lack of sensitivity of acetylcholine and substance P to inhibition by glibenclamide; (2) the endothelium-dependence of both the relaxant responses and tracer efflux induced by acetylcholine and substance P; (3) the relative lack of sensitivity of acetylcholine-induced efflux to inhibition by Ba^{2+} and TEA; (4) the rapid inactivation kinetics of the $^{86}Rb^+/^{42}K^+$ efflux induced by endothelium-dependent vasodilators and (5) differences in the magnitude of efflux induced by acetylcholine and substance P as compared to cromakalim, although each of these substances were equally effective as relaxants.

Origin of acetylcholine-induced efflux

In rat aortic rings, acetylcholine produced a small transient increase in $^{86}Rb^+$ and $^{42}K^+$ efflux. Taylor *et al.* (1988) had previously shown that acetylcholine transiently hyperpolarized this tissue and elicited a concomitant increase in $^{86}Rb^+$ efflux. To investigate whether the small increases in $^{86}Rb^+$ efflux observed here with acetylcholine stem from the endothelial cells or the smooth muscle cells, donor/acceptor experiments were performed. The rationale for these experiments was derived from the observation that in endothelium-denuded tissues no tracer efflux in response to acetylcholine

was observed. Therefore, it was possible that acetylcholine was acting either directly to increase the permeability of the endothelium for K^+ or indirectly, by the release of factors from the endothelium, which lead to an increase in $^{86}Rb^+$ efflux from either the endothelium or the underlying smooth muscle. In endothelium-denuded preparations which were loaded with $^{86}Rb^+$, efflux of this tracer was only observed in the presence of an endothelium-intact donor (unlabelled) tissue. Thus, the efflux must have been a result of a factor(s) released from the endothelium which acts on the smooth muscle.

In these donor/acceptor experiments the observed efflux was considerably smaller than that obtained from endothelium-intact tissues. This may reflect the small amounts or the short half-life of EDRF/EDHF released by the endothelium, the effects of which are normally facilitated by the close association with the underlying smooth muscle. Alternatively, a component of the efflux induced by acetylcholine from tissues with intact endothelium, may be derived from the endothelium itself. It has recently been shown that activation of cultured endothelial cells by endothelium-dependent vasodilators leads to hyperpolarization due to the opening of Ca^{2+}-dependent K^+-channels (Lueckhoff & Busse, 1990), and this may therefore, contribute to the increased efflux observed after exposure to acetylcholine.

Nature of acetylcholine-induced efflux

Some information on the nature of the endothelium-derived hyperpolarizing factor in rat aorta was obtained with the EDRF synthesis inhibitor, L-NMMA at concentrations that inhibit endothelium-mediated vasorelaxation maximally (250 μM, Palmer *et al.*, 1988). Acetylcholine (10 μM)-induced $^{86}Rb^+$ efflux was inhibited by L-NMMA (but not D-NMMA) by 37%, suggesting that these effects were specific for the L-enantiomer. In contrast, cromakalim-induced effects were not significantly inhibited by L-NMMA.

The observation that part of the response was resistant to the actions of L-NMMA, suggested that at least two factors may be released from the endothelium. It also suggests that in the presence of acetylcholine at least part of the observed efflux is induced by EDRF. The present result is consistent with those of Tare *et al.* (1990) who showed that EDRF (NO) could hyperpolarize guinea-pig uterine artery. However, in other vessels there is evidence against a role of EDRF (NO) in acetylcholine-induced hyperpolarization, for example in canine mesenteric arteries (Komori *et al.*, 1988), and in rabbit femoral (Huang *et al.*, 1988) and cerebral arteries (Brayden, 1990). These differences may relate to the vessel studied and this matter clearly requires further clarification. In the present study, L-NMMA inhibited the relaxant and efflux responses to acetylcholine (10 μM) by 50% and 37%, respectively. Thus, at this acetylcholine concentration, EDRF and EDHF appear to make approximately equal contributions to the observed responses. At the lower concentration of acetylcholine (1 μM) it seems likely that of the two factors released, EDRF plays a greater role in mediating relaxation (EDRF release has previously been estimated to produce 60–80% of the acetylcholine-induced relaxation; Chen & Suzuki, 1989).

Substance P produces an endothelium-dependent hyperpolarization of pig coronary artery (Bény *et al.*, 1986), and also hyperpolarizes pig coronary artery endothelial cells in primary culture (Brunet & Bény, 1989). In support of these results, substance P increased the efflux of $^{86}Rb^+$ from pig coronary artery. However, in contrast to acetylcholine, the effects of substance P on tracer efflux (and agonist-induced tone) were not antagonized by L-NMMA. This suggests that substance P acts to release factors from the endothelium which hyperpolarize (EDHF) and/or relax (EDRF) the coronary artery but which are synthesized via pathways different from those leading to EDRF (= nitric oxide, NO) synthesis in other vessels, and are therefore probably different from NO.

The nature of the $^{86}Rb^+$ efflux induced by acetylcholine was also investigated with K^+-channel blocking agents and ouabain, an inhibitor of Na^+/K^+ ATPase. Tetraethylammonium, a K^+-channel blocker with broad specificity, inhibited the acetylcholine-stimulated efflux suggesting that at least a part of this response was a result of K^+ ($^{86}Rb^+$) flux through ion channels. The results obtained with ouabain are, on the other hand, more difficult to interpret. Activation of Na^+/K^+ ATPase leads to membrane hyperpolarization (see Fleming, 1980, for review) and has been implicated in endothelium-dependent hyperpolarization of smooth muscle (De Mey & Vanhoutte, 1980; Rapoport *et al.*, 1985; Feletou & Vanhoutte, 1988). Conversely, inhibition of the pump leads to cell depolarization (Fleming, 1980) and increases in intracellular Ca^{2+} via Na^+/Ca^{2+} exchange (see Brading & Lategan, 1985, for discussion). Accordingly, exposure to ouabain alone produced a slowly-developing increase in the baseline efflux of $^{86}Rb^+$. However, although activation of the pump by acetylcholine may provide an explanation as to the relatively small amount of induced efflux, it does not reconcile the fact that in the presence of ouabain, acetylcholine-induced flux was reduced, when theoretically, due to an increase in the electrochemical driving force for $^{86}Rb^+$ to leave the cell, an increase in $^{86}Rb^+$ efflux would be predicted. As similar qualitative results were obtained with cromakalim, i.e. the cromakalim-induced efflux was also inhibited by ouabain, these observations may reflect that in the presence of ouabain there is a decreased release of the hyperpolarizing factor(s) (in the case of acetylcholine) due to depolarization of the endothelium or may reflect the voltage- and Ca^{2+}-dependence of the Rb^+-permeable channels opened by these hyperpolarizing factors and by cromakalim. It has been suggested that Ca^{2+}-influx or extracellular Ca^{2+} may be involved in the closure of K^+-channels opened by cromakalim, in accordance with this suggestion (Coldwell & Howlett, 1988).

Pharmacology of acetylcholine-induced effects

The most interesting observation made was that the sulphonylurea glibenclamide, a potent and selective blocker of K_{ATP} in pancreatic β-cells and related cell lines (Trube *et al.*, 1986; Schmidt-Antomarchi *et al.*, 1987; Sturgess *et al.*, 1988) was completely without effect on both the relaxant and efflux-inducing properties of both acetylcholine and substance P in rat aorta and pig coronary, respectively. In rabbit middle cerebral artery, however, glibenclamide has previously been shown to inhibit the hyperpolarization induced by acetylcholine suggesting that in this vessel this effect is mediated by an opening of K_{ATP} (Standen *et al.*, 1989; Brayden, 1990). This difference may relate to the inherent physiological properties of the vessels studied since we have used large, tonic vessels, in contrast to Standen *et al.* (1989) and Brayden (1990), who used small (resistance) vessels.

In contrast to the lack of effect of glibenclamide (10 μM) on the relaxant and tracer efflux stimulating effects of acetylcholine and substance P in rat aorta and pig coronary artery, respectively, the effects of cromakalim in these vessels were antagonized by concentrations of glibenclamide up to 200 times lower. This is in agreement with others, who suggest that the effects of cromakalim may be exerted at an ATP-dependent K^+-channel in smooth muscle (see e.g. Quast & Cook, 1989b; Standen *et al.*, 1989).

In addition to the differential effects of glibenclamide, on the cromakalim- and acetylcholine-induced $^{86}Rb^+/^{42}K^+$ efflux in the rat aorta, differences were obtained with non-selective K^+-channel blockers. TEA, at 10 mM, fully inhibited the $^{86}Rb^+$-efflux induced by cromakalim whereas responses to acetylcholine were only partially inhibited (by 65%), thus demonstrating that the ion channels involved in these two responses differ in their sensitivity to TEA. Similarly Ba^{2+} (100 μM) was unable to reduce appreciably acetylcholine-induced $^{86}Rb^+$ efflux, whereas cromakalim-induced efflux was much more sensitive to inhibition. Ba^{2+}, at these concentra-

tions, is an effective and relatively specific inhibitor of K_{ATP} in skeletal (Quayle *et al.*, 1988) and vascular smooth muscle (Standen *et al.*, 1989). Thus, the relative insensitivity of the acetylcholine-induced $^{86}Rb^+$ efflux in rat aorta to blockade by Ba^{2+} provides additional support for the contention that the channels involved in this response differ from K_{ATP} channels, and from the channels opened by cromakalim in smooth muscle.

To show that the lack of effect of glibenclamide on endothelium-dependent vasodilators was not restricted to the actions of acetylcholine in the rat aorta, similar experiments were carried out with substance P in the pig coronary artery. Like acetylcholine in aorta, substance P produced a concentration- and endothelium-dependent increase in $^{42}K^+$ and $^{86}Rb^+$ efflux and relaxation of $PGF_{2\alpha}$-induced contractions. These responses were completely unaffected by glibenclamide suggesting that the relaxant response and $^{42}K^+$ and $^{86}Rb^+$-efflux produced by substance P were not associated with the activation of glibenclamide-sensitive K^+-channels.

In conclusion, in the present experiments clear differences in the profiles of action of cromakalim and acetylcholine in rat aorta, were observed. While both agents opened K^+-channels permeable to both $^{86}Rb^+$ and $^{42}K^+$, only cromakalim opened K^+-channels which were inhibited by glibenclamide. A similar pattern emerged in pig coronary artery where substance P increased the efflux of $^{86}Rb^+/^{42}K^+$ an effect which was also unaffected by glibenclamide. In both tissues, the effects of cromakalim were inhibited by glibenclamide in a concentration-dependent manner. These results together with the differential sensitivity of the stimulated $^{86}Rb^+$ effluxes in rat aorta to TEA and Ba^{2+} strongly suggest that the K^+-channel opened by endothelium-dependent vasodilators such as acetylcholine and substance P is distinct from that opened by cromakalim, at least in the two large vessels studied here.

References

ASHFORD, M.J.L., STURGESS, N.C., TROUT, N.J., GARDNER, N.J. & HALES, C.N. (1988). Adenosine-5'-triphosphate-sensitive ion channels in neonatal rat cultured central neurones. *Pflügers Arch.*, **412**, 297–304.

BEECH, D.J. & BOLTON, T.B. (1989). Properties of the cromakalim-induced potassium conductance in smooth muscle cells isolated from the rabbit portal vein. *Br. J. Pharmacol.*, **98**, 851–864.

BÉNY, J.-L., BRUNET, P.C. & HUGGEL, H. (1986). Effect of mechanical stimulation, substance P and vasoactive intestinal peptide on the electrical and mechanical activities of circular smooth muscles from pig coronary arteries contracted with acetylcholine: role of endothelium. *Pharmacology*, **33**, 61–68.

BRADING, A.F. & LATEGAN, T.W. (1985). Na-Ca exchange in vascular smooth muscle. *J. Hypertens.*, **3**, 109–116.

BRAYDEN, J.E. (1990). Membrane hyperpolarisation is a mechanism of endothelium-dependent cerebral vasodilation. *Am. J. Physiol.*, (in press).

BRUNET, P.C. & BÉNY, J.L. (1989). Substance P and bradykinin hyperpolarize pig coronary artery endothelial cells in primary culture. *Blood Vessels*, **26**, 228–234.

BUCKINGHAM, R.E., HAMILTON, T.C., HOWLETT, D.R., MOOTOO, S. & WILSON, C. (1989). Inhibition by glibenclamide of the vasorelaxant action of cromakalim in the rat. *Br. J. Pharmacol.*, **97**, 57–64.

CAVERO, I., MODOT, S. & MESTRE, M. (1989). Vasorelaxant effects of cromakalim in rats are mediated by glibenclamide-sensitive potassium channels. *J. Pharmacol. Exp. Ther.*, **248**, 1261–1268.

CHEN, G. & SUZUKI, H. (1989). Some electrical properties of the dependent hyperpolarization recorded from rat arterial smooth muscle cells. *J. Physiol.*, **410**, 91–106.

COLDWELL, M.C. & HOWLETT, D.R. (1988). Potassium efflux enhancement by cromakalim (BRL 34915) in rabbit mesenteric artery: an indirect effect independent of calcium. *Biochem. Pharmacol.*, **37**, 4105–4110.

COOK, D.L. & HALES, C.N. (1984). Intracellular ATP directly blocks K channels in pancreatic cells. *Nature*, **311**, 271–273.

DE MEY, J. & VANHOUTTE, P.M. (1980). Interaction between Na^+, K^+ exchanges and the direct inhibitory effect of acetylcholine on canine femoral arteries. *Circ. Res.*, **46**, 826–836.

FELETOU, M. & VANHOUTTE, P.M. (1988). Endothelium-dependent hyperpolarisation of canine coronary smooth muscle. *Br. J. Pharmacol.*, **93**, 515–524.

FLEMING, W.W. (1980). The electrogenic Na^+, K^+ pump in smooth muscle. Physiologic and pharmacologic significance. *Annu. Rev. Pharmacol. Toxicol.*, **20**, 129–149.

FURCHGOTT, R.F. & ZAWADSKI, J.V. (1980). The obligatory role of endothelial cells in the relaxtion of arterial smooth muscle by acetylcholine. *Nature*, **299**, 373–376.

GELBAND, C.D., LODGE, N.J., TALVENHEIMO, J.A. & VAN BREEMEN, C. (1988). BRL 34915 increases P_{open} of the large conductance Ca^{2+} activated K^+ channel isolated from rabbit aorta in planar lipid bilayers. *Biophys. J.*, **53**, 149a.

HAMILTON, T.C., WEIR, S.W. & WESTON, A.H. (1986). Comparison of the effects of BRL34915 and verapamil on electrical and mechanical activity in rat portal vein. *Br. J. Pharmacol.*, **88**, 103–111.

HU, S., KIM, H.S., OKOLIE, P. & WEISS, G.B. (1990). Alterations by glyburide of effects of BRL 34915 and P1060 on contraction, ^{86}Rb efflux and the maxi-K^+ channel in rat portal vein. *J. Pharmacol. Exp. Ther.*, **253**, 771–777.

HUANG, A.H., BUSSE, R. & BASSENGE, E. (1988). Endothelium-dependent hyperpolarisation of smooth muscle cells in rabbit femoral arteries is not mediated by EDRF (nitric oxide). *Naunyn Schmiedebergs Arch. Pharmacol.*, **338**, 438–442.

KOMORI, K., LORENZ, R.R. & VANHOUTTE, P.M. (1988). Nitric oxide, ACh, and electrical and mechanical properties of canine arterial smooth muscle. *Am. J. Physiol.*, **255**, H207–H212.

LUECKHOFF, A. & BUSSE, R. (1990). Calcium influx into endothelial cells and formation of endothelium-derived relaxing factor is controlled by the membrane potential. *Pflügers Arch.*, **416**, 305–311.

NOMA, A. (1983). ATP-regulated K-channels in cardiac muscle. *Nature*, **305**, 147–148.

PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988). L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251–1256.

QUAST, U. & BAUMLIN, Y. (1988). Comparison of the effluxes of $^{42}K^+$ and $^{86}Rb^+$ elicited by cromakalim (BRL 34915) in tonic and phasic vascular tissue. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **338**, 319–326.

QUAST, U. & BAUMLIN, Y. (1990). Der durch das Endothel vermittelte $^{86}Rb^+$ Ausfluss in der Aorta der Ratte und der Koronararterie des Schwiens wird nicht dirch Glibenclamid inhibiert. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, (suppl) R 63.

QUAST, U. & BRAY, K. (1990). Glibenclamide-insensitivity of endothelium-mediated $^{86}Rb^+$ and $^{42}K^+$ efflux and vasorelaxation in rat aorta and pig coronary artery. *Eur. J. Pharmacol.*, **183**, 1123.

QUAST, U. & COOK, N.S. (1989a). *In vitro* and *in vivo* comparison of two K channel openers, diazoxide and cromakalim, and their inhibition by glibenclamide. *J. Pharmacol. Exp. Ther.*, **250**, 261–271.

QUAST, U. & COOK, N.S. (1989b). Moving together: K^+ channel openers and ATP-sensitive K^+ channels. *Trends Pharmacol. Sci.*, **10**, 431–435.

QUAYLE, J.M., STANDEN, N.B. & STANFIELD, P.R. (1988). The voltage-dependent block of ATP-sensitive potassium channels of frog skeletal muscle by caesium and barium ions. *J. Physiol.*, **405**, 677–697.

RAPOPORT, R.M., SCHWARTZ, K. & MURAD, F. (1985). Effect of Na^+, K^+ pump inhibitors and membrane depolarizing agents on acetylcholine induced endothelium dependent relaxation and cyclic GMP accumulation in rat aorta. *Eur. J. Pharmacol.*, **110**, 203–209.

REES, D.D., PALMER, R.M.J., HODSON, H.F.H. & MONCADA, S. (1989). A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br. J. Pharmacol.*, **96**, 418–424.

SCHMID-ANTOMARCHI, H., DEWEILLE, J., FOSSET, M. & LAZDUNSKI, M. (1987). The receptor for antidiabetic sulfonylureas controls the activity of the ATP-modulated K^+ channel in insulin-secreting cells. *J. Biol. Chem.*, **262**, 15840–15844.

SPRUCE, A.E., STANDEN, N.B. & STANFIELD, P.R. (1985). Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature*, **316**, 736–738.

SPRUCE, A.E., STANDEN, N.B. & STANFIELD, P.R. (1987). Studies on the unitary properties of adenosine-5'-triphosphate-regulated potassium channels of frog skeletal muscle. *J. Physiol.*, **382**, 213–237.

STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators

activate ATP-sensitive K channels in arterial smooth muscle. *Science*, **245**, 177–180.

STURGESS, N.C., ASHFORD, M.L.J., COOK, D.L. & HALES, C.N. (1985). The sulphonylurea receptor may be an ATP-sensitive potassium channel. *Lancet*, **ii**, 474–475.

STURGESS, N.C., KOZLOWSKI, R.Z., CARRINGTON, C.A., HALES, C.N. & ASHFORD, M.L.J. (1988). Effects of sulfonylureas and diazoxide on insulin secretion and nucleotide-sensitive channels in an insulin-secreting cell line. *Br. J. Pharmacol.*, **95**, 83–94.

TARE, M., PARKINGTON, H.C., COLEMAN, H.A., NIELD, T.O. & DUSTING, G.J. (1990). Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. *Nature*, **346**, 69–71.

TAYLOR, S.G., SOUTHERTON, J.S., WESTON, A.H. & BAKER, J.R.J. (1988). Endothelium-dependent effects of acetylcholine in rat aorta: a comparison with sodium nitroprusside and cromakalim. *Br. J. Pharmacol.*, **94**, 853–863.

TRUBE, G. & HESCHELER, J. (1983). Potassium channels in isolated patches of cardiac cell membrane (Abstract). *Naunyn-Schmiedebergs Arch. Pharmacol.*, **322**, R64.

TRUBE, G., RORSMAN, P. & OHNO-SHOSAKU, T. (1986). Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channel in mouse pancreatic β-cells. *Pflügers Arch.*, **407**, 493–499.

ZAWADSKI, J.V., FURCHGOTT, R.F. & CHERRY, P.D. (1983). Endothelium-dependent relaxation of arteries by octa-substance P, kassinin and octacholecystokinin. *Fed. Proc.*, **42**, 619.

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Local application of drugs to sympathetic nerve terminals: an electrophysiological analysis of the role of prejunctional α-adrenoceptors in the guinea-pig vas deferens

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1 Focal extracellular recording techniques were used to study the effects of clonidine, yohimbine and tyramine on the intermittent transmitter release mechanism in the guinea-pig vas deferens *in vitro*. Drugs were applied locally to the varicosities located within the recording electrode. Statistical methods were used to determine whether noradrenaline (NA) acts locally to inhibit secretion from the same or a closely related release site (local regulation) on an impulse-to-impulse basis.

2 The α-adrenoceptor agonist, clonidine, inhibited transmitter release, an effect reversed by the α-adrenoceptor antagonist, yohimbine. Yohimbine alone increased action potential-evoked transmitter release, findings consistent with the idea that transmitter release is regulated through prejunctional α-adrenoceptors.

3 The indirectly acting sympathomimetic, tyramine, powerfully inhibited evoked transmitter release, an effect reversed by both yohimbine and phentolamine. The inhibitory effects of tyramine were greatly reduced in tissues taken from animals pretreated with reserpine. Clonidine powerfully inhibited transmitter release in reserpinized tissues showing that prejunctional α-adrenoceptors were functionally intact. The inhibitory effects of tyramine on transmitter release are therefore mediated indirectly through the release of endogenous NA.

4 Paradoxically, when transmitter release from a small population of variscosities on a single nerve fibre was studied in the absence of α-adrenoceptor antagonists, no evidence was found for local regulation of transmitter release.

5 The intermittent character of the transmitter release process makes it difficult to envisage how impulse-to-impulse regulation could occur. Furthermore, it is unlikely that NA will accumulate to any appreciable extent in the vicinity of the secreting varicosity.

6 The pharmacological evidence clearly supports the view that NA released from sympathetic nerve terminals by nerve impulses modulates subsequent transmitter release. However, the evidence does not support the view that released NA acts locally to inhibit secretion from recently activated varicosities.

Introduction

The idea that transmitter release from postganglionic sympathetic nerve terminals can be modulated through prejunctional α-adrenoceptors is widely accepted (see Starke, 1987) but the exact mechanism of inhibition remains to be established. Studies of the effects of α-adrenoceptor agonists and antagonists on transmitter release from electrically field stimulated tissues *in vitro* provides good evidence for the existence but little information on the anatomical location of prejunctional α-adrenoceptors. In the majority of studies, nerves are field-stimulated 'supramaximally' so that synchronous activation of many release sites occurs. This may well be an artificial situation which tends to exaggerate the importance of α-autoinhibition in regulating transmitter release.

It is usually assumed that transmitter released from one site, presumed to be the varicosity, acts locally to inhibit subsequent release from the same or a closely related site. However, electrophysiological studies at the sympathetic neuroeffector junction have shown that transmitter release from individual varicosities is highly intermittent and monoquantal (Blakeley & Cunnane, 1979; Hirst & Neild, 1980; Cunnane & Stjärne, 1984; Brock & Cunnane, 1987; 1988; Åstrand & Stjärne, 1988), findings which make it highly unlikely that transmitter will accumulate in the vicinity of its site of release. Furthermore, if release from a particular varicosity occurs only once every hundred stimuli, it is difficult to envisage how local regulation of the releasing varicosity could occur.

Studies of α-autoinhibition are further complicated by the recent finding that several transmitters are secreted from sympathetic nerve terminals. Indeed, there is good reason to believe that the transmitter responsible for the excitatory junction potential (e.j.p.) and therefore the excitatory junction current (e.j.c.) in the rodent vas deferens is adenosine 5'-triphosphate (ATP) rather than NA. However, previous studies have shown that the e.j.p. is modulated by drugs in a manner consistent with the α-autoregulation hypothesis (Blakeley *et al.*, 1981; Illes & Starke, 1983; Brock *et al.*, 1990).

Recently a focal extracellular recording technique has been developed which can monitor transmitter release from a small population of varicosities located on a single sympathetic axon on an impulse-to-impulse basis (Brock & Cunnane, 1987; 1988). This approach has been used to study the effects on transmitter release of the local application of the α-adrenoceptor agonist, clonidine, the α-adrenoceptor antagonist, yohimbine and the indirectly acting sympathomimetic amine, tyramine. Statistical techniques were also used to determine whether local regulation of transmitter release occurs when α-adrenoceptor antagonists are not present in the physiological saline.

Methods

Extracellular recording

Male guinea-pigs (Duncan Hartley, 300–400 g) were killed by a blow to the head and bled. Vasa deferentia were removed and individual preparations pinned to the Sylgard (Dow Corning) covered base of a 4 ml Perspex organ bath, which was mounted on the stage of a Zeiss ACM microscope. The organ bath was perfused continuously at 1–3 ml min^{-1} with Krebs solution of the following composition (mM): NaCl 118.4, $NaHCO_3$ 25.0, NaH_2PO_4 1.13, $CaCl_2$ 1.8, KCl 4.7, $MgCl_2$ 1.3 and glucose 11.0. The solution was gassed with a

mixture of 95% O_2 and 5% CO_2 (to pH 7.4) and maintained at 36–37°C. Postganglionic sympathetic nerve fibres were excited by electrical stimulation of the hypogastric nerve trunk close to the prostatic end of the vas deferens. The stimulus parameters were varied as required throughout the experiment (pulse width 0.01–0.5 ms, 7–30 V).

A window was cut in the connective tissue sheath enveloping the vas deferens, and a beveled glass electrode (tip diameter ~50 μm) filled with Krebs solution applied to the muscle surface with slight suction (~20–30 mmHg, seal resistance <1 MΩ). A sintered Ag/AgCl pellet in the organ bath served as the indifferent electrode. Electrical activity was recorded through an a.c. amplifier (Neurolog NL104, low frequency cut-off 0.1 Hz) and the output fed through a low pass filter (Neurolog NL125) with the cut-off set at 3 kHz. Drugs were applied by internal perfusion of the suction electrode (25–50 μl min^{-1}).

Reserpine pretreatment

Guinea-pigs received a single dose of 5 mg kg^{-1} reserpine (i.p.) 18 h before the experiment, a procedure which depleted the NA content of the vas deferens below levels detectable by high performance liquid chromatography.

Data collection and analysis

A system based on a Zenith 386 microcomputer and a Data Translation DT2801A analogue to digital converter card was used to digitize and analyse electrophysiological signals previously recorded on magnetic tape (see Brock & Cunnane, 1988). The signal detection level was set at about twice the noise level of the recording system which was usually about 10 μV peak-to-peak.

The stochastic features of electrically evoked transmitter release were analysed by the correlogram method (see Rotshenker & Rahamimoff, 1970). A correlogram gives an estimate of the probability of occurrence of an e.j.c. at any time after a given e.j.c. A correlogram is constructed by calculating the number of e.j.c.s (fx) occurring 1, 2, 3, etc. pulses following each e.j.c. recorded during a train of stimuli. The expected number of e.j.cs in each interval class (f) was determined by averaging the number of e.j.cs (fx) occurring 16 to 25 pulses (1 Hz) or 91 to 100 pulses (4 Hz) following each e.j.c., during which period it was assumed that e.j.cs occurred independently. The different periods of sampling to determine fx were chosen to ensure that comparative periods of time after the occurrence of an e.j.c. were analysed. The probability of e.j.c.s occurring 1, 2, 3 etc. pulses following an e.j.c. relative to that expected if e.j.c.s occurred independently of one another was determined by dividing fx by f. If e.j.cs occur independently (i.e. randomly) then the relative probability of occurrence is one.

Drugs

Clonidine hydrochloride (Sigma), phentolamine mesylate (Ciba), reserpine (Sigma), tyramine hydrochloride (Sigma) and yohimbine hydrochloride (Sigma). Reserpine was made up in 20% (w/v) ascorbic acid. The remaining drugs were made up as concentrated stock solutions in distilled water and serially diluted in Krebs solution to the required final concentration.

Results

Extracellular recording

A detailed description of the pattern of electrical activity recorded by use of the focal extracellular recording method has been described elsewhere (Brock & Cunnane, 1988). For the purpose of the present inquiry it is useful to state briefly the key features of the transmitter release mechanism. When a small suction electrode is applied to the surface of the vas deferens, in the absence of stimulation, spontaneous excitatory junction currents (s.e.j.cs) are recorded. Following electrical field stimulation of the hypogastric nerve trunk with trains of pulses at 0.1–4 Hz, stimulus-locked excitatory junction currents (e.j.c.) are evoked intermittently, the amplitude and time course of which are similar to those of spontaneous excitatory junction currents (s.e.j.cs). Evoked events are always preceded by a non-intermittent nerve impulse. For a number of reasons we believe that the nerve impulse preceding the e.j.c. is the extracellular equivalent of the nerve terminal action potential (Brock & Cunnane, 1988). Intermittence therefore results from a low probability of transmitter release in the invaded varicosity. Examples of nerve impulses and e.j.cs are shown in Figure 1. Negative-going e.j.cs reflect the quantal release of transmitter from varicosities located under the recording electrode. Positive-going activity reflects the depolarization of smooth muscle cells outside the recording electrode (see Brock & Cunnane, 1988).

Local application of clonidine and yohimbine

Clonidine (0.1 μM) applied by internal perfusion of the focal recording electrode (6 experiments) markedly reduced the number of e.j.cs recorded within 5-10 min of application (Figure 2a,b). Inhibition of transmitter release by clonidine occurred without any apparent alteration in the nerve terminal impulse (Figure 1a) and was readily reversed by wash. Clonidine did not significantly alter the size or the frequency of occurrence of s.e.j.cs. Yohimbine (0.1–1 μM) applied by the

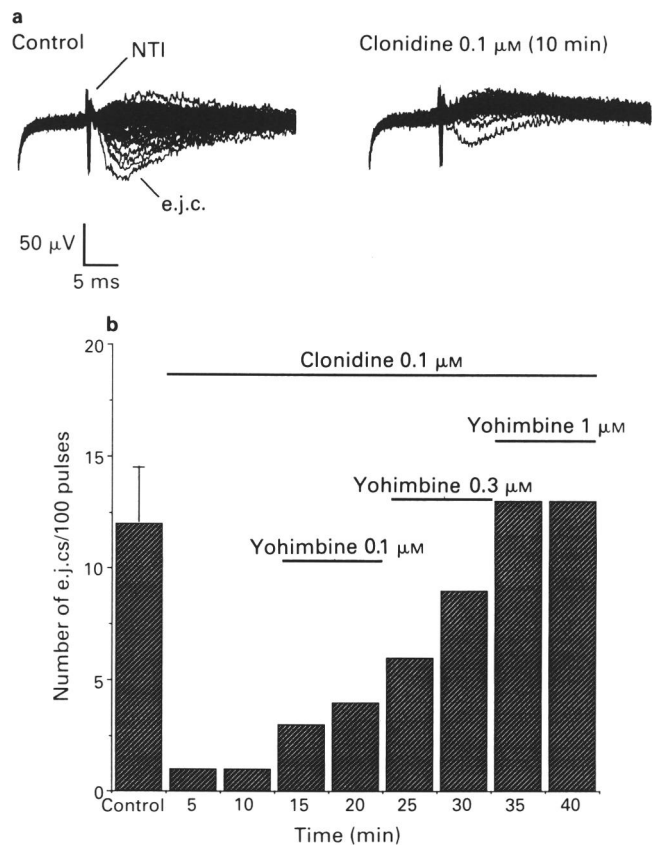

Figure 1 Effects of local application of clonidine on evoked electrical activity in the guinea-pig vas deferens. (a) Nerve impulses and excitatory junction currents (e.j.cs) evoked by trains of 100 stimuli at 1 Hz before and 10 min after the addition of clonidine (0.1 μM). Clonidine inhibited the occurrence of e.j.cs without altering the nerve terminal impulse (NTI). (b) Histogram showing the number of e.j.cs recorded during trains of 100 pulses at 1 Hz delivered at 5 min intervals before, and during the addition of clonidine (0.1 μM) and yohimbine (0.1–1 μM). The control value is the mean number of e.j.cs recorded during 5 periods of stimulation (vertical bar shows s.e.mean). The inhibitory effect of clonidine was reversed by yohimbine.

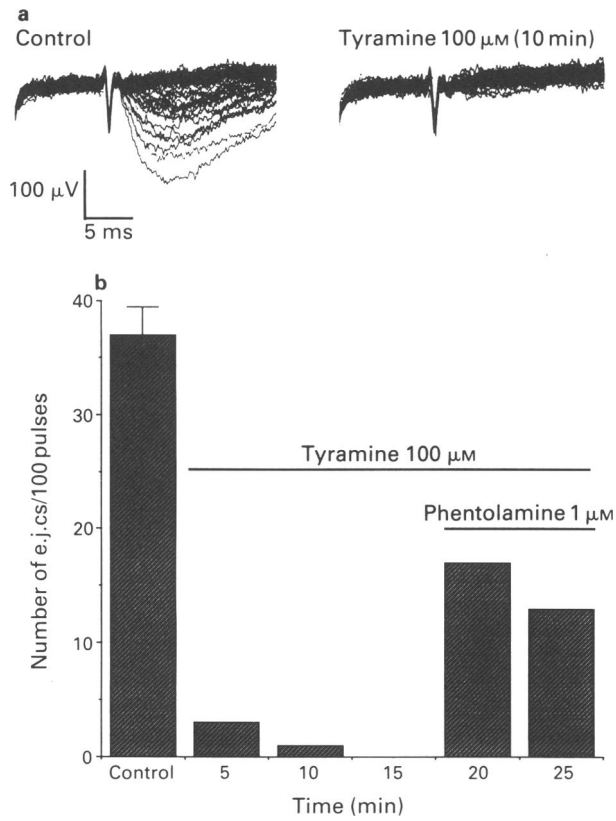

Figure 2 Inhibition of transmitter release by locally applied tyramine in the guinea-pig vas deferens. (a) Nerve impulses and excitatory junction currents (e.j.cs) evoked by trains of 50 stimuli at 2 Hz before and 10 min after the addition of tyramine (100 μM). Tyramine inhibited transmitter release without altering the nerve terminal impulse. (b) Histogram showing the number of e.j.cs recorded during trains of 100 pulses at 2 Hz delivered at 5 min intervals before, and during the addition of tyramine (100 μM) and phentolamine (1 μM). The control value is the mean number of e.j.cs recorded during 5 periods of stimulation (vertical bar shows s.e.mean). The inhibitory effect of tyramine was reversed by phentolamine.

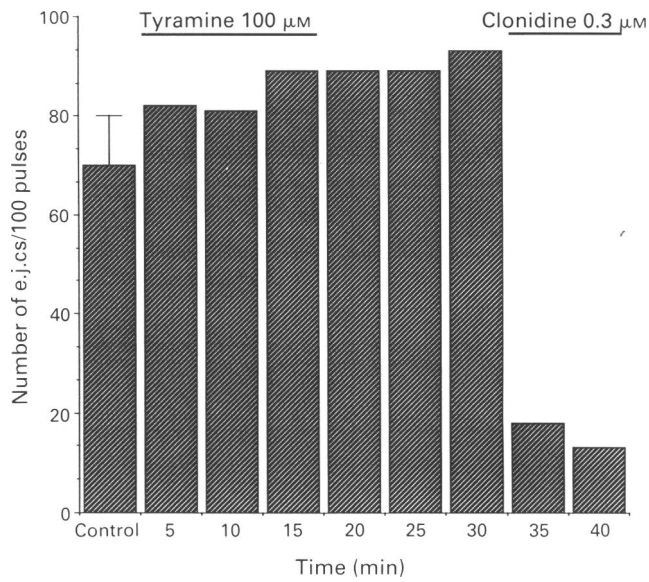

Figure 3 Lack of effect of tyramine (100 μM) on electrically evoked transmitter release in reserpinized guinea-pig vas deferens (5 mg kg^{-1} i.p., 18 h earlier). The histogram shows the number of excitatory junction currents (e.j.cs) recorded during trains of 100 pulses at 1 Hz delivered at 5 min intervals before, and during the addition of both tyramine and clonidine. The control value is the mean number of e.j.cs recorded during 4 periods of stimulation (vertical bar shows s.e.mean). Note that although tyramine was without effect in the reserpinized tissue, clonidine (0.3 μM) powerfully inhibited transmitter release.

same route (4 experiments) reversed the inhibitory effects of clonidine (Figure 1b). The effects of yohimbine alone, when applied by internal perfusion of the suction electrode, were also examined. At a concentration of 0.1 μM, which previously had been shown to antagonize the effects of clonidine (Figure 1b), yohimbine failed to cause a consistent increase in the size or number of e.j.cs (7 experiments). We have subsequently found, however, that at higher concentrations yohimbine increases the frequency of occurrence of e.j.cs during trains of stimuli, and this effect is enhanced by desipramine which prevents the released NA from being taken up into sympathetic nerve terminals (Brock *et al.*, 1990).

Effects of locally applied tyramine

It was interesting to determine whether endogenous NA released locally by the indirectly acting sympathomimetic amine, tyramine, could activate prejunctional α-adrenoceptors and thus inhibit quantal transmitter release. In 8 experiments tyramine (10–100 μM) caused a marked inhibition of release without altering the nerve terminal impulse (Figure 2a,b). The inhibitory effect of tyramine was reversed by introducing yohimbine (1 μM) or phentolamine (1 μM) into the recording suction electrode, suggesting that the inhibition was mediated through prejunctional α-adrenoceptors. It is possible that tyramine was directly stimulating prejunctional α-adrenoceptors at the concentrations used (10–100 μM). However, in reserpinized tissues (4 experiments) the inhibitory actions of tyramine (100 μM) on transmitter release were absent or markedly reduced, whereas the local application of clonidine (0.1–0.3 μM) still markedly inhibited transmitter release (Figure 3). These observations demonstrate that tyramine inhibits electrically evoked transmitter release by releasing endogenous NA from sympathetic nerve terminals.

Does transmitter release inhibit subsequent evoked release?

Previous studies have clearly shown that exogenously applied drugs can modify the transmitter release process but have failed to demonstrate that the endogenous transmitter can regulate its own release locally in the absence of prejunctional α-adrenoceptor antagonists (Blakeley *et al.*, 1982). Using the focal extracellular recording technique, we have tried to establish whether the occurrence of an e.j.c. alters the probability of observing subsequent e.j.cs by analysing the stochastic features of the transmitter release process (see Methods). Correlograms were constructed for trains of 500 pulses delivered at various frequencies (0.5–4 Hz) in five separate experiments. In no case was there any evidence for a decreased probability of occurrence of evoked transmitter release immediately following an e.j.c. Indeed, the occurrence of an e.j.c. had no obvious effect on the probability of observing subsequent e.j.cs, suggesting that transmitter release events occur independently of one another. Examples of two correlograms constructed from data obtained in a single attachment are shown in Figure 4. It is clear that the relative probability that an e.j.c. would occur in the next 20 pulses following an e.j.c., during trains of 500 stimuli delivered at 1 and 4 Hz, is unchanged. If α-autoinhibition were operating locally to any appreciable extent, then one would expect previous transmitter release to inhibit subsequent release in the field of the recording electrode.

Discussion

The results of the present inquiry have a bearing on the mechanisms involved in α-autoinhibition. While the pharmacological evidence clearly supports the hypothesis that trans-

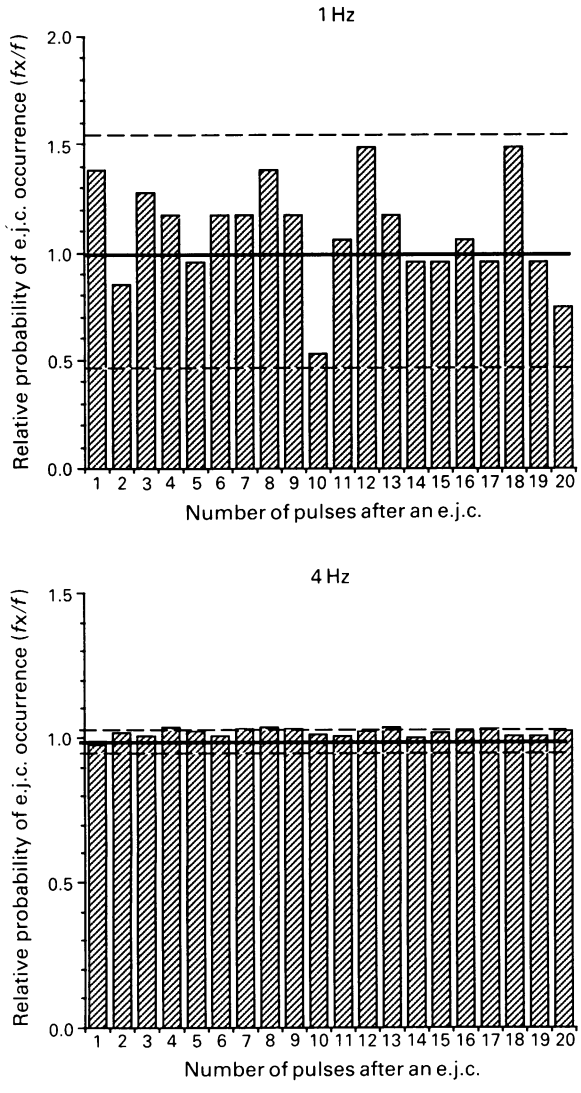

Figure 4 Correlograms of excitatory junction current (e.j.c.) interval data for trains of 500 stimuli at 1 and 4 Hz in a single attachment in the guinea-pig vas deferens. The continuous line is the curve predicted if e.j.cs occur independently and the broken lines represent the 95% confidence limits of this prediction. The occurrence of an e.j.c. does not inhibit the occurrence of subsequent e.j.cs on an impulse-to-impulse basis.

mitter release is regulated by the activation of prejunctional α-adrenoceptors, surprisingly little is known about the precise mechanisms involved in the regulation of release at the level of the individual varicosity. To clarify further these mechanisms the secretory activity of a small group of varicosities located on a single sympathetic axon has been studied, in the absence and presence of α-adrenoceptor agonists and antagonists.

The effects of clonidine and yohimbine on the e.j.c. are similar to those previously reported both for NA release and for the e.j.p. (see Starke, 1987). Clonidine powerfully inhibited electrically evoked transmitter release, an effect reversed by yohimbine. Moreover, yohimbine alone potentiated transmitter release (Brock *et al.*, 1990). These findings are in accord with the hypothesis that NA inhibits transmitter release by activating prejunctional α-adrenoceptors. It should be stressed that α-autoinhibition is not the major cause of intermittent transmitter release, since α-adrenoceptor antagonists only increase release by about one to two fold, i.e. on average from one, to two or three secretory events per hundred stimuli per varicosity (Brock *et al.*, 1990).

The e.j.p. (and therefore the e.j.c.) in many sympathetically innervated tissues including the guinea-pig vas deferens is probably mediated by ATP or a related purine nucleotide rather than by NA (see White, 1988). E.j.ps can be recorded in tissues taken from animals pretreated with reserpine to deplete NA stores (Burnstock & Holman, 1961; Sneddon & Westfall, 1984) and when NA synthesis was also inhibited with α-methyl *p*-tyrosine (Cunnane & Manchanda, 1989). Much evidence suggests that NA and ATP are co-released from the same sympathetic nerve terminal and therefore it is reasonable to assume that the e.j.c. is an indirect measure of the quantal release of NA. It should be noted that Ellis & Burnstock (1989) have recently argued on the basis of overflow studies that NA and ATP may be stored and/or released separately. Nevertheless, the present pharmacological observations support the view that ATP release is modified by exogenous α_2-adrenoceptor agonists and endogenous NA (see below). In the mouse vas deferens and in vascular smooth muscle, the e.j.p. is similarly modified by α_2-adrenoceptor agonists and antagonists (Blakeley *et al.*, 1981; Illes & Starke, 1983; Ramme *et al.*, 1987).

In a previous investigation no evidence was found for local regulation of transmitter release in the absence of drugs when release from a small population of varicosities was studied (Blakeley *et al.*, 1982). Similarly, the occurrence of an e.j.c. had no apparent inhibitory effect on the occurrence of subsequent e.j.cs. Although not rigorously investigated, there was some evidence to suggest that pairs of e.j.cs, with a similar amplitude and time course, were evoked within a few pulses of one another during trains of stimuli even though release was intermittent (see Brock & Cunnane, 1988). Similar observations have been made for discrete events in this tissue (Cunnane & Stjärne, 1984). Discrete events and e.j.cs of similar amplitude and time course probably represent transmitter release from the same or a closely related group of transmitter release sites. Therefore, following transmitter release there appears to be, if anything, a transient increase in the probability of subsequent transmitter release from the same site rather than the expected inhibition. We have not yet investigated whether β-adrenoceptors are involved in this apparent enhancement of transmitter release. Thus when a focal extracellular electrode is used to monitor transmitter release on an impulse-to-impulse basis from a few release sites no evidence for local α-autoinhibition can be found.

Further evidence against the idea of local regulation of release is provided by the intermittent nature of the transmitter release mechanism. It is difficult to envisage how appreciable concentrations of transmitter could accumulate locally if release from a particular varicosity only occurs once or twice per 100 stimuli. It might seem surprising that a transient high concentration of transmitter at the releasing varicosity would fail to activate the local prejunctional receptors. One explanation might be that the interval between successive releases from the same varicosity is sufficiently long to allow the transmitter release mechanism to recover from any transient inhibitory effects produced by the released transmitter. Further, intermittence makes it difficult to envisage how local regulation could occur on an impulse-to-impulse basis if there are long intervals between successive secretory events. There can be no doubt however that α-adrenoceptor antagonists increase electrically evoked transmitter release during short trains of low frequency stimuli (Story *et al.*, 1981; Blakeley *et al.*, 1981; Illes & Starke, 1983; Brock *et al.*, 1990).

We are therefore left with the following observations: (1) exogenous agonists readily inhibit transmitter release locally, (2) antagonists reveal inhibition of transmitter release by endogenous NA and (3) the inhibition escapes detection when release is recorded on an impulse-to-impulse basis in the absence of drugs. It was therefore of some interest to determine whether the endogenous NA released from sympathetic nerve terminals by locally applied tyramine could activate prejunctional α-adrenoceptors and thus inhibit transmitter release. Ambache *et al.* (1972) were the first to demonstrate the inhibitory effects of tyramine on electrically evoked contractions of the guinea-pig vas deferens. In the present experiments it has clearly been demonstrated that locally applied

tyramine readily inhibits electrically evoked transmitter release, indirectly, by releasing endogenous NA. The inhibitory actions of tyramine were greatly attenuated in tissues taken from reserpinized animals but clonidine still powerfully inhibited transmitter release showing that reserpine had not interfered with prejunctional α-adrenoceptor transduction mechanisms. Interestingly, in reserpinized tissues, yohimbine did not potentiate the amplitude of the e.j.ps evoked by trains of stimuli presumably because α-autoinhibition was no longer operative (Brock *et al.*, 1990). Thus endogenous NA released from nerves by tyramine can inhibit transmitter release locally but action potential evoked NA release apparently cannot.

Some further explanation is required for the paradoxical inability of NA released by nerve impulses to inhibit transmitter release locally. First, the neurotransmitter may act at sites remote from its own site of release. It is important to remember that the question addressed by the statistical approach was – does previous release inhibit subsequent release from the same or closely related release sites? It does not address the question whether the absolute probability of release at the release sites under investigation is determined by the influence of NA spreading from neighbouring varicosities (whose activity is not detected by the recording electrode). Thus, one can account for the effects of locally applied yohimbine by assuming that it removes the lateral inhibition produced by the spread of NA from distant release sites. The idea of lateral inhibition of transmitter release fits well with the intermittent nature of the transmitter release process, since the biophase concentration of NA 'seen' by the prejunctional α-adrenoceptors will depend on the amount of NA diffusing from the sites of transmitter release and not on the concentration in the junctional cleft. Second, prejunctional α-adrenoceptors may have an anatomical location at some point remote from the sites of transmitter release, for example at some point upstream or downstream from the site of release (Stjärne, 1989). However, the local application of clonidine and the local release of NA by focally applied tyramine both powerfully inhibited transmitter release, suggesting that α-adrenoceptors are located close to the sites of transmitter release and certainly within the recording electrode ($\sim 50\,\mu m$ diameter). Third, NA and ATP may not be co-released. If one abandons the assumption that NA and ATP are co-released from the same nerve terminal then these electrophysiological studies, which measure ATP release, will give no indication as to the prejunctional site(s) of action of NA. What these results clearly demonstrate is that ATP (or a related breakdown product) does not regulate its own release locally. The present and previous results do, however, clearly demonstrate that the release of ATP is regulated by endogenous NA.

A working hypothesis which encompasses most observations can be briefly stated: when a particular varicosity releases a packet of transmitter, the probability of subsequent release from that varicosity may be enhanced and this effect overrides local α-autoinhibition. The released NA spreading to varicosities not yet activated stimulates prejunctional α-adrenoceptors decreasing the probability that these varicosities will release transmitter when invaded by a nerve action potential. Thus the concept of lateral inhibition rather than local inhibition of transmitter release emerges, one function being to limit the overall magnitude of transmitter release from the entire nerve terminal network. (Sjärne, 1981; 1989; Starke, 1987). It may well be that the terminal network of the sympathetic nerve fibres has a complex three dimensional branching anatomical arrangement the function of which is not yet fully appreciated.

In conclusion, postganglionic sympathetic nerve terminals are endowed with α-adrenoceptors which limit transmitter release during short trains of stimuli. Local regulation of transmitter release by previously released NA was not detected. It would seem that α-autoinhibition operates at a point remote from the immediate site of transmitter release (lateral inhibition), presumably by reducing the probability that varicosities will release transmitter when invaded by the nerve action potential. However, the interpretation of some of these observations would have to be reviewed if it can be demonstrated that NA and ATP are released independently of one another and this is a question for future study.

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References

AMBACHE, N., DUNK, L.P., VERNEY, J. & ZAR, M.A. (1972). Inhibition of post-ganglionic motor transmission in the vas deferens by indirectly acting sympathomimetic drugs. *J. Physiol.*, **227**, 433–456.

ÅSTRAND, P. & STJÄRNE, L. (1988). On the secretory activity of single varicosities in the sympathetic nerves innervating the rat tail artery. *J. Physiol.*, **409**, 207–220.

BLAKELEY, A.G.H. & CUNNANE, T.C. (1979). The packeted release of transmitter from the sympathetic nerves of the guinea-pig vas deferens: an electrophysiological study. *J. Physiol.*, **296**, 85–96.

BLAKELEY, A.G.H., CUNNANE, T.C. & PETERSEN, S.A. (1981). An electropharmacological analysis of the effects of some drugs on neuromuscular transmission in the vas deferens of the guinea-pig. *J. Autonom. Pharmacol.*, **1**, 367–375.

BLAKELEY, A.G.H., CUNNANE, T.C. & PETERSEN, S.A. (1982). Local regulation of transmitter release from rodent sympathetic nerve terminals? *J. Physiol.*, **325**, 93–109.

BROCK, J.A. & CUNNANE, T.C. (1987). Relationship between the nerve action potential and transmitter release from sympathetic postganglionic nerve terminals. *Nature*, **326**, 605–607.

BROCK, J.A. & CUNNANE, T.C. (1988). Electrical activity at the sympathetic neuroeffector junction in the guinea-pig vas deferens. *J. Physiol.*, **399**, 607–632.

BROCK, J.A., CUNNANE, T.C., STARKE, K. & WARDELL, C. (1990). α_2-Adrenoceptor-mediated autoinhibition of sympathetic transmitter release in guinea-pig vas deferens studied by intracellular and focal extracellular recording of junction potentials and currents. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **342**, 45–52.

BURNSTOCK, G. & HOLMAN, M.E. (1982). Effect of denervation and of reserpine treatment on transmission at sympathetic nerve endings. *J. Physiol.*, **160**, 461–496.

CUNNANE, T.C. & MANCHANDA, R. (1989). Effects of reserpine pretreatment on neuroeffector transmission in the vas deferens. *Clin. Exp. Pharmacol. Physiol.*, **16**, 351.

CUNNANE, T.C. & STJÄRNE, L. (1984). Transmitter secretion from individual varicosities of guinea-pig and mouse vas deferens: highly intermittent and monoquantal. *Neuroscience*, **13**, 1–20.

ELLIS, J.L. & BURNSTOCK, G. (1989). Angiotensin neuromodulation of adrenergic and purinergic co-transmission in the guinea-pig vas deferens. *Br. J. Pharmacol.*, **97**, 1157–1164.

HIRST, G.D.S. & NEILD, T.O. (1980). Some properties of spontaneous excitatory junction potentials recorded from arterioles of guinea-pigs. *J. Physiol.*, **303**, 43–60.

ILLES, P. & STARKE, K. (1983). An electrophysiological study of presynaptic α-adrenoceptor in the vas deferens of the mouse. *Br. J. Pharmacol.*, **78**, 365–373.

RAMME, D., REGENOLD, J.T., STARKE, K., BUSSE, R. & ILLES, P. (1987). Identification of the neuroeffector transmitter in jejunal branches of the rabbit mesenteric artery. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **336**, 267–273.

ROTSHENKER, S. & RAHAMIMOFF, R. (1970). Neuromuscular synapse: stochastic properties of spontaneous release of transmitter. *Science*, **170**, 648–649.

SNEDDON, P. & WESTFALL, D.P. (1984). Pharmacological evidence that adenosine triphosphate and noradrenaline are co-transmitters in the guinea-pig vas deferens. *J. Physiol.*, **347**, 561–580.

STARKE, K. (1987). Presynaptic α-adrenoceptors. *Rev. Physiol. Biochem. Pharmacol.*, **107**, 73–146.

STJÄRNE, L. (1981). On sites and mechanisms of presynaptic control of noradrenaline secretion. In *Chemical Neurotransmission 75 Years*. ed. Stjärne, L., Hedqvist, P., Lagercrantz & Wennmalm, Å. pp. 257–272. London, New York, Sydney, San Francisco: Academic Press.

STJÄRNE, L. (1989). Basic mechanisms and local modulation of nerve impulse-induced secretion of neurotransmitters from individual sympathetic nerve varicosities. *Rev. Physiol. Biochem. Pharmacol.*, **12**, 1–137.

STORY, D.F., McCULLOCH, M.W., RAND, M.J. & STANDFORD-STARR, C.A. (1981). Conditions required for the inhibitory feedback loop in noradrenergic transmission. *Nature*, **293**, 62–65.

WHITE, T.D. (1988). Role of adenine compounds in autonomic neurotransmission. *Pharmacol. Ther.*, **38**, 129–168.

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Beneficial effects of N-acetylcysteine and cysteine in stunned myocardium in perfused rat heart

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1 The objective of this study was to evaluate the effects of three sulphydryl (SH) compounds, N-acetylcysteine (NAC), cysteine (Cys) and cystamine, on functional recovery and ventricular arrhythmias (VF) in stunned myocardium in the isolated perfused heart of the rat.

2 Hearts ($n = 7$–8 per group) were perfused by the Langendorff procedure for 20 min to stabilize and then assigned to one of five groups: saline, sham, NAC, Cys and cystamine. After the stabilizing period, the drugs (at 3.6 μM min^{-1}) or their vehicle (saline) were infused into coronary vessels throughout the experimental period. Ten min after administration of drugs, the left anterior descending coronary artery (LAD) was ligatured for 20 min and then untied to reperfuse for 30 min. In the sham group, a ligature was placed around the LAD but not tied.

3 NAC and Cys had a significant effect in attenuating myocardial stunning: the percentage recovery of rate-pressure product measured 30 min after reperfusion as an index of heart function, was improved with the NAC (98.3 ± 4.5) and Cys groups (104.0 ± 6.5) compared with the saline (only 73.6 ± 3.8, $P < 0.01$) group. Cystamine did not show these beneficial effects. This may be due to the difference in chemical structure between NAC, Cys and cystamine since the latter does not have a free SH group with a disulphide bond formed. This phenomenon suggests that a free SH group is essential for the protective effects of compounds like NAC and Cys in myocardial injury.

4 NAC and Cys prevented the fall in coronary flow during the LAD occlusion and enhanced coronary flow during reperfusion but cystamine did not have such a beneficial effect.

5 The incidence of VF in the saline, cystamine, Cys and NAC groups was 6/8 (75.0%), 4/7 (57.1%), 3/8 (37.5%) and 2/7 (28.6%), respectively, and no significant differences ($P > 0.05$) were noted between the saline- and drug-treated groups.

6 An *in vitro* study with electron spin resonance indicated that Cys effectively scavenged the hydroxyl radical (·OH) generated by Fenton's reaction but did not scavenge superoxide generated in an irradiated riboflavin system. NAC and cystamine showed a scavenging effect on ·OH to a certain extent but this effect did not reach statistical significance ($P > 0.05$ vs saline).

7 Our results demonstrate that NAC and Cys treatment before ischaemia and reperfusion can reduce myocardial stunning. This beneficial effect may be mainly due to their ability to preserve and enhance coronary flow during coronary occlusion and reperfusion and in part due to scavenging ·OH and/or replenishing intracellular glutathione. The results also indicate that the condition of coronary perfusion can produce a great impact on postischaemic ventricular performance.

Introduction

Re-establishment of the blood flow to ischaemic myocardium is essential for tissue survival and functional recovery. However, many studies have demonstrated that reperfusion also brings about some deleterious effects, i.e., reperfusion injury (Braunwald & Kloner, 1985; Becker & Ambrosio, 1987; Opie, 1989). Recent evidence suggests that postischaemic myocardial dysfunction (stunning) may represent a functional or nonlethal form of reperfusion injury (Braunwald & Kloner, 1982; Kloner *et al.*, 1989) and oxygen free radicals may contribute to the pathogenesis of myocardial stunning (Gross *et al.*, 1986; Bolli *et al.*, 1987; 1989; Forman *et al.*, 1988; Faber *et al.*, 1988). Bolli and his colleagues (1988) using electron paramagnetic resonance spectroscopy detected a burst of free radical formation within the coronary venous effluent in dogs subjected to 15 min of coronary occlusion and reperfusion; other studies found that production of free radicals increased markedly in isolated hearts of rabbit or rat undergoing global ischaemia and reperfusion (Zweier *et al.*, 1987; Garlick *et al.*, 1987; Kramer *et al.*, 1987) and pretreatment with the oxygen radical-scavenging agents superoxide dismutase and catalase reduced the degree of left ventricular dysfunction in canine models of stunned myocardium (Gross *et al.*, 1986; Ambrosio *et al.*, 1987; Przyklenk & Kloner, 1989). Nevertheless, recent studies also indicate that other factors independent of free radicals are involved in the pathogenesis of myocardial stunning such as: calcium overload (Kusuoka *et al.*, 1987; Lee *et al.*, 1987; Steenbergen *et al.*, 1990; Sun & Lin, 1990), heterogeneous impairment of myocardial perfusion (Stahl *et al.*, 1986) and neutrophil accumulation in the postischaemic myocardium (Westlin & Mullane, 1989) and so on.

NAC and Cys are sulphydryl (SH) compounds which have been used recently as antioxidant agents in myocardial ischaemia and reperfusion and have shown some beneficial effects (Forman *et al.*, 1988; Ceconi *et al.*, 1988; Tang & Tang, 1991). Previously, SH compounds have been shown to have anti-inflammatory properties and can protect against irradiation and drug-induced disease states in which free radicals have been implicated as mediators of tissue injury (Suguhara *et al.*, 1977; Doroshow *et al.*, 1980; Forman *et al.*, 1983; Betts *et al.*, 1984) through such effects as: reacting directly with free radicals, promoting the resynthesis of glutathione (GSH) or

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acting as an alternative substrate for glutathione peroxidase. It has also been shown that NAC potentiates both the peripheral and coronary vasodilator effects of nitroglycerin (Horowitz et al., 1983; Winniford et al., 1986). The latter findings suggest that NAC may work through mechanisms other than as an oxidant scavenger to benefit the ischaemic myocardium.

The purpose of the present study is to examine whether the SH compounds: NAC, Cys and cystamine have therapeutic actions in a rat heart model of brief ischaemia. If these compounds have these actions, how do they produce the effects, as free radical scavengers or via other actions? In order to understand better the mechanisms by which the drugs in this study might be capable of acting as oxidant scavengers *in vivo*, we used the electron spin resonance (ESR) method to evaluate the scavenging effects of NAC, Cys and cystamine on superoxide ($\cdot\bar{O}_2$) and hydroxyl radicals ($\cdot$OH) *in vitro*. Furthermore, since cystamine does not have a free SH group for a formed disulphide link, we could also determine whether the free SH group is necessary for the prevention of ischaemia and reperfusion injury.

Methods

Heart perfusion

Wistar rats of either sex weighing between 250–350 g were given 1000 iu kg^{-1} of heparin intraperitoneally (i.p.) 15 min prior to the induction of anaesthesia with sodium pentobarbitone (40 mg kg^{-1}, i.p.). Following a thoracotomy, the hearts were rapidly excised and placed in ice-cold oxygenated (95% O_2:5% CO_2) Krebs-Henseleit (K-H) solution which contained (in mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.1, $CaCl_2 \cdot 2H_2O$ 2.5, $NaHCO_3$ 25 and glucose 11. Within 30 s, the hearts were perfused retrogradely via the aorta with oxygenated K-H solution according to the Langendorff technique as described earlier (Sun & Lin, 1990) and by Roth et al. (1985). The ventricular pressure was monitored by placing a plastic catheter with a small perforated ball tip into the left ventricle via the mitral valve. The catheter was filled with perfusate and connected to a Statham P50 pressure transducer. Coronary perfusion pressure (CPP) was measured in a side branch of the inflow line with a Statham P50 pressure transducer. Left ventricular pressure, left ventricular *dp/dt* and (−) *dp/dt* and CPP were recorded on a polygraph recorder (RM-46, Nihon Kohden). The rate-pressure product (RPP) was determined by calculating heart rate times left ventricular developed pressure (LVDP, systolic minus diastolic pressure). The coronary flow rate (CFR) was measured from collection of the coronary effluent every minute.

Protocol

At all times, the hearts were maintained at 37°C and perfused with normal K-H buffer at a constant pressure of 60 mmHg. After a 25 min equilibration period, hearts were assigned to one of five groups: NAC, Cys, cystamine, saline and sham. The baseline recordings were taken at the end of the equilibration period (designated time 0) and ischaemia was produced by tying a previously placed reversible ligature around the left anterior descending coronary artery (LAD) at a point close to its origin. Infusion of NAC, Cys and cystamine (each at 18 mM) or their vehicle (0.9% NaCl), via a peristalic pump (model HL-2, Shanghai) into the aortic inflow cannula at a rate of 0.2 ml min^{-1}, corresponding to a final concentration of 3.6 μM min^{-1}, was started 10 min before coronary artery occlusion and continued for the duration of the experiment. The concentrations of drugs chosen were based on data obtained in our previous experiments in which NAC and Cys at the above dose showed a significant effect in reducing reperfusion-induced arrhythmias in the transient ischaemic model of rat heart (Tang & Tang, 1991) and also is comparable with the concentration used by Ceconi et al. (1988). In a sham group, all procedures were the same as the other groups except that the ligature around the LAD was not tied and no drug was infused.

Assays of scavenging effects of the drugs

The ESR spin trapping methods were used to assess the ability of the drugs to react with $\cdot\bar{O}_2$ and $\cdot$OH which were produced by the following methods. The assays for each drug were performed at least three times.

The irradiated riboflavin reaction: the reaction solution contained: 3×10^{-4} M riboflavin, 0.1 M 5,5-dimethyl-1-pyrroline N-oxide (DMPO, Sigma Chemical, St. Louis, Missouri, U.S.A.), 5×10^{3} M ethylenedinitrolotetraacetic acid (EDTA), 3.6×10^{-4} M of the drug dissolved in saline (these concentrations of drugs were equivalent to the concentrations of drugs which were infused into the coronary vessels in this experiment) and 0.05 M Na_2HPO_4–NaH_2PO_4 buffer at pH 5.0; this solution was mixed evenly and then a sample from the mixture was placed in an optical cavity (ER 4104 OR), irradiated with a Xenon lamp (150 W, distance: 30 cm) for 10 s and immediately thereafter ESR spectra were recorded with the ESR spectrograph (Bruke ESP-300, F.R.G.) under the following conditions: microwave power: 10 mW, modulation frequency and amplitude: 100 kHz and 0.2 G, time constant: 20 ms, at 25°C.

Fenton's reaction: the reaction mixture contained: 1.25% H_2O_2, 2×10^{-4} M $(NH_4)_2FeSO_4$, 0.1 M DMPO, 3.6×10^{-4} M drug dissolved in saline, 0.05 M Na_2HPO_4-NaH_2PO_4 buffer at pH 7.4; this was shaken quickly for 4 min. A sample from the mixture was put into a TE102 cavity (ER 4102 ST) and then ESR spectra were recorded as above, except that modulation frequency and amplitude were 25 kHz and 1.0 G, respectively.

Statistics

The results are presented as mean ± s.e.mean. Differences between experimental groups were analysed by one-way analysis of variance and when this test indicated statistical significance, the results from different groups were analysed by Student's nonpaired *t* test for multiple comparison to the cystamine or saline group. The incidence of ventricular fibrillation (VF) was analysed by Fisher's exact test. The probability was considered significant if less than 0.05.

Chemicals

Three compounds: NAC, Cys and cystamine were provided by the Pharmaceutical Laboratory in our institute. Their chemical structures are illustrated in Figure 1.

Results

Changes of heart function parameters

In the period of reperfusion after exposure to 20 min of LAD occlusion, ventricular function was severely depressed in saline and cystamine groups; however, the recovery of ventricular function indicated by the percentage recovery of RPP was significantly better in the cys and NAC groups (Figure 2). LVDP and (±)*dp/dt* also showed improvement to a varying extent in the Cys and NAC groups as compared with saline and cystamine groups (Table 1). Heart rate (HR) appeared to be

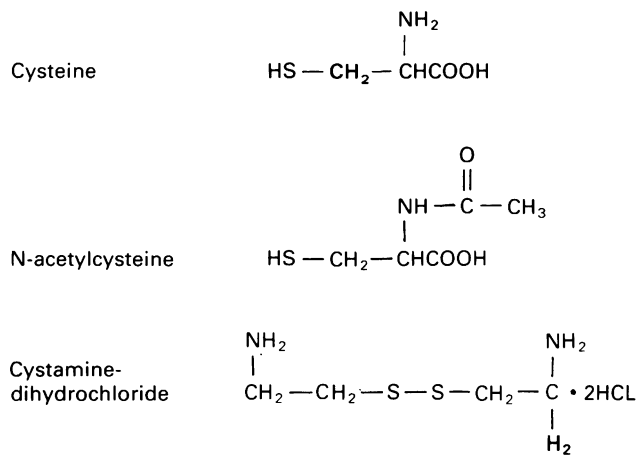

Figure 1 Structure of the drugs used in the experiments.

decreased during occlusion and reperfusion in the saline and cystamine groups though significant differences were not noted between groups ($P > 0.05$, Table 1). Obviously, both LVDP and HR contributed to changes in RPP; however, it was mainly drug-induced changes in LVDP that underlay the change in RPP.

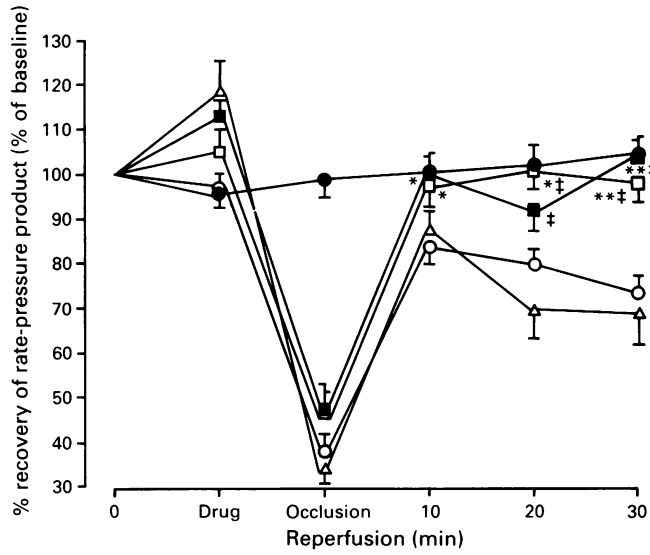

Figure 2 Rate-pressure product expressed as a percentage of the baseline value. Drug: 10 min after infusion of saline or drugs. Occlusion: 20 min after the LAD occlusion. (●) Sham; (■) cysteine; (□) N-acetylcysteine; (○) saline; (△) cystamine. Each point is mean with s.e.mean shown by vertical bars. * $P < 0.05$; ** $P < 0.01$ vs saline; † $P < 0.05$; ‡ $P < 0.01$ vs cystamine.

Table 1 Effects of N-acetylcysteine (NAC), cysteine (Cys) and cystamine on left ventricular function in stunned myocardium in perfused rat heart

	HR (beats min^{-1})	*LVDP* (mmHg)	dp.dt^{-1} (mmHg s^{-1} × 10^{-3})	(−)dp.dt^{-1} (mmHg s^{-1} × 10^{-3})	*CFR* (ml min^{-1})
0 (Baseline)					
Saline	248 ± 18	67 ± 1	1.55 ± 0.08	1.08 ± 0.05	9.1 ± 0.6
Sham	235 ± 14	64 ± 3	1.47 ± 0.07	1.20 ± 0.07	8.9 ± 0.6
NAC	232 ± 16	69 ± 2	1.50 ± 0.09	1.06 ± 0.08	9.1 ± 0.6
Cys	253 ± 27	61 ± 3	1.36 ± 0.08	0.92 ± 0.09	10.2 ± 0.9
Cystamine	223 ± 13	70 ± 7	1.52 ± 0.12	1.13 ± 0.11	8.7 ± 0.5
10 min after drugs infusion					
Saline	239 ± 17	67 ± 2	1.56 ± 0.08	1.10 ± 0.06	9.3 ± 0.6
Sham	226 ± 11	63 ± 3	1.49 ± 0.06	0.97 ± 0.06	8.9 ± 0.7
NAC	235 ± 19	72 ± 4	1.61 ± 0.15	1.12 ± 0.11	10.6 ± 0.9
Cys	256 ± 25	69 ± 5	1.61 ± 0.11	1.07 ± 0.11	11.3 ± 1.0
Cystamine	253 ± 11	73 ± 8	1.69 ± 0.18	1.26 ± 0.13	13.0 ± 1.3*
20 min after occlusion					
Saline	209 ± 16	30 ± 3	0.64 ± 0.06	0.36 ± 0.06	6.6 ± 0.5
Sham	239 ± 10	64 ± 3	1.48 ± 0.06	1.02 ± 0.06	9.5 ± 0.9**
NAC	222 ± 13	34 ± 5	0.76 ± 0.07†	0.44 ± 0.06	8.9 ± 0.5**
Cys	229 ± 29	33 ± 5	0.72 ± 0.07	0.42 ± 0.04	9.1 ± 0.7**
Cystamine	220 ± 12	24 ± 3	0.51 ± 0.07	0.31 ± 0.04	8.5 ± 0.9
10 min after reperfusion					
Saline	242 ± 20	59 ± 3	1.32 ± 0.05	0.86 ± 0.05	10.3 ± 0.6
Sham	243 ± 11	61 ± 4	1.41 ± 0.06	1.01 ± 0.05	9.4 ± 0.7
NAC	244 ± 15	66 ± 5	1.51 ± 0.13†	1.06 ± 0.10	13.1 ± 1.1*
Cys	234 ± 30	63 ± 3	1.39 ± 0.08	0.94 ± 0.06	13.1 ± 1.3
Cystamine	240 ± 15	44 ± 10	1.10 ± 0.12	0.77 ± 0.08	11.5 ± 0.9
20 min after reperfusion					
Saline	231 ± 22	58 ± 4	1.36 ± 0.06	0.88 ± 0.06	9.6 ± 0.8
Sham	246 ± 11	61 ± 4	1.50 ± 0.06	1.01 ± 0.07	9.5 ± 0.6
NAC	237 ± 17	70 ± 4*†	1.58 ± 0.13‡	1.09 ± 0.10†	12.6 ± 1.1*
Cys	237 ± 28	60 ± 3	1.35 ± 0.07†	0.88 ± 0.09	12.5 ± 1.2
Cystamine	226 ± 16	45 ± 8	0.92 ± 0.16*	0.66 ± 0.12	10.7 ± 0.8
30 min after reperfusion					
Saline	212 ± 23	58 ± 4	1.28 ± 0.07	0.78 ± 0.06	8.7 ± 0.6
Sham	257 ± 16	61 ± 3	1.54 ± 0.03	1.06 ± 0.08	9.6 ± 0.9
NAC	229 ± 17	70 ± 4†	1.59 ± 0.13†	1.08 ± 0.10*†	12.0 ± 1.1*
Cys	230 ± 30	62 ± 4	1.33 ± 0.09	0.88 ± 0.09	11.8 ± 1.1*
Cystamine	206 ± 17	54 ± 4	0.93 ± 0.17	0.61 ± 0.14	9.8 ± 1.0

$n = 7$–8 in each group.
HR, heart rate, LVDP, left ventricular developed pressure, i.e., the difference between systolic and end-diastolic pressure, *dp/dt* and (−)*dp/dt*, rate of left ventricular pressure rising and falling, CFR, coronary flow rate. * $P < 0.05$, ** $P < 0.01$ vs saline; † $P < 0.05$, ‡ $P < 0.01$ vs cystamine.

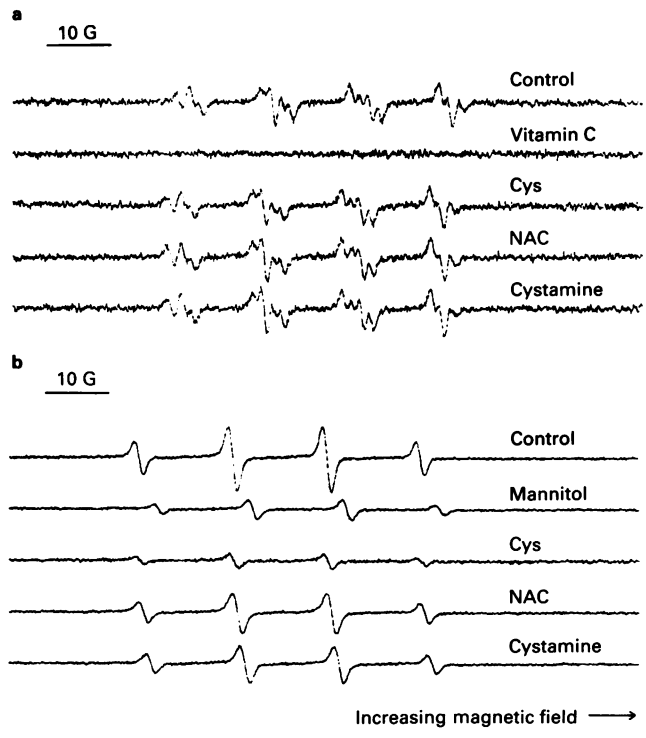

Figure 3 (a) ESR spectra of $\cdot\bar{O}_2$ generated from aqueous solution system by spin trapping of 5,5-dimethyl-1-pyrroline N-oxide (DMPO). Control: 3×10^{-4} M riboflavin, 0.1 M DMPO, 5×10^{-3} M EDTA, 0.05 M PBS, pH = 5.0. Cysteine (Cys), N-acetylcysteine (NAC), cystamine and vitamin C: the conditions were the same as control except that Cys, NAC, cystamine or vitamin C were added. Cys, NAC and cystamine as in Table 1. (b) ESR spectra of ·OH generated from aqueous solution by spin trapping of DMPO. Control: 1.25% H_2O_2, 2×10^{-4} M $(NH_4)_2$ $FeSO_4$, 0.1 M DMPO, 0.05 M PBS, pH = 7.4. Mannitol, cysteine (Cys), N-acetylcysteine (NAC) and cystamine: the conditions were the same as control except that mannitol, Cys, NAC and cystamine were added respectively. In this study, vitamin C and mannitol were used as a positive control for scavenging $\cdot O_2$ and ·OH, respectively. In this figure, centre field and sweep width are 3420.00 and 100.0 Gauss, respectively.

The haemodynamic profiles of the five groups, suggested that NAC and Cys had beneficial effects on recovery of heart function.

Changes of coronary flow rate (CFR)

After 10 min of cystamine administration, CFR increased significantly ($P < 0.05$ vs saline). This indicated that cystamine could enhance CFR in the perfused rat heart under non-ischaemic conditions. During the LAD occlusion period, CFR decreased in the saline group but this fall was prevented in the NAC and Cys groups ($P < 0.05$ vs saline); during the 30 min reperfusion period, CFR increased significantly in the NAC and Cys groups ($P < 0.05$ vs saline), indicating that NAC and Cys can improve coronary flow during both coronary occlusion and reperfusion periods. Cystamine did not show the beneficial effect on CFR throughout the occlusion and reperfusion periods (Table 1).

Effect of the drugs on arrhythmias

In this experiment, the incidence of ventricular fibrillation (VF) after reperfusion was compared between groups. There was no VF in the sham group during the reperfusion period. The incidence of VF after reperfusion in the saline, cystamine Cys and NAC groups was 6/8 (75.0%), 4/7 (57.1%), 3/8 (37.5%) and 2/7 (28.6%), respectively and no significant differences ($P > 0.05$) were noted between the NAC, Cys, cystamine and saline groups though NAC and Cys showed a strong tendency to reduce the incidence of VF in this study.

Scavenging effects of the drugs on $\cdot\bar{O}_2$ and ·OH in vitro

Effect on $\cdot\bar{O}_2$ The ability of the drugs to react with $\cdot\bar{O}_2$ generated by the irradiated riboflavin system was used to evaluate the effectiveness of the drugs in scavenging $\cdot\bar{O}_2$. As illustrated in Figure 3a, NAC, Cys and cystamine all had no significant effect on scavenging $\cdot\bar{O}_2$.

Effect on ·OH The intensity of the ESR signal (which is proportional to the concentration of spin adducts in the sample) was determined by measuring the height of the first line of the second doublet of the spectrum and expressed in signal unit. As shown in Figure 3b, ESR signals characteristic of radical adducts of DMPO appeared in the control mixture (saline); however, DMPO adduct production in the mixture in which Cys, NAC, cystamine and mannitol were added was reduced to 2.18 ± 0.22 (Cys, $P < 0.05$ vs saline), 4.62 ± 0.85 (NAC), 4.01 ± 0.91 (cystamine) and 2.82 ± 0.47 (mannitol, $P < 0.05$ vs saline) respectively from 7.66 ± 1.32 (saline control). From this *in vitro* study, Cys scavenged ·OH significantly, however, both NAC and cystamine (at 3.6×10^{-4} M) did not have a significant scavenging effect on ·OH compared with the saline control ($P > 0.05$).

Discussion

The major finding in this study is that SH compounds, NAC and Cys which were administered before ischaemia and reperfusion, can improve the recovery of ventricular function in a rat model of stunned myocardium. This beneficial effect of NAC and Cys on stunned myocardium is probably through the following mechanisms.

(a) NAC and Cys do benefit the ischaemic and reperfused myocardium by significantly enhancing CFR during occlusion and reperfusion after a brief period of ischaemia. This may be the major mechanism by which NAC and Cys protect the myocardium against ischaemic and reperfusion injury. Winniford *et al.* (1986) have shown that NAC could potentiate the coronary vasodilator effect of nitroglycerin in man, as shown by an increase in coronary sinus blood flow. Blaustein *et al.* (1989) demonstrated that GSH administration could prevent an increase in coronary vascular resistance during 30 min of reperfusion after 20 min of ischaemia in isolated rat heart, suggesting that GSH may be particularly important in protecting the coronary vasculature. Other studies also show that some of the microvascular damage may be caused by free radicals because this damage is decreased by free radical scavengers (Przyklenk & Kloner, 1989; Zweier *et al.*, 1988). Conceivably, during a brief period of ischaemia and ensuing reperfusion, NAC and Cys may have a protective effect on both the myocardium and coronary vasculature through such mechanisms as: preserving or even potentiating coronary vasodilator reaction to some ischaemia-induced accumulated metabolites which can dilate coronary vessels; donating a SH group, which probably is involved in a common intermediate reaction through which all vasodilators act other than via 'specific receptors' (Needleman *et al.*, 1973), and replenishing endogenous GSH and/or scavenging ·OH.

The finding that NAC and Cys attenuated myocardial stunning through their actions in enhancing CFR was consistent with our previous reports in which a progressive decrease in CFR was found to be occurring in the early period of reperfusion following 20 min of global ischaemia and in which hyperosmotic mannitol, given on reperfusion, prevented this decrease and therefore reduced myocardial dysfunction (Sun & Lin, 1990; Sun *et al.*, 1991).

(b) The *in vitro* studies described in this paper demonstrate the ability of Cys to scavenge ·OH rather than $\cdot\bar{O}_2$. This suggests that the beneficial effects of Cys on stunned myocardium are due, at least in part, to prevention of ·OH production. This result is comparable with the reports by Bolli *et al.* (1987, 1988) and Faber *et al.* (1988), who have shown that the ·OH

radical or one of its reactive products is a mediator of post-ischaemic dysfunction.

NAC did not show a significant effect in scavenging ·OH ($P > 0.05$ vs saline) but it may exert its protective effects through mechanisms other than its beneficial effect on CFR, such as maintaining intracellular GSH concentration. NAC is a low molecular weight precursor of GSH and has been used successfully to replenish intracellular GSH stores in several diseased states (Olson *et al.*, 1980; Rumack *et al.*, 1981; Bernard *et al.*, 1984; Moldeus *et al.*, 1986). Furthermore, NAC may increase cytoplasmic superoxide dismutase activity and interfere with autocatalytic lipid peroxidation (Papaccio, 1986; Tribble *et al.*, 1987). Therefore, we suggest that NAC may work either through promoting the resynthesis of GSH or acting as an alternative substrate for glutathione peroxidases in this experimental model. These actions would limit the cytotoxic effects of free radicals and lipid peroxides.

(c) The chemical structure of three compounds, NAC, Cys and cystamine, were similar except that Cys and NAC have a free SH group but cystamine does not. However, the effects of NAC and Cys on myocardial stunning were significantly different from that of cystamine. Both NAC and Cys attenuated ventricular dysfunction in the postischaemic period whereas cystamine did not. This suggests that the free SH group is necessary in order to be effective in providing protection to the myocardium against ischaemia and reperfusion injury, particularly in improving coronary flow during ischaemia and reperfusion.

A number of studies in isolated perfused hearts (Woodward & Zakaria, 1985; Bernier *et al.*, 1986; Heuer *et al.*, 1988) and in rat models of transient regional ischaemia *in vivo* (Manning *et al.*, 1984; 1988) have supported the concept that oxygen free radicals play a role in the development of reperfusion arrhythmias. Superoxide dismutase, catalase, L-methionine, mannitol, GSH and desferrioxamine all reduced the incidence of reperfusion-induced VF (Bernier *et al.*, 1986). From our previous study (Tang & Tang, 1990), the antioxidant compounds, NAC and Cys, also showed beneficial effects in reducing the incidence of VF. However, in this experiment, although NAC and Cys showed a strong tendency to reduce reperfusion-induced VF, this effect did not reach statistical significance ($P > 0.05$ vs saline), which was probably due to the small size of the groups used in this study.

In summary, the data presented in this paper demonstrated that NAC and Cys improve the functional recovery of stunned myocardium. This ability was most likely due to their beneficial effect in preventing the fall in CFR on coronary occlusion and increase CFR on reperfusion, although they may also have a beneficial effect in scavenging ·OH and replenishing the intracellular GSH store. These protective effects appear to be correlated closely with their free SH group because when this group is replaced by a disulphide bond in the compound cystamine, the beneficial effect in the stunned myocardium was not observed. These results also suggest that the pathogenesis of myocardial stunning involves a multifactorial process; free radicals may play an important role in this process, however, other factors, such as coronary perfusion can greatly affect the recovery of postischaemic ventricular function as well.

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References

AMBROSIO, G., WEISFELDT, M.L., JACOBUS, W.E. & FLAHERTY, J.T. (1987). Evidence for a reversible oxygen radical-mediated component of reperfusion injury: reduction by recombinant human superoxide dismutase administered at the time of reflow. *Circulation*, **75**, 282–291.

BECKER, L.C. & AMBROSIO, G. (1987). Myocardial consequences of reperfusion. *Prog. Cardiovasc. Dis.*, **30**, 23–44.

BERNARD, G.R., LUCHT, W.D., NIEDERMEYER, M.E., SNAPPER, J.R., OGLETREE, M.L. & BRIGHAM, K.L. (1984). Effect of N-acetylcysteine on pulmonary response to endotoxin in the awake sheep and upon *in vitro* granulocyte function. *J. Clin. Invest.*, **73**, 1772–1784.

BERNIER, M., HEARSE, D.J. & MANNING, A.S. (1986). Reperfusion-induced arrhythmias and oxygen-derived free radicals: Studies with "anti-free radical" interventions and a free radical-generating system in the isolated perfused rat heart. *Circ. Res.*, **83**, 149–151.

BETTS, W.H., CLELAND, L.G., GEE, D.J. & WHITEHOUSE, M.W. (1984). Effects of D-pencillamine on a model of oxygen-derived free radical mediated tissue damage. *Agents Actions*, **14**, 283–290.

BLAUSTEIN, A., DEUEKE, S.M., STOLZE, R.I., BAXTER, D., HEALEY, N. & FANBURG, B.L. (1989). Myocardial glutathione depletion impairs recovery after short periods of ischemia. *Circulation*, **80**, 1449–1457.

BOLLI, R., ZHU, W-X., HARTLEY, C.J., MICHAEL, L.H., REPINE, J., HESS, M.L., KUKREJK, R.C. & ROBERTS, R. (1987). Attenuation of dysfunction in the postischemic "stunned" myocardium by dimethylthiourea. *Circulation*, **76**, 458–568.

BOLLI, R., PATEL, B.S., JEROUDI, M.O., LAI, E.K. & McCAY, P.B. (1988). Demonstration of free radical generation in "stunned" myocardium of intact dogs with the use of the spin trap α-phenyl N-tert-butyl nitrone. *J. Clin. Invest.*, **82**, 476–485.

BOLLI, R., JEROUDI, M.O., PATEL, B.S., ARUOMA, O.I., HALLIWELL, B., LAI, E.K. & McCAY, P.B. (1989). Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial "stunning" is a manifestation of reperfusion injury. *Circ. Res.*, **65**, 607–622.

BRAUNWALD, E. & KLONER, R.A. (1982). The stunned myocardium: Prolonged, postischemic ventricular dysfunction. *Circulation*, **66**, 1146–1149.

BRAUNWALD, E. & KLONER, R.A. (1985). Myocardial reperfusion: A double-edged sword? *J. Clin. Invest.*, **76**, 1713–1719.

CECONI, C., CURELLO, S., CARGNONI, A., FERRARI, R., ALBERTINI, A. & VISIOL, O. (1988). The role of glutathione status in the protection against ischaemic and reperfusion damage: effects of N-acetyl cysteine. *J. Mol. Cell. Cardiol.*, **20**, 5–13.

DOROSHOW, J.H., LOCKER, G.Y., IFRIM, I. & MYERS, C.E. (1980). Prevention of duxorubicin cardiac toxicity in the mouse by N-acetylcysteine. *J. Clin. Invest.*, **68**, 1053–1064.

FABER, N.E., VERCELLOTTI, G.M., JACOB, H.S., PIEPER, G.H. & GROSS, G.L. (1988). Evidence for a role of iron-catalyzed oxidants in functional and metabolic stunning in the canine heart. *Circ. Res.*, **63**, 351–360.

FORMAN, H.J., ROTMAN, E.I. & FISHER, A.B. (1983). Roles of selenium and sulfur-containing amino acids in protection against oxygen toxicity. *Lab. Invest.*, **49**, 148–153.

FORMAN, M.B., PUETT, D.W., CATES, C.U., McCROSKEY, D.E., BECKMAN, J.K., GREENE, H.L. & VIRMANI, R. (1988). Glutathione redox pathway and reperfusion injury. Effect of N-acetylcysteine on infarct size and ventricular function. *Circulation*, **78**, 202–213.

GARLICK, P.P., DAVIES, M.J., HEARSE, D.J. & SLATER, T.F. (1987). Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. *Circ. Res.*, **61**, 757–760.

GROSS, D.J., FARBER, N.E., HARDMAN, H.F. & WARLTIER, D.C. (1986). Beneficial actions of superoxide dismutase and catalase in stunned myocardium of dogs. *Am. J. Physiol.*, **250**, H372–H377.

HEUER, H.J., MULLER, E. & BERNAUER, W. (1988). Differential effects of superoxide dismutase on high energy phosphates, creatinine kinase release, and arrhythmias during post-ischemic reperfusion in isolated rat hearts. *Basic Res. Cardiol.*, **83**, 149–151.

HOROWITZ, J.D., ANTMAN, E.M., LORELL, B.H., BARRY, W.H. & SMITH, T.W. (1983). Potentiation of the cardiovascular effects of nitroglycerin by N-acetylcysteine. *Circulation*, **68**, 1247–1253.

KLONER, R.A., PRZYKLENK, K. & PETEL, B. (1989). Altered myocardial states: The stunned and hibernating myocardium. *Am. J. Med.*, **86**, Suppl., **1**, 31–42.

KRAMER, J.H., ARROYO, C.M., DICKENS, B.F. & WEGLICKI, W.B. (1987). Spin-trapping evidence that graded myocardial ischemia alters post-ischemic superoxide production. *Free Radical Biol. Med.*, **3**, 153–159.

KUSUOKA, H., PORTERFIDLD, J.K., WEISMAN, H.F., WEISFELDT, M.L. & MARBAN, E. (1987). Pathophysiology and pathogenesis of stunned myocardium: depressed Ca^{2+} activation of contraction as a consequence of reperfusion-induced cellular calcium overload in ferret hearts. *J. Clin. Invest.*, **79**, 950–961.

LEE, H-C., SMITH, N., MOHABIR, R. & CLUSIN, W.T. (1987). Cytosolic calcium transients from the beating mammalian heart. *Proc. Natl. Acad. Sci., U.S.A.*, **84**, 7793–7797.

MANNING, A.S., COLTART, D.J. & HEARSE, D.J. (1984). Ischemia and reperfusion-induced arrhythmias in the rat and effects of xanthine oxidase inhibition with allopurinol. *Circ. Res.*, **55**, 545–548.

MANNING, A.S., BERNIER, M., CROME, R., LITTLE, S. & HEARSE, D. (1988). Reperfusion-induced arrhythmias: a study of the role of xanthine-oxidase derived free radicals in the rat heart. *J. Mol. Cell. Cardiol.*, **20**, 35–45.

MOLDEUS, P., COTGREAVE, I.A. & BERGGREN, M. (1986). Lung protection by a thiol-containing antioxidant: N-acetylcysteine. *Respiration*, **50**, Suppl., **1**, 31–42.

NEEDLEMAN, P., JAKSCHIK, B. & JOHNSON, E.M. (1973). Sulfhydryl requirement for relaxation vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **187**, 324–331.

OLSON, R.D., MACDONALD, J.S., VAN BOXHL, C.J., BOERTH, R.C., HARBISON, R.D., SLONIM, A.E., FREEMAN, R.W. & OATES, J.A. (1980). Regulatory role of glutathione and sulfhydryl groups in the toxicity of adriamycin. *J. Pharmacol. Exp. Ther.*, **315**, 450–454.

OPIE, L.H. (1989). Reperfusion injury and its pharmacologic modification. *Circulation*, **80**, 1049–1062.

PAPACCIO, G., PISANTI, F.A. & FRASCATORE, S. (1986). Acetylhomocyteine-thiolactone-increase of superoxide dismutase counteracts the effect of subdiabetogenic doses of streptozocin. *Diabetes*, **35**, 470–474.

PRZYKLENK, K. & KLONER, R.A. (1989). "Reperfusion injury" by oxygen-derived free radicals: Effect of superoxide dismutase plus catalase, given at the time of reperfusion, on myocardial infarct size, contractile function, coronary microvasculature, and regional myocardial blood flow. *Circ. Res.*, **64**, 86–96.

ROTH, D.M., LEFER, D.J., HOCK, C.E. & LEFER, A.M. (1985). Effects of peptide leukotrienes on cardiac dynamics in rat, cat, and guinea pig hearts. *Am. J. Physiol.*, **249**, H477–H484.

RUMACK, B.H., PETERSON, R.C., KOCH, G.G. & AMARA, J.A. (1981). Acetaminophen overdose: 662 cases with evaluation of oral acetylcysteine treatment. *Arch. Intern. Med.*, **141**, 380–385.

STAHL, L.D., AVERSANO, T.R. & BECKER, L.C. (1986). Selective enhancement of function of stunned myocardium by increased flow. *Circulation*, **74**, 843–851.

STEENBERGEN, C., MURPHY, E., WATTS, J.A. & LONDON, R.E. (1990). Correlation between cytosolic free calcium, contracture, ATP, and irreversible ischemic injury in perfused rat heart. *Circ. Res.*, **66**, 135–146.

SUGUHARA, T., HORIKAWA, M., HIKITA, M. & NAGATA, H. (1977). Studies on a sulphydryl radioprotector of low toxicity. *Experientia*, **27**, Suppl., 53–61.

SUN, J.Z. & LIN, C.Z. (1990). Changes in calcium and water contents of stunned myocardium and effects of hyperosmotic mannitol in the rat heart. *Acta Physiol. Sinica*, **42**, 155–162.

SUN, J.Z., YANG, H.B., MU, Z.M. & LIN, C.Z. (1991). Prevention of myocardial stunning by hyperosmotic mannitol or low calcium solution perfusion in rats. *Chinese J. Physiol. Sci.*, **7**, 61–71.

TANG, L.D. & TANG, Z.M. (1991). The protective actions of sulfhydryl compounds against arrhythmias induced by reperfusion in isolated rat heart. *Acta Pharmacol. Sinica*, (in press).

TRIBBLE, D.L., AW, T.Y. & JONES, D.P. (1987). The pathophysiological significance of lipid peroxidation in oxidative cell injury. *Hepatology*, **7**, 377–386.

WESTLIN, W. & MULLANE, K.M. (1989). Alleviation of myocardial stunning by leukocyte and platelet depletion. *Circulation*, **80**, 1828–1836.

WINNIFORD, M.D., KENNEDY, P.L., WELLS, P.J. & HILLS, L.D. (1986). Potentiation of nitroglycerin-induced coronary dilatation by N-acetylcysteine. *Circulation*, **73**, 138–142.

WOODWARD, B. & ZAKARIA, M.V.M. (1985). Effect of some free radical scavengers on reperfusion induced arrhythmias in the isolated rat heart. *J. Mol. Cell. Cardiol.*, **17**, 485–493.

ZWEIER, J.L., FLAHERTY, J.T. & WEISFELDT, M.L. (1987). Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc. Natl. Acad. Sci., U.S.A.*, **84**, 1404–1407.

ZWEIER, J.L., KUPPUSAMY, P. & LUTTY, G.A. (1988). Measurement of endothelial cell free radical generation: evidence for a central mechanism of free radical injury in postischemic tissue. *Proc. Natl. Acad. Sci., U.S.A*, **85**, 4046–4050.

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Heterogeneity of thromboxane A_2 (TP-) receptors: evidence from antagonist but not agonist potency measurements

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1 Thromboxane A_2 (TP-) receptors in human, rat and rabbit platelets and in smooth muscle of guinea-pig trachea, rat aorta and rabbit aorta have been characterized by measurement of the potencies of agonists and antagonists having considerable variations in chemical structure.

2 On each washed platelet system, eight prostanoids induced maximal irreversible aggregation (full agonists) and the potency ranking was EP 171 > STA_2 > 9,11-azo PGH_2 > 9,11-endoxy-10a-homo PGH_2 > U-46619 (standard) > PGH_2 = 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$ > 16,16-dimethyl $PGF_{2\alpha}$. Correlations between the three platelet preparations for both absolute and relative potencies were good. On human platelets, STA_2, at concentrations above that required for maximum aggregation, exerted an inhibitory effect which was independent of its interaction with the TP-receptor.

3 Five prostanoids, EP 109, EP 167, EP 204, PTA_2 and 16,20-methano PTA_2, exhibited partial agonist activity on the platelet and smooth muscle preparations. There was good agreement between absolute potencies on the six preparations; on platelets potency was assessed from shape change measurements, since aggregation, when present, always showed a very shallow concentration-response relationship. The magnitude of the maximum response induced by each compound decreased in the order listed above, to the extent that 16,20-methano PTA_2 could be treated as a pure antagonist.

4 With U-46619 as agonist, the pA_2 values of seven antagonists were found to be very similar on human and rat platelets. The potency ranking was EP 169 > AH 23848 > EP 092 > ONO 11120 > EP 115 = 16,20-methano PTA_2 > BM 13177. There was a similar trend on rabbit platelets but pA_2 values were 1.0–1.5 log units smaller; the exception was BM 13177 which had similar affinities. The antagonism produced by EP 169 and AH 23848 was surmountable on rabbit platelets but not on human and rat platelets.

5 None of the antagonists was highly potent on the rabbit aorta (pA_2 values < 7.5 by Schild analysis). Affinities on the guinea-pig trachea and the rat aorta were higher and in the same range as those obtained for human and rat platelets. However the correlations of pA_2 values between any pair of smooth muscle preparations and between any pair of platelet/smooth muscle preparations were either weak or not significant ($P > 0.05$).

6 The excellent agreement for both full and partial agonist potencies between the six preparations provides no evidence for TP-receptor subtypes and further suggests that the agonist recognition sites of the TP-receptors could be very similar, if not identical, in nature. In contrast, the different antagonist affinities found in this and other published studies indicate heterogeneity of TP-receptors. However, classification into TP_1-, TP_2-receptors, etc. on the basis of the limited antagonist data available does not appear appropriate at this time.

Introduction

This paper addresses the possible existence of subtypes of the thromboxane A_2 (TP-) receptor. Our early *in vitro* studies with the TP-receptor antagonist EP 045 revealed a less effective block of the contractile actions of TXA_2 and U-46619 (a stable PGH_2 analogue, Figure 1) on rabbit aorta compared with guinea-pig trachea and dog saphenous vein (Jones *et al.*, 1982). In a preliminary report Anderson & MacIntyre (1982) also showed that EP 045 and several other TP-receptor antagonists were less potent as inhibitors of U-46619-induced aggregation in rabbit platelet-rich plasma (PRP) compared with human PRP. More extensive studies (Narumiya *et al.*, 1986) have shown that the TP antagonist, ONO 11120 (Katsura *et al.*, 1983) (Figure 1), is a less effective inhibitor of aggregation induced by STA_2 (a stable TXA_2 analogue, Figure 1) in rabbit PRP than in human PRP. In addition, radiolabelled I-PTA-OH, a close relative of ONO 11120, was found to bind with high affinity to TP-receptors on human and dog washed platelets but not rabbit washed platelets. In order to throw light on these potential differences between TP-receptors, we decided to measure pA_2 values for a number of TP antagonists on rabbit aorta and rabbit washed platelets and to compare these with corresponding values for guinea-pig trachea, rat aorta, and human and rat washed platelets.

STA_2 is a highly potent agonist at TP-receptors in smooth muscle with EC_{50} values in the 1–5 nM range (Mais *et al.*, 1985; Toda *et al.*, 1986). Its aggregatory potency appears to be lower: EC_{50} = 0.4 μM (Katsura *et al.*, 1983) and 1.1 μM (Mais *et al.*, 1985) for human PRP, 0.68 μM (Mais *et al.*, 1985) for dog PRP and 8 μM for rabbit PRP (Narumiya *et al.*, 1986). Although plasma protein binding in PRP could dramatically reduce the potency of this highly lipophilic molecule, we felt that true potency differences might be involved, particularly with respect to the rabbit platelet. We have therefore compared the potencies of STA_2 and seven other full agonists on washed platelets from man, rat and rabbit.

Finally we have compared the activities of several partial agonists, including the novel prostanoids EP 167 and EP 204 (Figure 1), on the platelet and smooth muscle preparations.

Methods

Washed platelet preparations

Blood was collected into acid-citrate-dextrose (ACD) anticoagulant (1 vol : 5 vol blood) from a forearm vein of human

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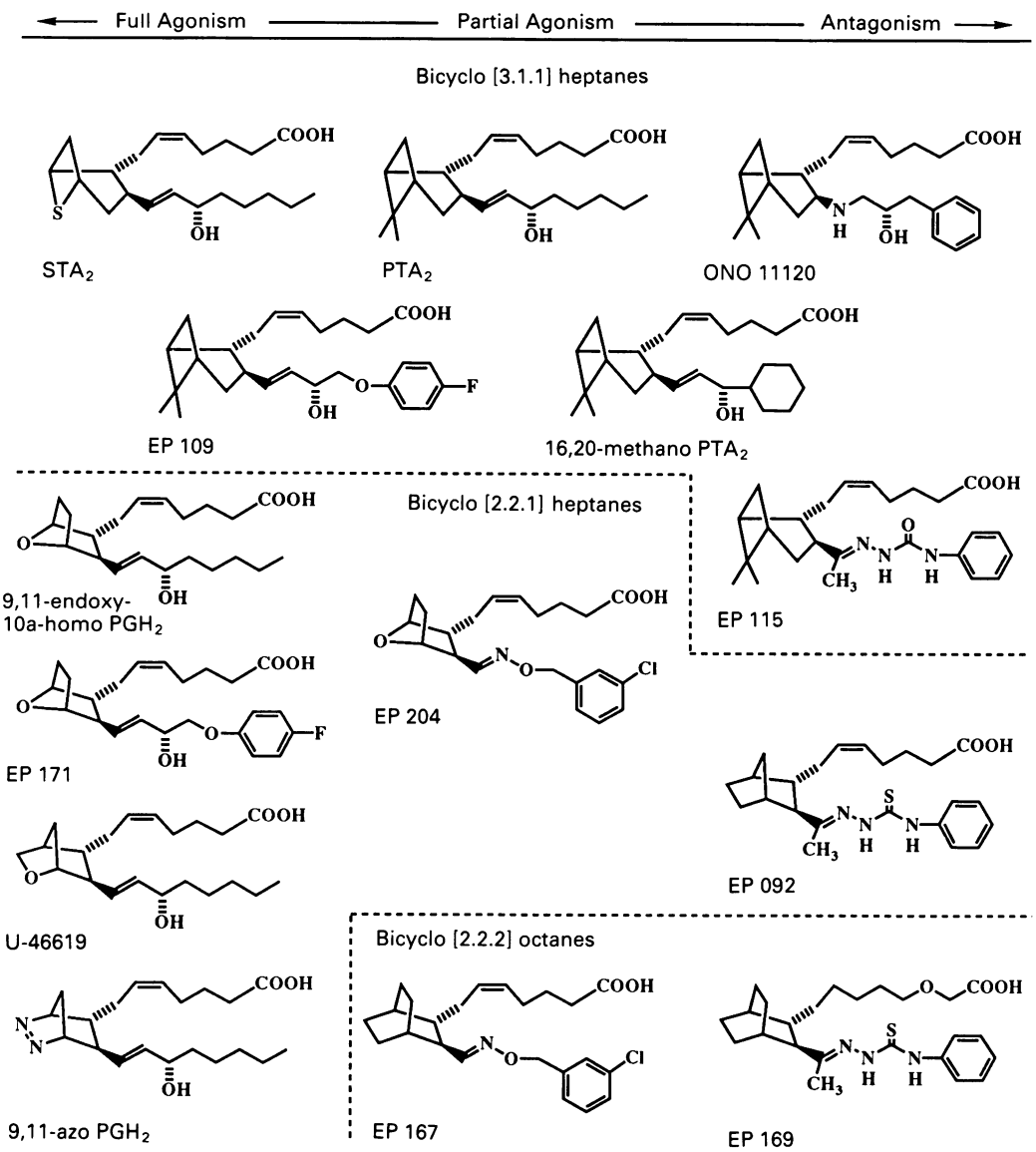

Figure 1 Synthetic analogues of thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2) arranged according to their ring structures and whether they are TP-receptor full agonists, partial agonists or antagonists. All compounds have the natural upper side chain with the exception of EP 169 and all have the side chains in a *trans* relationship to the ring system. U-46619, 9,11-azo PGH_2 and all the bicyclo[3.3.1]heptane analogues are chiral; the remaining compounds are racemic.

volunteers who had not taken non-steroidal anti-inflammatory drugs for at least 7 days previously, from the common carotid arteries of rabbits (2–4 kg) under pentobarbitone anaesthesia and from the abdominal aorta of rats (200–350 g) under ether anaesthesia. In a few experiments blood was obtained from human donors who had ingested 600 mg of aspirin 12 and 2 h before collection. Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 200 *g* for 20 min. PGI_2 was added to the PRP (about 30 nM for human and 300 nM for rat and rabbit) and a platelet pellet prepared by centrifugation at 600 *g* for 20 min. The pellet was suspended in calcium-free Krebs solution (composition given below) at 37°C and the space above the suspension vented with 95% O_2/5% CO_2. At least 30 min was allowed for the PGI_2 to decay.

Platelet shape change and aggregation were measured with a Chrono-Log dual-channel aggregometer (Born, 1962). Each cuvette (siliconised glass) contained 0.4 ml washed platelet suspension, 0.02 ml 25 mM $CaCl_2$ solution and 0.06 ml 0.9% NaCl solution (in which inhibitors were dissolved). Stirring was achieved with a Teflon-coated magnetic follower revolving at 1000 r.p.m. Aggregating agents were added in 0.02 ml 0.9% NaCl solution.

To measure the relative potency of a full agonist in a single experiment, concentration-aggregation relationships for the standard agonist (U-46619) followed by the test agonist were obtained on one channel of the aggregometer, with duplicate measurements being made on the second channel, test agonist before standard. Each aggregation response was measured as the maximum pen deflection occurring within 1 min of agonist addition and was expressed as a percentage of the U-46619 maximum. Log concentration-response curves (log CRCs) were plotted using the mean data and an equi-effective molar ratio (EMR) calculated at the 50% level. In the case of EP 171, aggregation waves had slower time courses than those of other agonists and responses were measured at 2 min after drug addition (see Jones *et al.*, 1989).

For partial agonists (and EP 171) shape change responses were measured using a similar protocol. Large shape change responses were often curtailed by the onset of primary aggregation and the resulting log concentration-response curves were bell-shaped. The effect was most marked with U-46619 and EP 109, their apparent maxima being slightly less than those of EP 167 and EP 204. In each experiment the EC_{50} value for each agonist was measured at the 50% maximum shape change level of the agonist producing the highest

maximum. This means that shape change EC_{50} values presented here are likely to be smaller than 'true values' from experiments in which aggregation was specifically suppressed. However, potency ranking is unlikely to be drastically affected by this limitation. The partial agonists were also tested for their ability to inhibit maximal aggregation induced by U-46619 (300 nM) and either platelet-activating factor (PAF, 100 and 10 nM on human and rabbit platelets) or adenosine 5'-diphosphate (ADP, 1 μM on rat platelets), the cyclo-oxygenase inhibitor flurbiprofen (1 μM) being present in the latter two tests. Each compound was added with minimal agitation to the platelet suspension at room temperature and 15 s later the cuvette was placed in the heated cell block of the aggregometer and normal stirring started. After 2 min the aggregating agent was added and the extent of aggregation at 1 min recorded as described previously. With this procedure the partial agonist alone induced shape change but no detectable aggregation. Log concentration-inhibition curves were constructed and IC_{50} values calculated. EP 109 was not tested in this manner since aggregation usually occurred as the cuvette contents heated up.

Antagonists were usually incubated at 37°C for 2 min before agonist addition and aggregation responses were measured at 1 min as above. In each experiment a control CRC for U-46619 followed by a CRC for U-46619 in the presence of a concentration of antagonist giving a dose-ratio of about 5 were obtained on one channel. On the second channel a CRC in the presence of an antagonist concentration some 3 to 4 times higher (intended to give a dose ratio not greater than 20) followed by a control CRC were obtained. In successive experiments with any one antagonist the order of the low and high antagonist treatments was reversed. Dose-ratios were calculated from log CRCs at the 50% maximum aggregation level. pA_2 values (calculated as the sum of log (dose ratio − 1 and −log [antagonist]) for the two antagonist concentrations were averaged, since they did not differ by more than 0.2 log units. In the case of EP 169 and AH 23848, maximum block was not achieved within 2 min and incubation periods of 5 and 10 min respectively were used. Aggregation waves were also slower at concentrations of antagonist producing dose-ratios in excess of 5; all responses were therefore measured at 2 min after agonist addition.

Smooth muscle preparations

Thoracic aortae were obtained from male rats and rabbits and tracheae from male and female guinea-pigs killed by stunning and exsanguination. Rings of tissue (3 mm wide) were suspended between stainless steel hooks in 10 ml organ baths. The Krebs bathing solution (NaCl 118, KCl 5.4, $MgSO_4$ 1.0, $CaCl_2$ 2.5, NaH_2PO_4 1.1, $NaHCO_3$ 25, dextrose 10 mmol l^{-1}) was gassed with 95% O_2/5% CO_2 and maintained at 37°C. Tension was recorded with Grass FT03 force-displacement transducers linked to a Grass polygraph. The spontaneous tone of the guinea-pig trachea was abolished by inclusion of 1 μM indomethacin and 20 nM atropine sulphate in the bathing solution.

Cumulative concentration-response relationships were obtained for the agonists and partial agonists, with U-46619 used as the standard agonist. With antagonists, the preparation was exposed to two series of cumulative doses of U-46619, followed by exposure to the antagonist (50 min) and then a further series of U-46619 doses. Dose-ratios at three antagonist concentrations (1:5:25) from 3 or 4 preparations were obtained. pA_2 values were calculated for individual dose-ratios and the regression of pA_2 on log [antagonist] determined. If the regression coefficient, r, is not statistically significant from zero ($P = 0.05$), then the Schild equation for competitive antagonism, log (dose-ratio − 1) versus log [antagonist] (Arunlakshana & Schild, 1959), is obeyed and the best estimate of pA_2 is the mean value (Mackay, 1978). Slopes of best fit for the Schild plots were also determined.

Compounds

The following prostanoids were prepared in our laboratory and essential details of the synthetic methods are to be found in Wilson *et al.* (1982, 1988): *rac* 10a-homo-15S-hydroxy-9α, 11α-epoxy-prosta-5Z,13E-dienoic acid (9,11-endoxy-10a-homo PGH_2) and its 16*p*-fluorophenoxy-ω-tetranor (EP 171) and 13-(*m*-chlorobenzyloxyimino)-ω-heptanor (EP 204) analogues; *rac* 9α,11α-ethano-ω-heptanor-13-methyl-13-phenyl-thio-carbamoyl-hydrazino-prosta-5Z-enoic acid (EP 092); 11a-carba-15S-hydroxy-9α,11α-isopropylideno-prosta-5Z,13E-dienoic acid (PTA_2) and its 16*p*-fluorophenoxy-ω-tetranor (EP 109), ω-heptanor-13-methyl-13-phenylecarbamoylhydrazino (EP 115) and 16,20-methano (16,20-methano PTA_2) analogues; 9α,11α-ethano-ω-heptanor-10a-homo-13-(*m*-chlorobenzyl-oxyimino)prosta-5Z-enoic acid (EP 167) and 4a,10a-dihomo-9α,11α-ethano-ω-heptanor-3-oxa-13-phenyl-thio-carbamoyl-hydrazino-prostanoic acid (EP 169); 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$. PGH_2 was prepared from $PGF_{2\alpha}$ by the method of Porter *et al.* (1979).

The following compounds were gifts and are gratefully acknowledged: 9α,11α-thia-11a-carba-prosta-5Z,13E-dienoic acid (STA_2) and 11a-carba-12-(2'S-hydroxy-3'-phenylpropylamino)-9α,11α-isopropylideno-ω-octanor-prost-5Z-enoic acid (ONO 11120) from ONO, Japan; *p*-(2-benzenesulphonamidoethyl)-phenoxyacetic acid (BM 13177) from Boehringer-Mannheim, W. Germany; *rac* 9α-(biphenylyl)methoxy-12β-(N-morpholinyl)-ω-octanor-11-oxo-prost-4Z-enoic acid (AH 23848), 9α-(biphenylyl)methoxy-11β-hydroxy-12β-(N-piperidinyl)-ω-octanorprost-4Z-enoic acid (GR 32191) and 6-isopropoxy-9-oxoxanthen-2-carboxylic acid (AH 6809) from Glaxo, UK; *rac* 15S-hydroxy-9-oxo-16-phenoxy-ω-tetranorprost-13E-enoic acid (MB 28767) from Rhone-Poulenc, U.K.; *rac* 16-*p*-chlorophenoxy-ω-tetranor PGE_2 (ICI 80205) from ICI Pharmaceuticals, U.K.; PGI_2 from Schering AG, Berlin, W. Germany; dazoxiben from Pfizer, U.K.; flurbiprofen from Boots, U.K.

11α,9α-Epoxymethano-15S-hydroxy-prosta-5Z,13E-dienoic acid (U-46619), 9α,11α-azo-15S-hydroxy-prosta-5Z,13E-dienoic acid (9,11-azo PGH_2), 16,16-dimethyl PGE_2, 16,16-dimethyl $PGF_{2\alpha}$ and 15-methyl $PGF_{2\alpha}$ were purchased from Upjohn Diagnostics Ltd., U.S.A. Indomethacin, PAF and ADP were purchased from Sigma.

Prostanoids were usually dissolved in absolute ethanol to give concentrations of 5–20 mM and stored at −20°C. Aqueous stock solutions (100–500 μM) were prepared by adding an equimolar equivalent of NaOH to a portion of the ethanol solution, evaporating the ethanol with a nitrogen stream and dissolving the sodium salt residue in 0.9% NaCl solution. AH 23848, supplied as the calcium salt, was dissolved with gentle warming in 0.9% NaCl solution containing a trace of solid $NaHCO_3$. Aqueous stocks of EP 092, EP 115, EP 169 and AH 23848 were freshly prepared for each experiment.

Results

Washed platelet suspensions

U-46619 always produced complete and irreversible aggregation in washed platelet suspensions from man, rat and rabbit and was used as the standard agonist in agonist and antagonist potency determinations. Mean EC_{50} values for shape change on human, rat and rabbit platelets were 4.8 (range 4.1–6.5), 6.0 (3.9–8.7) and 7.3 (5.3–11) nM respectively (n = 24–26) and for aggregation 82 (55–130), 145 (108–310) and 65 (42–120) nM respectively (n = 96–108).

Full agonists In addition to U-46619, seven other prostanoids behaved as full agonists (see Figure 1 for structures) and the same order of potency was found on the three platelet preparations: EP 171 > STA_2 > 9,11-azo PGH_2 >

Table 1 Potencies of TP-receptor full agonists on human, rat and rabbit platelets

Agonist	*Platelet aggregation: equi-effective molar ratios*		
	Man	*Rat*	*Rabbit*
EP 171	0.044 ± 0.007	0.032 ± 0.007	0.045 ± 0.005
	(0.025 ± 0.005)*	(0.022 ± 0.005)*	(0.026 ± 0.004)*
STA_2	0.30 ± 0.05	0.19 ± 0.04	0.21 ± 0.03
9,11-Azo PGH_2	0.46 ± 0.06	0.50 ± 0.04	0.30 ± 0.06
9,11-Endoxy-10a-homo PGH_2	0.97 ± 0.05	0.99 ± 0.07	0.52 ± 0.04
U-46619	1.00	1.00	1.00
PGH_2 §	1.8 ± 0.1	1.5 ± 0.1	1.3 ± 0.1
16-*p*-Fluorophenoxy ω-tetranor $PGF_{2\alpha}$	3.2 ± 0.2	1.1 ± 0.1	1.4 ± 0.2
16,16-Dimethyl $PGF_{2\alpha}$	7.3 ± 0.6	8.2 ± 0.8	12.5 ± 2.1

Values are means (± s.e.mean) for 4 determinations.
* Equi-effective molar ratios for shape change.
§ Dazoxiben 80 μM present.

9,11-endoxy-10a-homo PGH_2 > U-46619 > PGH_2 = 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$ > 16,16-dimethyl $PGF_{2\alpha}$. The activity of PGH_2 was measured in the presence of 80 μM dazoxiben (Randall *et al.*, 1981) to prevent its conversion to TXA_2 by platelet thromboxane synthetase. In individual experiments, log concentration-aggregation curves for test agonists were parallel to that of U-46619. Mean equi-effective molar ratios (EMR) derived from 4 separate experiments are given in Table 1. In view of the difficulty of quantifying the slow aggregation waves produced by EP 171, shape change responses were used to obtain an additional potency comparison (Table 1); EP 171 was about 40 times more potent than U-46619 on each platelet preparation.

On human platelets, increasing the concentration of STA_2 above that required for maximal aggregation (100 nM) led to a reduced response (Figure 2); a similar depression of the maximum response was not seen on rat or rabbit platelets. This effect was studied further in platelet suspensions pretreated with a high concentration (6 μM) of the TP-receptor antagonist EP 092; at this concentration the antagonism is unsurmountable (U-46619 dose-ratio > 200). On human platelets STA_2 inhibited aggregation induced by PAF (100 nM) over the concentration-range corresponding to the depression of its own maximum when acting alone (Figure 2). In contrast, STA_2 (up to 3 μM) had little inhibitory effect (< 10%) on PAF (10 nM)-induced aggregation in rabbit platelets (n = 4) and on ADP (1 μM)-induced aggregation in rat platelets (which are insensitive to PAF) (n = 4). Under similar conditions U-46619 (up to 3 μM) did not affect PAF action on human platlets.

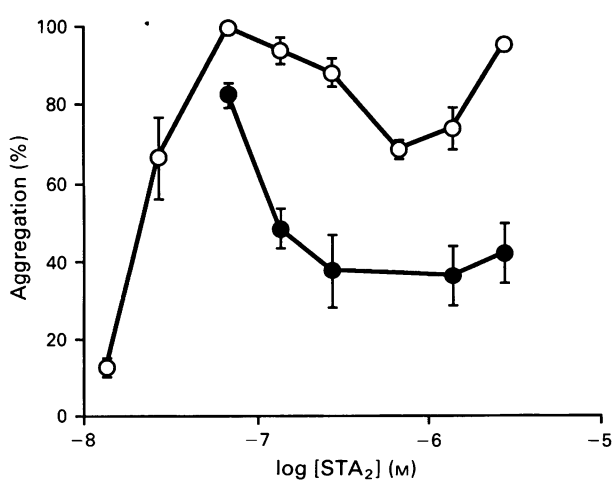

Figure 2 Washed human platelets: log concentration-response curves for STA_2 as an aggregating agent (○) and as an inhibitor of aggregation induced by platelet-activating factor (PAF) (100 nM) (●). The TP-receptor antagonist EP 092 (6 μM) was present in the PAF experiments. Each value is the mean from 4 experiments; vertical bars show s.e.mean.

Partial agonists Five prostanoids, EP 109, EP 167, EP 204, PTA_2 and 16,20-methano PTA_2 behaved in general as partial agonists, eliciting only shape change (EC_{50} values given in Table 2) or shape change and reversible aggregation (relative maxima in Table 2). Similar profiles were observed in platelet suspensions from aspirin-treated human donors. Of the three bicyclo[3:1.1]heptane analogues, EP 109 had the strongest agonist activity. It induced maximal shape change in all three platelet systems and was about twice as potent as U-46619. On rabbit platelets, EP 109 was also about twice as active as U-46619 with respect to aggregation and can be classed as a full agonist. Although EP 109 produced irreversible aggregation on some samples of human and rat platelets, it always had a shallower log CRC than U-46619 in these experiments; on other platelet samples EP 109 could only induce reversible aggregation waves (maximum concentration tested = 2 μM).

EP 167, EP 204 and PTA_2 inhibited irreversible aggregation induced by U-46619 with IC_{50} values respectively of 0.64 ± 0.08, 0.94 ± 0.06 and 0.10 ± 0.09 μM on human platelets, 0.55 ± 0.07, 1.11 ± 0.10 and 1.25 ± 0.05 μM on rat platelets and 0.95 ± 0.03, 0.98 ± 0.05 and 9.8 ± 1.5 μM on rabbit platelets. With the exception of PTA_2 on human platelets, these inhibitory effects were specific for U-46619, aggregation responses to PAF on human and rabbit platelets (100 and 10 nM respectively) and to ADP (1 μM) on rat platelets being unaffected (EP analogues at 1 μM and PTA_2 at 1–20 μM). PTA_2 at 50–500 nM markedly inhibited PAF-induced aggregation in human platelets.

16,20-methano PTA_2 (0.5–5 μM) elicited small shape change responses in each of the three platelet systems. Since the maximum effects were never greater than 50% of the U-46619 maxima it was decided to treat the compound as an antagonist (see later).

Antagonists Five of the seven antagonists studied, EP 092, ONO 11120, 16,20-methano PTA_2, EP 115 and BM 13177 inhibited aggregation induced by U-46619 without changing the nature of aggregation waves. In individual experiments parallel shifts in the U-46619 log concentration-response curve were obtained. Mean pA_2 values from 4 or 5 experiments together with ranges are given in Table 3. It can be seen that antagonist potencies are very similar on human and rat platelets and, with the exception of BM 13177, considerably lower on rabbit platelets.

Surmountable block of U-46619-induced aggregation, with parallel displacement of log concentration-response curves was also observed for the other two antagonists, EP 169 and AH 23848, on rabbit platelets. However these two compounds produced an unsurmountable block on both human (Figure 3) and rat platelets. In order to obtain at least some estimate of blocking potency, a pA_2 value was calculated for the lowest concentrations of EP 169 (10 nM) and AH 23848 (15 nM), where little change in the U-46619 aggregation profile was seen and parallel shifts of the U-46619 log concentration-response curves were obtained. These values are denoted by

Table 2 Potencies of TP-receptor partial agonists on human, rat and rabbit platelets and on guinea-pig trachea, rat aorta and rabbit aorta

	EC_{50} *for shape change* (nM) *and relative aggregation maximum*			EC_{50} *for contraction* (nM) *and relative maximum*		
Prostanoid	*Human platelets*	*Rat platelets*	*Rabbit platelets*	*Guinea-pig trachea*	*Rat aorta*	*Rabbit aorta*
U-46619 (full agonist)	4.8 ± 0.2 (100%)	5.5 ± 0.5 (100%)	7.2 ± 0.5 (100%)	7.8 ± 1.0 (100%)	20.2 ± 3.1 (100%)	12.3 ± 3.8 (100%)
EP 109	1.6 ± 0.4 (62–95%)	3.7 ± 0.6 (10–100%)	3.1 ± 0.5 (100%)	2.6 ± 0.9 (46–70%)	8.3 ± 0.7 (38–63%)	4.4 ± 1.2 (75–94%)
EP 167	21 ± 6 (3–22%)	33 ± 6 (12–16%)	76 ± 31 (5–47%)	57 ± 10 (62–78%)	80 ± 18 (55–59%)	61 ± 5 (58–66%)
EP 204	47 ± 7 (4–11%)	210 ± 17 (10–14%)	96 ± 23 (5–34%)	104 ± 25 (84–88%)	590 ± 270 (50–61%)	119 ± 6 (65–76%)
PTA_2	145 ± 43 (0%)	170 ± 55 (0%)	290 ± 44 (0–3%)	~500 (4–13%)	~400 (4–16%)	172 ± 33 (47–63%)
16,20-methano PTA_2	>5000* (0%)	>5000* (0%)	>5000* (0%)	—* (0%)	—* (0%)	640 ± 170 (22–42%)

EC_{50} values are means (± s.e.mean) for 5 experiments; on smooth muscle preparations EC_{50} values relate to the compound's own maximum. Relative maxima are expressed as ranges.
* pA_2 estimated by the Schild method (see Tables 3 and 4).

an asterisk in Table 3 to indicate that we have little, if any, evidence for reversible competitive antagonism by these two compounds.

The previously unreported compounds, 16,20-methano PTA_2, EP 115 and EP 169, each at 10 μM, did not inhibit PAF-induced aggregation of human and rabbit platelets nor ADP-induced aggregation of rat platelets.

Isolated smooth muscle preparations

Full agonists The potencies of a number of analogues on guinea-pig trachea, rat aorta and rabbit aorta were estimated in order to supplement published data. On guinea-pig trachea (5 μM AH 6809 present to block EP_1-receptors), rat aorta and rabbit aorta, mean EMR (U-46619 = 1.0, $n = 4$) were as follows: 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$ 1.2, 1.2, 1.6; MB 28767 4.7, 5.9, 3.0; 16,16-dimethyl PGE_2 (no value), 54, 30; 15-methyl $PGF_{2\alpha}$ 177, 180, 155.

Partial agonists EP 109, EP 167, EP 204 and PTA_2 behaved as partial agonists on guinea-pig trachea, rat aorta and rabbit aorta. EC_{50} values and relative maxima are given in Table 2. Established responses to each prostanoid of about 80% of its own maximum were abolished within 20 min of addition of 1 μM EP 092. 16,20-methano PTA_2 (0.1–10 μM) elicited no contractile activity on guinea-pig trachea and rat aorta and was treated as a pure antagonist (see next section). However on the rabbit aorta this compound consistently behaved as a partial agonist (Table 2).

Table 3 Potencies of TP-receptor antagonists on human, rat and rabbit platelets

	pA_2		
Antagonist	*Human*	*Rat*	*Rabbit*
EP 169	8.30* (8.22–8.37)	8.48* (8.32–8.61)	7.24 (7.20–7.28)
AH 23848	8.05* (7.92–8.33)	8.19* (8.02–8.39)	6.88 (6.86–6.97)
EP 092	7.73 (7.31–7.95)	7.80 (7.65–7.99)	6.71 (6.67–6.85)
ONO 11120	7.49 (7.41–7.57)	7.38 (7.35–7.81)	5.95 (5.93–5.96)
EP 115	6.98 (6.94–7.03)	7.00 (6.96–7.05)	6.15 (6.07–6.25)
16,20-methano-PTA_2	7.24 (7.17–7.35)	6.85 (6.77–6.95)	5.70 (5.66–5.78)
BM 13177	6.21 (6.15–6.27)	6.11 (6.08–6.11)	5.87 (5.83–5.91)

Values are means and ranges for 5 experiments on human and 4 experiments on rat and rabbit platelets. The agonist was U-46619.
* Indicates that the pA_2 is calculated for a single low concentration of antagonist at which block is surmountable; higher concentrations produce unsurmountable block.

Antagonists On individual preparations of guinea-pig trachea, rat aorta and rabbit aorta, six antagonists, EP 169, AH 23848, EP 092, ONO 11120, 16,20-methano PTA_2 and BM 13177, produced parallel shifts of the log concentration-

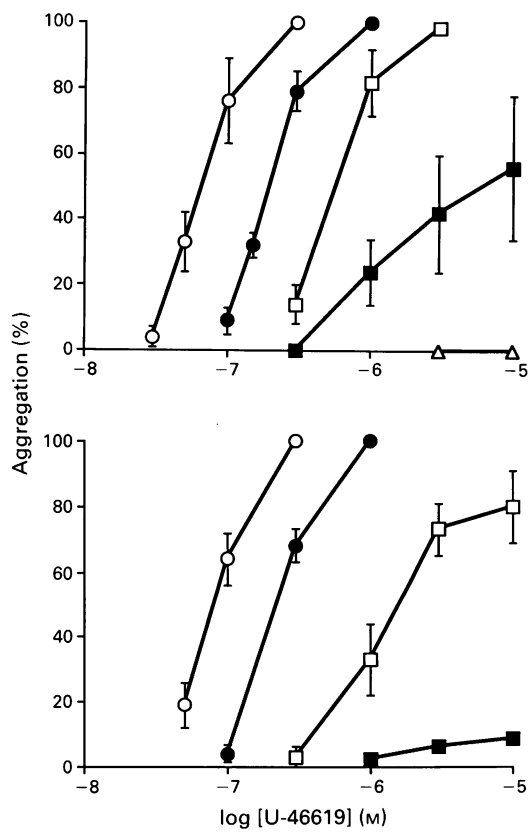

Figure 3 Unsurmountable antagonism of U-46619-induced aggregation in washed human platelets. In (a) the antagonist is EP 169 at 0 (○), 10 (●), 20 (□), 40 (■) and 200 (△) nM; in (b) the antagonist is AH 23848 at 0 (○), 15 (●), 30 (□) and 75 (■) nM. Each value is the mean of 4 experiments; vertical bars show s.e.mean.

Table 4 Potencies of TP-receptor antagonists on guinea-pig trachea, rat aorta and rabbit aorta

	Guinea-pig trachea		*Rat aorta*		*Rabbit aorta*	
Antagonist	pA_2	*Slope of Schild plot*	pA_2	*Slope of Schild plot*	pA_2	*Slope of Schild plot*
EP 169	8.77	1.00 ± 0.17	8.73	1.00 ± 0.17	7.16	1.27 ± 0.30
AH 23848	9.76	1.12 ± 0.20	8.47	0.96 ± 0.14	7.30	0.97 ± 0.17
GR 32191	9.43	0.97 ± 0.19	8.41	1.00 ± 0.20	—	—
EP 092	8.02	1.05 ± 0.12	8.55	0.95 ± 0.20	7.48	1.06 ± 0.14
ONO 11120	8.07	0.99 ± 0.21	7.14	0.89 ± 0.13	5.96	1.08 ± 0.19
16,20-methano PTA_2	7.14	1.01 ± 0.22	7.83	1.01 ± 0.15	partial agonist ($-\log EC_{50} = 6.2$)	
BM 13177	6.30	0.89 ± 0.17	6.25	1.12 ± 0.14	6.24	0.94 ± 0.11

U-46619 was used as the agonist.
The slope of the Schild plot is that of the least squares regression line (±95% confidence limits).

response curve to U-46619 with no suppression of the maximum response. For each antagonist/preparation no significant regression ($P > 0.05$) of pA_2 on log [antagonist] was found, indicating that the slope of the Schild plot did not differ from unity; pA_2 values and slopes of best fit (±95% confidence limits) for the Schild plot are given in Table 4. With the exception of BM 13177, pA_2 values were higher on the guinea-pig trachea and rat aorta than the rabbit aorta.

In view of the high potency of AH 23848 on guinea-pig trachea we decided to measure the potency of the chemically similar antagonist, GR 32191. Again very high TP-receptor antagonist potency was found on guinea-pig trachea (pA_2 = 9.43, Table 4). Our attempts to measure the potency of GR 32191 on rabbit aorta were thwarted by the complete insensitivity of this preparation to TP-receptor agonists over a period of about 6 months. It is of interest that jugular vein preparations from the same rabbits were highly responsive to TP-receptor agonists (U-46619 EC_{50} = 3 nM): GR 32191 block was surmountable and a pA_2 value of 7.22 was obtained.

Correlation of potencies

Potencies (log EMR) for full agonists on both the platelet and smooth muscle preparations are compared in Figure 4. The data include our previously published results for the smooth muscle preparations used in this study and for the dog saphenous vein (Jones *et al.*, 1982; 1989). Linear regression analysis gave highly significant correlations for all the 21 possible pairs of data sets: $r = 0.90$–0.99, $P < 0.01$.

For antagonists used in this study, pA_2s on human and rat platelets were highly correlated ($r = 0.98$, $P < 0.001$) and rabbit and rat platelet values were also well correlated ($r = 0.89$, $P < 0.01$). pA_2 values for rabbit aorta *versus* guinea-pig trachea, or human platelets or rat platelets, rat aorta *versus* rabbit platelets, and guinea-pig trachea *versus* rabbit platelets were not correlated ($P > 0.05$). The remaining 8 pairs of data sets showed weak correlations ($r = 0.77$–0.91, $P < 0.05$).

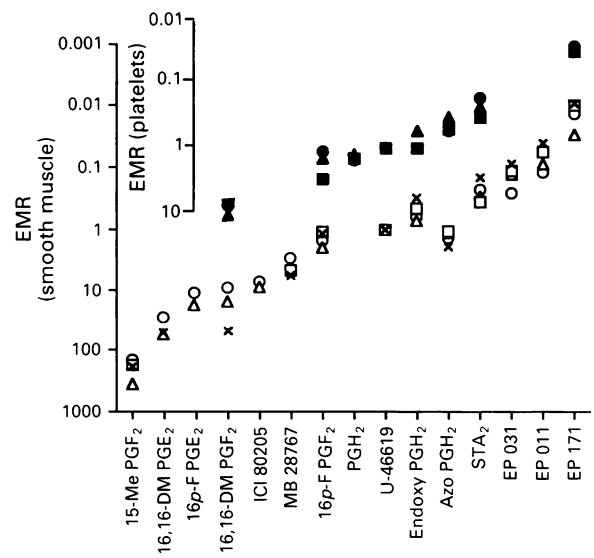

Figure 4 Comparison of TP-agonist potencies (equi-effective molar ratio, EMR, on log scale) for rabbit aorta (○), rat aorta (×), dog saphenous vein (△), guinea-pig trachea (□) and human (■), rat (●) and rabbit (▲) platelets. The platelet data have been displaced upwards for the sake of clarity.

Discussion

We contend that our results for full and partial agonist potencies on both the platelet and smooth muscle preparations (Jones *et al.*, 1982, 1989; these studies) provide no real evidence for the existence of TP-receptor subtypes.

With respect to the full agonists, both absolute and relative potencies are well correlated, particularly on the smooth muscle preparations where the EC_{50} values of the least (15-methyl $PGF_{2\alpha}$) and most potent (EP 171, Jones *et al.*, 1989) prostanoids differ by four orders of magnitude. Fewer agonists were tested on the platelet preparations, since some of the PGE analogues (ICI 80205, 16,16-dimethyl PGE_2 and MB 28767) with medium agonist potency at TP-receptors also potentiate both primary and secondary aggregation by an agonist action at a different prostanoid receptor (Jones & Wilson, 1990) (see Shio & Ramwell, 1972; Shio *et al.*, 1972 for the activity of PGE_2 in this respect).

STA_2 was found to have EC_{50} values for aggregation in the 15–30 nM range on human, rat and rabbit washed platelets and to be 3–5 times more potent than U-46619. These potencies agree well with data obtained on smooth muscle (Mais *et al.*, 1985; Toda *et al.*, 1986; Jones *et al.*, 1989) and thus there appear to be no major discrepancies in the agonist profile of STA_2. The only additional action (inhibition of PAF-induced aggregation in the presence of flurbiprofen) which might reduce its TP-agonist potency on platelets was found on human platelets and not rat or rabbit platelets. We believe this action is unlikely to reduce greatly the TP agonist potency of STA_2 on human platelets in view of the relative positions of the aggregatory and inhibitory log CRCs (Figure 2). However, if STA_2 were to be used as an agonist in antagonist studies on human platelets, its additional inhibitory action at the higher concentrations required to overcome the TP-receptor block could augment the block and the pA_2 would then be in error; U-46619 is clearly a more suitable agonist for this purpose. It is of interest that three TXA_2 analogues, CTA_2 and PTA_2 (Armstrong *et al.*, 1985) and STA_2 should show non-TP-receptor-mediated inhibitory actions on human platelets.

Turning to the stimulant actions of the partial agonists examined, there is also a close correspondence of the potency parameters (EC_{50} and relative maximum) of individual compounds on the six preparations investigated. On human platelets, irreversible inhibition of cyclo-oxygenase with aspirin did not significantly alter the partial agonist profiles. This is compatible with our previous experiments with the cyclo-oxygenase flurbiprofen on human PRP, where we showed that only a minor part of the irreversible aggregation and associated release reaction induced by U-46619 is due to endogenous prostaglandin endoperoxides and TXA_2 (Armstrong *et al.*, 1985). The novel analogues EP 167 and EP 204 differ from the usual prostanoids which exhibit TP-receptor agonist activity in not having a 15-hydroxyl group in the w-chain (Figure 1). In terms of inhibitory activity, EP 167 and EP 204 specifically blocked U-46619-induced aggregation on human, rat and rabbit platelets, with similar IC_{50} values. We have already mentioned the non-TP receptor inhibitory activity of PTA_2 on human platelets; this must preclude the use of its IC_{50} value from our comparisons. On rat and rabbit platelets however the inhibitory action of PTA_2 appears to be specific for TP-receptors and its greater inhibitory potency against U-46619 on rat as opposed to rabbit platelets would appear to reflect a genuine difference in its affinity for the respective TP-receptors.

The strongest evidence however for TP-receptor heterogeneity comes from our pA_2 measurements for TP antagonists. Of the 15 possible pairs of data sets, only human and rat platelets showed very close agreement of affinities. Although there was significant correlation of pA_2 values for rabbit platelets versus human or rat platelets, absolute values, with the exception of BM 13177, were lower on rabbit platelets. It is of interest that Swayne *et al.* (1988) have reported a good correlation between the potencies of nine TP-receptor antagonists on human and rabbit platelets and have used this result to postulate homogeneity of TP-receptors. However, although a highly significant correlation ($P < 0.01$ using a multiple linear correlation matrix) was obtained, actual IC_{50} values for inhibition of U-46619-induced aggregation were always much higher (13–600 fold!) on rabbit platelets than on human platelets. There is obviously a danger of using statistical correlation in this way to decide on the identity of receptors. It is almost certain that plasma protein binding contributes to these differences since the rabbit platelets were suspended in native plasma whereas the human platelets were in a buffer solution. It is difficult to make use of the Swayne data as the antagonist potencies are unlikely to be offset by a constant amount due to plasma protein binding.

Correlations between antagonist pA_2 values for all other pairs of preparations were found to be weak or not significant and the pA_2 values for some antagonists varied by 2 log units or more. Examination of the literature shows that other groups have also found widely differing affinities for TP antagonists on different preparations. On smooth muscle, pA_2 values for GR 32191 on 8 preparations varied between 7.14 and 8.72 (a range of 1.58 log units) (Lumley *et al.*, 1989); for the non-prostanoid TP-receptor antagonist SK&F 88046 on 4 preparations from 6.07 to 8.84 (2.77 log units) (Ohlstein *et al.*, 1988); for ICI 192605 on 6 preparations from 8.0 to 9.6 (1.6 log units) (Jessup *et al.*, 1988). In relation to comparing TP-receptors in smooth muscle and platelets, Mais *et al.* (1985) also demonstrated poor agreement of pA_2 values for 12 analogues of ONO 11120 on human washed platelets and dog saphenous vein (e.g. 8.74 and 6.78 respectively for the 16,17-thia analogue of ONO 11120). These authors state that 'the current evidence strongly supports the notion that the TXA_2/PGH_2 receptor in platelets is distinct from that in blood vessels'. We believe that a generalisation of this nature is of no value, since the differences in pA_2 values within each tissue category are themselves large enough to indicate TP-receptor heterogeneity.

An alternative explanation for these antagonist affinity differences is that of differential access to the receptor biophase. This concept is easier to apply to smooth muscle preparations than platelets, where the densely packed cell mass of the former may contain physical barriers or concentrating processes which could drastically reduced ligand concentrations in the extracellular fluid. There are however considerable similarities between the properties of the TP agonists, whose actions suggest receptor homogeneity, and the TP antagonists where the opposite is true. For example, the affinity of the most potent agonist EP 171 (Jones *et al.*, 1989) is of the same order as the more potent antagonists. All the ligands are of similar bulk (Figure 1) and cover a similar range of lipophilicity. For example STA_2 and EP 167 are highly lipophilic whereas 16,16-dimethyl $PGF_{2\alpha}$ is hydrophilic; EP 169 is lipophilic whereas BM 13177 is hydrophilic. The only obvious difference is that three of the antagonists, AH 23848 (Brittain *et al.*, 1985), GR 32191 and ONO 11120, will exist mainly as zwitterions in neutral aqueous solution. It seems more likely that small variations in the accessory binding sites for antagonists (say the substitution of a single amino acid) could account for the affinity differences: these structural differences would have minimal effect on the potencies of the full and partial agonists. Leff & Martin (1986) have discussed similar propositions in relation to the characterisation of 5-HT_2-like receptors and have raised the question as to whether antagonists chemically related to the natural agonist are of more use in defining subtypes as opposed to antagonists which bear little chemical relation. It is of interest that in our studies, the TP-receptor antagonist which is least prostanoid-like in structure, BM 13177 (Patscheke & Stegmeier, 1984), shows least variation in antagonist affinity. At present it would seem prudent not to subclassify the TP-receptor (TP_1-, TP_2-, etc.) on the basis of the rather limited antagonist potency data available.

Finally we wish to comment on the different antagonist profile of EP 169 and AH 23848 on human and rat platelets compared to rabbit platelets. It is possible that the apparently non-competitive nature of the block on human and rat platelets can be accounted for in terms of genuine reversible competition between agonist and antagonist, if agonist occupancy is incomplete at the time of response measurement due to slow dissociation of the antagonist. We have previously postulated that a similar mechanism could account for the slow irreversible aggregation waves to U-46619 seen on human platelets after treatment with high concentrations of EP 092 (Armstrong *et al.*, 1985). EP 169 and AH 23848 are more potent than EP 092 and they may well have smaller dissociation rate constants (k_2) than EP 092. Ligand binding studies indicate that potent TP antagonists can have half-lives for dissociation from TP-receptors compatible with our hypothesis. Armstrong *et al.* (1989) have shown that GR 32191 has a dissociation $t_{1/2}$ of at least 8 min in human washed platelets. GR 32191 also produces a marked slowing of U-46619 aggregation on human platelets, at concentrations slightly lower than those of AH 23848 used in the present study (Lumley *et al.*, 1989). These workers allowed up to 20 min for the agonist to overcome the block and analysed the results by the Schild procedure ($pA_2 = 8.78$, slope = 1.31). However, it is our experience of working with washed platelets that prolonged stirring alone can often lead to spontaneous aggregation and therefore one can never be entirely sure that the apparent surmountability of the block is entirely due to the dissociation of antagonist from TP-receptors. Clearly there are major difficulties in accurately estimating the pA_2 values of potent TP-receptor antagonists on platelet preparations using aggregation as the response.

References

ANDERSON, L. & MACINTYRE, D.E. (1982). Differences between thromboxane A_2 receptors on rabbit and human platelets. *Br. J. Pharmacol.*, **77**, 546P.

ARMSTRONG, R.A., JONES, R.L., PEESAPATI, V., WILL, S.G. & WILSON, N.H. (1985). Competitive antagonism at thromboxane receptors in human platelets. *Br. J. Pharmacol.*, **84**, 595–607.

ARMSTRONG, R.A., LUMLEY, P. & HUMPHREY, P.P.A. (1989). Characteristics of [^{3}H] GR 32191 binding to the thromboxane (TP) receptor of human platelets. *Br. J. Pharmacol.*, **98**, 843P.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonism. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BORN, G.R.V. (1962). Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature*, **194**, 927–929.

BRITTAIN, R.T., BOUTAL, L., CARTER, M.C., COLEMAN, R.A., COLLINGTON, E.W., GEISOW, H.P., HALLETT, P., HORNBY, E.J., HUMPHREY, P.P.A., JACK, D., KENNEDY, I., LUMLEY, P., MCCABE, P.J., SKIDMORE, I.F., THOMAS, M. & WALLIS, C.J. (1985). AH 23848: a thromboxane receptor-blocking drug that can clarify the pathophysiological role of thromboxane A_2. *Circulation*, **72**, 1208–1218.

JESSUP, C.L., JESSUP, R. & WAYNE, M. (1988). ICI 192605; a potent selective thromboxane A_2 receptor antagonist on smooth muscle. *Br. J. Pharmacol.*, **95**, 675P.

JONES, R.L., PEESAPATI, V. & WILSON, N.H. (1982). Antagonism of the thromboxane-sensitive systems of the rabbit aorta, dog saphenous vein and guinea-pig trachea. *Br. J. Pharmacol.*, **76**, 423–438.

JONES, R.L. & WILSON, N.H. (1990). An EP_3-receptor may mediate prostaglandin E-induced potentiation of aggregation in human platelets. *Br. J. Pharmacol.*, **101**, 522P.

JONES, R.L., WILSON, N.H. & LAWRENCE, R.A. (1989). EP 171: a high affinity thromboxane A_2 mimetic, the actions of which are slowly reversed by receptor blockade. *Br. J. Pharmacol.*, **96**, 875–887.

KATSURA, M., MIYAMOTO, T., HAMANAKA, N., KONDO, K., TERADA, T., OHGAKI, Y., KAWASAKI, A. & TSUBOSHIMA, M. (1983). *In vitro* and *in vivo* effects of new powerful thromboxane antagonists (3-alkylamino pinane derivatives). *Adv. Prostaglandin Thromboxane Leukotriene Res.*, **11**, 327–332.

LEFF, P. & MARTIN, G.R. (1986). Peripheral 5-HT_2-like receptors. Can they be classified with the available antagonists? *Br. J. Pharmacol.*, **88**, 585–593.

LUMLEY, P., WHITE, B.P. & HUMPHREY, P.P.A. (1989). GR 32191, a highly potent and specific thromboxane blocking drug on platelets and vascular and airways smooth muscle *in vitro*. *Br. J. Pharmacol.*, **97**, 783–794.

MACKAY, D. (1978). How should values of pA_2 and affinity constants for pharmacological competitive antagonists be calculated? *J. Pharm. Pharmacol.*, **30**, 312–313.

MAIS, D.E., SAUSSY, D.L., CHAIKHOUNI, A., KOCHEL, P.J., KNAPP, D.R., HAMANAKA, N. & HALUSHKA, P.V. (1985). Pharmacologic characterisation of human and canine thromboxane A_2/prostaglandin H_2 receptors in platelets and blood vessels: evidence for different receptors. *J. Pharmacol. Exp. Ther.*, **233**, 418–424.

NARUMIYA, S., OKUMA, M. & USHIKUBI, F. (1986). Binding of a radio-iodinated 13-azapinane thromboxane antagonist to platelets: correlation with antiaggregatory activity in different species. *Br. J. Pharmacol.*, **88**, 323–331.

OHLSTEIN, E.H., KOPIA, G.A., ZEID, R.L., VALOCIK, R.W., HOROHONICH, S., HIEBLE, J.P. & WASSERMAN, M.A. (1988). Effects of the thromboxane receptor antagonist SK&F 88046 in the canine, monkey and human coronary vasculature. *Prostaglandins*, **36**, 69–84.

PATSCHEKE, H. & STEGMEIER, K. (1984). Investigations on the selective nonprostanoic thromboxane antagonist BM 13177 in human platelets. *Thromb. Res.*, **33**, 277–288.

PORTER, N.A., BYERS, J.D., HOLDEN, K.M. & MENZYL, D.B. (1979). Synthesis of prostaglandin H_2. *J. Amer. Chem. Soc.*, **101**, 4319–4323.

RANDALL, M.J., PARRY, M.J., HAWKESWOOD, E., CROSS, P.E. & DICKINSON, R.P. (1981). UK-37248, a novel selective thromboxane synthetase inhibitor with anti-aggregatory and anti-thrombotic activity. *Thromb. Res.*, **23**, 145–162.

SHIO, H. & RAMWELL, P.W. (1972). Effect of prostaglandin E_2 and aspirin on the secondary aggregation of human platelets. *Nature, (New Biol.)*, **236**, 45–46.

SHIO, H., RAMWELL, P.W. & JESSUP, J. (1972). Prostaglandin E_2: Effects on aggregation, shape change and cAMP of rat platelets. *Prostaglandins*, **1**, 29–35.

SWAYNE, G.T.G., MAGUIRE, J., DOLAN, J., RAVAL, P., DANE, G., GREENER, M. & OWEN, D.A.A. (1988). Evidence for homogeneity of thromboxane A_2 receptor using structurally different antagonists. *Eur. J. Pharmacol.*, **152**, 311–319.

TODA, N., NAKAJIMA, M., OKAMURA, T. & MIYAZAKI, M. (1986). Interactions of thromboxane A_2 analogs and prostaglandins in isolated dog arteries. *J. Cardiovasc. Pharmacol.*, **8**, 818–825.

WILSON, N.H., JONES, R.L., MARR, C.G. & MUIR, G. (1988). Synthesis of oxabicyclo[2.2.1]heptane prostanoids having thromboxane-like activity at subnanomolar concentrations. *Eur. J. Med. Chem.*, **23**, 359–364.

WILSON, N.H., PEESAPATI, V., JONES, R.L. & HAMILTON, K. (1982). Synthesis of prostanoids with bicyclo[2.2.1]heptane, bicyclo[3.1.1]heptane, and bicyclo[2.2.2]octane ring systems. Activities of 15-hydroxy-epimers on human platelets. *J. Med. Chem.*, **25**, 495–500.

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Evidence that the 5-HT_3 receptors of the rat, mouse and guinea-pig superior cervical ganglion may be different

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1 Using grease-gap recordings from the isolated superior cervical ganglion of mouse, rat and guinea-pig, we have compared the depolarization evoked by 5-hydroxytryptamine (5-HT) with that evoked by the selective 5-HT_3 receptor agonist 2-methyl-5-HT (2-Me-5-HT).

2 The maximum depolarization induced by 2-Me-5-HT was smaller than that induced by 5-HT in all three species, and particularly in the guinea-pig.

3 The 5-HT_2 receptor antagonist ketanserin (1 μM) caused a clear rightward shift of the dose-response curve to 5-HT on the guinea-pig ganglion, but not on the mouse or rat ganglion. Spiperone (0.03 μM) had a quantitatively similar action to ketanserin (0.1 μM) on the 5-HT dose-response curve of the guinea-pig ganglion. Ketanserin had no significant effect on the dose-response curve to 2-Me-5-HT on any of these ganglia.

4 Using 2-Me-5-HT as the agonist, we determined the pA_2 values for two 5-HT_3 receptor antagonists. The potency of ICS 205-930 varied by approximately 100 fold between the species and that of (+)-tubocurarine varied by over 1000 fold. The differences in the pA_2 values of these compounds varied independently among the species.

5 We conclude that 5-HT_3 receptors are present on the superior cervical ganglion from the rat, mouse and guinea-pig, but these receptors may be pharmacologically distinct from each other. In addition, the depolarization of the guinea-pig superior cervical ganglion by low concentrations of 5-HT is largely mediated by ketanserin-sensitive receptors.

Introduction

The actions of 5-hydroxytryptamine (5-HT) on mammalian sympathetic ganglia were among its first effects on neurones to be reported (e.g. Trendelenburg, 1956). On the rabbit superior cervical ganglion, a 5-HT-induced fast depolarization was shown to be associated with the opening of channels permeable to sodium and potassium ions (Wallis & Woodward, 1975; Wallis & North, 1978). The fast depolarization of such ganglia is generally considered to be mediated by 5-HT_3 receptors given its sensitivity to the antagonists MDL 72222 (Fozard, 1984b) and ICS 205-930 (Richardson *et al.*, 1985). 5-HT_3 receptors also occur on parasympathetic, sensory and enteric neurones (Fozard, 1984a; Bradley *et al.*, 1986; Richardson & Engel, 1986), and there have been a number of recent reports of the presence of 5-HT_3 binding sites in the central nervous system (Kilpatrick *et al.*, 1987; Watling, 1988).

A number of reports has indicated that 5-HT_3 receptors may be heterogeneous (Bradley *et al.*, 1986; Round & Wallis, 1987; Richardson & Engel, 1986). The principal reasons for such conclusions are the differing pA_2 values of particular 5-HT_3 receptor antagonists on different tissues. For instance, MDL 72222 had a pA_2 of approximately 9 on the rabbit heart (Fozard, 1984b), about 8 on both the rabbit vagus and rabbit superior cervical ganglion (Azami *et al.*, 1985; Donatsch *et al.*, 1984), but on the guinea-pig ileum it was lower than 6 (Fozard, 1984b). Another antagonist ICS 205–930 had a pA_2 of approximately 10 on the heart, superior cervical ganglion and vagus nerve of the rabbit (Donatsch *et al.*, 1984; Round & Wallis, 1986), but between 7 and 8 on the guinea-pig ileum (Donatsch *et al.*, 1984; Richardson *et al.*, 1985; Lattimer *et al.*, 1989). The above evidence was cited by Richardson & Engel (1986) when they proposed three subtypes of 5-HT_3 receptor (5-HT_{3A}, 5-HT_{3B} and 5-HT_{3C}).

Since (+)-tubocurarine antagonizes 5-HT responses (Gerschenfeld & Paupardin-Tritsch, 1974; Nash *et al.*, 1984), including those of the rat superior cervical ganglion (Newberry & Gilbert, 1989), it was interesting to note that (+)-tubocurarine antagonized the 5-HT_3 response of NG 108-15 and N1E-115 cells (Yakel & Jackson, 1988; Peters *et al.*, 1990) in nanomolar concentrations, but 10–500 μM was needed to antagonize the fast depolarization of guinea-pig submucous plexus neurones (Surprenant & Crist, 1988) and guinea-pig coeliac ganglion cells (Wallis & Dun, 1988).

It is not clear whether the different sensitivities of the above 5-HT responses to the 5-HT_3 receptor antagonists were due to the use of different tissues and/or different species. We have studied the action of 5-HT and 2-methyl-5-HT (2-Me-5-HT) on a neuronal preparation isolated from three species with the aim of detecting possible species differences in 5-HT_3 receptors.

Methods

Male Sprague-Dawley rats (150–180 g, Bantin and Kingman), Swiss-Webster mice (18–30 g, Bantin and Kingman) and Duncan-Hartley guinea-pigs (300–450 g, Interfauna or Graystone) were killed by a blow to the head followed by exsanguination. Each superior cervical ganglion (SCG, sometimes called the cranial cervical ganglion in the guinea-pig) was desheathed and submerged in a three compartment bath with the ganglion body in the central compartment and the pre- and postganglionic trunks (the cervical sympathetic trunk and the internal carotid nerve, respectively) protruding through greased gaps into the outer two compartments (Newberry & Priestley, 1987; Newberry, 1988). The central compartment (volume ca. 0.5 ml) was continually superfused at 2.0–2.5 ml min^{-1} with an aqueous medium, at 25°C. The same medium in the outer two compartments was static. The medium, pre-equilibrated with 5% CO_2/95% O_2, contained (in mM): NaCl 125, $NaHCO_3$ 25, (+)-glucose 10, $CaCl_2$ 2.5, KCl 2, KH_2PO_4 1 and $MgSO_4$ 1. The ganglionic potential was recorded between two Ag/AgCl electrodes, one in the central compartment and the other in the compartment containing the internal carotid nerve.

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5-Hydroxytryptamine or 2-Me-5-HT was applied to the ganglia for 1 min periods via the superfusing medium. At the start of each experiment, 100 μM 5-HT was applied 2 to 3 times, at 30 min intervals, to obtain two consecutive responses of a similar size. All subsequent response amplitudes (measured at the peak of the fast depolarization) were normalised to the amplitude of the last of these applications (given the arbitrary value of 1). Approximately 30 min after the 'last' application of 100 μM 5-HT (see above) the agonist dose-response curve was determined by superfusing 5-HT in increasing concentrations at intervals of 10–20 min (the longer intervals being used for the higher concentrations). The antagonist was added to the superfusate 15 min after the agonist application which evoked the maximum response. The antagonist was superfused for 1 h and the dose-response curve was re-determined in its presence; one concentration of antagonist was used for each ganglion. The same protocol was used for controls except that the antagonist was not added to the medium. On the guinea-pig ganglion, however, the determination of the 5-HT dose-response curve (10 nM–1 mM) took a considerable amount of time. Therefore, in order to investigate the action of ketanserin or spiperone against 5-HT on this preparation (e.g. Figure 2), the following protocol was used. The antagonist superfusion was started 30 min after the 'last' application of 100 μM 5-HT. After 1 h in the antagonist the agonist dose-response curve was determined. The effect of the antagonist was judged by comparing such dose-response curves with those determined on other ganglia where the same protocol had been used but no antagonist added to the medium. To be consistent on the guinea-pig SCG (for Figure 2), we used the same protocol to study the action of ketanserin on the dose-response curve to 2-Me-5-HT. However, full dose-response curves to 2-Me-5-HT were determined before and after the 5-HT_3 antagonists on all SCGs.

The potency of an agonist on a given preparation was expressed as its pEC_{50} value. The potency of an antagonist was expressed as a pA_2 value (Schild, 1947), the negative logarithm (base 10) of the molar concentration of the antagonist that would give a dose-ratio of 2. This was routinely estimated by the equation: $pA_2 = \log_{10}(DR - 1) - \log_{10}$ (molar concentration of the antagonist). Since in the majority of cases (see Figure 4) the maximum response to 2-Me-5-HT was reduced in the presence of the antagonist, the pA_2 value provides only a convenient means of quantifying the potency of an antagonist; however, by using the term 'pA_2' we neither imply that the antagonist acted competitively nor claim that this value is an accurate representation of the true binding affinity of the compound for the receptor (like a pK_B). Dose-ratios (DR) were obtained at the level of half the maximum response in the second dose-response curve. The dose-ratio values used in the calculation were usually between 2 and 10, but they were not used if the maximum response to 2-Me-5-HT was reduced by more than 60%. Dose-ratios were not possible with the methodology used for 5-HT on the guinea-pig ganglion. For these studies, apparent dose-ratios were obtained from the increase in the geometric mean of the concentration that produced half the response of the standard 100 μM 5-HT application i.e. a relative depolarization level of 0.5.

Statistical analyses of ratios (relative depolarizations and dose-ratios) were carried out with geometric ($\log_{10}$) values, since ratios are more normally distributed using this transformation. Comparisons were made by Student-Neuman-Keuls multiple range test or the unpaired t test (BMDP Statistical Software Inc.), with significance at $P < 0.05$. The number of determinations refers to the number of experiments on different ganglia.

2-Me-5-HT hydrobromide and ICS 205-930 hydrochloride ((3α-tropanyl)-1H-indole-3-carboxylic acid ester) were prepared at Merck Sharp & Dohme, Terlings Park by Clare Kneen. (+)-Tubocurarine chloride and 5-hydroxytryptamine hydrochloride were obtained from Sigma, 1-phenylbiguanide from Aldrich and spiperone and ketanserin tartrate from Research Biochemicals Inc. Stock solutions (1–100 mM) of each compound were prepared and diluted to the required concentrations on each experimental day. Stock solutions were prepared in distilled water, except for spiperone which was dissolved in 5 mM HCl.

Results

Agonists

At a concentration which was maximal on the rat SCG, 5-HT (100 μM) reproducibly depolarized the isolated SCG from all three species. This response was much larger on the mouse SCG than on SCG from the rat or guinea-pig (Figure 1). Following the 5-HT-induced fast depolarization of the rat and mouse SCG, but not the guinea-pig SCG, there was a hyperpolarization.

Two putative selective 5-HT_3 receptor agonists were also tested on these ganglia, initially at 100 μM since this concentration evoked a maximal response on the rat SCG. 2-Me-5-HT (Richardson *et al.*, 1985; Bradley *et al.*, 1986) depolarized the SCG from each species, but the response of the guinea-pig SCG was much smaller than that to 100 μM 5-HT (Figure 1). At 100 μM, phenylbiguanide (Fortune *et al.*, 1983) depolarized three mouse SCG by 0.88, 0.82 and 0.95 of the response to 100 μM 5-HT. On four rat SCG it evoked relative depolarizations of 1.21, 0.76, 0.93 and 0.93. By contrast, phenylbiguanide evoked no detectable depolarization of three guinea-pig

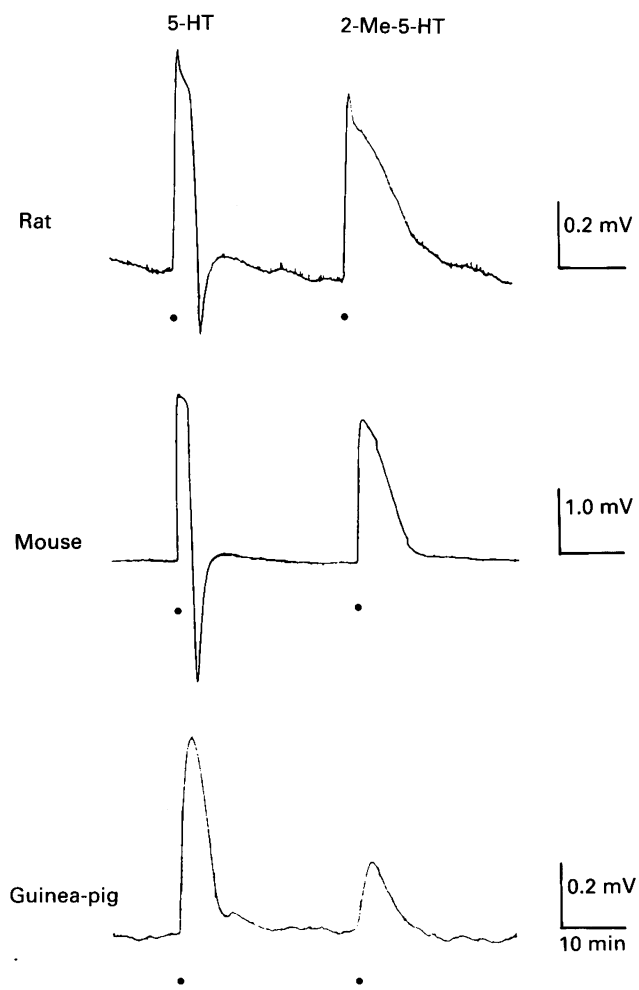

Figure 1 Chart recordings of the depolarizing responses to 5-hydroxytryptamine (5-HT) and 2-methyl-5-HT (2-Me-5-HT) on the isolated superior cervical ganglion from the indicated species. Each agonist was superfused at 100 μM for 1 min at the time indicated by the dot. The time calibration is the same for all records.

Table 1 The potencies and relative maximum responses to 5-hydroxytryptamine (5-HT) and 2-methyl-5-HT (2-Me-5-HT) on the superior cervical ganglia from three species

Agonist pharmacology of ganglionic depolarization

	Response to 100 μM 5-HT (mV)	*Relative maxima* 5-HT	*Relative maxima* 2-Me-5-HT	pEC_{50} 5-HT	pEC_{50} 2-Me-5-HT	*Repeat dose-ratios* 5-HT	*Repeat dose-ratios* 2-Me-5-HT
Rat							
Mean	**0.56**	**1.07**	**0.91**	**5.26**	**5.39**	**1.04**	**0.69**
+ s.d.	0.21	0.07	0.08	0.10	0.12	0.26	0.19
− s.d.	0.21	0.07	0.07	0.10	0.12	0.21	0.15
n	19	16	19	16	19	8	4
Mouse							
Mean	**2.44**	**1.10**	**0.90**	**5.32**	**5.16**	**1.13**	**0.81**
+ s.d.	1.00	0.09	0.08	0.26	0.15	0.48	0.29
− s.d.	1.00	0.08	0.07	0.26	0.15	0.34	0.21
n	39	11	28	11	28	7	8
Guinea-pig							
Mean	**0.62**	**1.27**	**0.81**	**5.08**	**4.17**	**ND**	**0.95**
+ s.d.	0.30	0.13	0.13	0.16	0.11	—	0.11
− s.d.	0.30	0.12	0.11	0.16	0.11	—	0.10
n	59	6	11	6	11	—	4

Potencies are expressed as pEC_{50} values ($-\log_{10}$ of the molar concentration required to evoke a half-maximal response). Relative maxima are in arbitrary units compared to the amplitude of the last response to 100 μM 5-HT at the start of the experiment (given the value of 1, see Methods). The range of absolute values of this particular response is indicated. The reproducibility of the 5-HT and 2-Me-5-HT dose-response curves are indicated by the dose-ratios obtained by repeating the dose-response protocol in the absence of an antagonist. ND = not done because of the different protocol (see Methods). Values are arithmetic means for *Response to 5-HT* and *pEC_{50}* and geometric means for *Relative maxima* and *Repeat dose-ratios*, hence the differences in the +s.d. and −s.d. values. The relative maxima and pEC_{50} values for 2-Me-5-HT and 5-HT, when compared for the ganglia from each individual species, were all significantly different.

SCG at this concentration. The phenylbiguanide responses of the rat and mouse SCG were both biphasic: a depolarization followed by a hyperpolarization (see Figure 6 of Newberry & Gilbert, 1989).

Dose-response curves to 5-HT and 2-Me-5-HT were determined on the SCG from each species. Their potencies in evoking depolarization and their relative maximum responses are shown in Table 1. The reproducibility of these dose-response curves was demonstrated by repeating the dose-response curve 1.25 h after the first determination. The 5-HT dose-response curve on the guinea-pig SCG occurred between 10 nM and 1 mM which was clearly a greater concentration-range than on the rat or mouse SCG. The maximum response to 2-Me-5-HT was significantly smaller than that to 5-HT on all SCG, especially that from the guinea-pig. In each species, the potency of 5-HT was significantly different from that of 2-Me-5-HT.

5-HT_2 receptor antagonists

We investigated the action of 1 μM ketanserin on the 5-HT dose-response curves of these ganglia. Its only clear action was against the 5-HT dose-response curve on the guinea-pig SCG where it caused a rightward shift of the dose-response curve (Figure 2). The resultant curve was steeper and had a reduced maximum. In contrast, the dose-response curves to 2-Me-5-HT on each SCG were not significantly affected by ketanserin.

Although not studied in great detail, the effect of ketanserin (1 μM) on the dose-response curve to 5-HT on the guinea-pig SCG was mimicked by spiperone (0.3 μM, $n = 5$, not shown). This similarity of action was also apparent at lower concentrations of spiperone and ketanserin (Figure 3). The geometric mean concentration (± s.d.) of 5-HT necessary to evoke a response level of 0.5 was 4.0 μM (+0.6, −0.5; $n = 6$) in the absence of an antagonist. This value increased to 34 μM (+14, −11; $n = 5$) in 0.1 μM ketanserin and to 32 μM (+11, −7; $n = 4$) in 0.03 μM spiperone.

5-HT_3 receptor antagonists

The antagonist potencies of two structurally unrelated 5-HT_3 receptor antagonists, ICS 205-930 and (+)-tubocurarine, were evaluated on the SCG from each species, with 2-Me-5-HT as agonist. Except on the guinea-pig SCG, tubocurarine produced a parallel shift of the dose-response curve. In contrast, ICS 205-930 flattened the dose-response curve on all SCGs. This type of action precluded the study of a wide range of concentrations. The calculated pA_2 values (see Methods) of both antagonists varied with species (Table 2). In a given species, however, the pA_2 values did not change significantly over the effective concentration-range. Real differences in the threshold concentration of an antagonist were apparent as the concentration-ranges tested often included a concentration that gave no significant shift of the dose-response curve. For instance, 1 nM ICS 205-930 had no effect on a mouse SCG, but it reproducibly antagonized 2-Me-5-HT on the rat SCG. The differences in potency of these compounds are clearly suggested in Figure 4 which shows graphs from individual experiments. There is a potency difference between the three species of approximately 100 fold for ICS 205-930 and greater than 1000 fold for (+)-tubocurarine. Since these differences could be due to the antagonist reaching equilibrium conditions at different rates on the three preparations, we tested the possibility on the rat and mouse SCG where ICS 205-930 had a relatively high pA_2. On these preparations a 2 h incubation of ICS 205-930 resulted in no greater antagonism compared to that observed after a 1 h pretreatment.

The presence of a ketanserin-sensitive response would have affected the calculated pA_2 values for a 5-HT_3 receptor antagonist against 5-HT or 2-Me-5-HT. This was clearly demonstrable on the guinea-pig SCG where 1 μM ICS 205-930 had no effect on the dose-response curve to 5-HT ($n = 2$) unless the preparation was superfused with ketanserin (1 μM) throughout the experiment. In three experiments in the presence of ketanserin, ICS 205-930 (1 μM) produced dose-ratios of 17, 15 and 7. From the data in Figure 2, it was possible that the response to 2-Me-5-HT on the guinea-pig SCG was

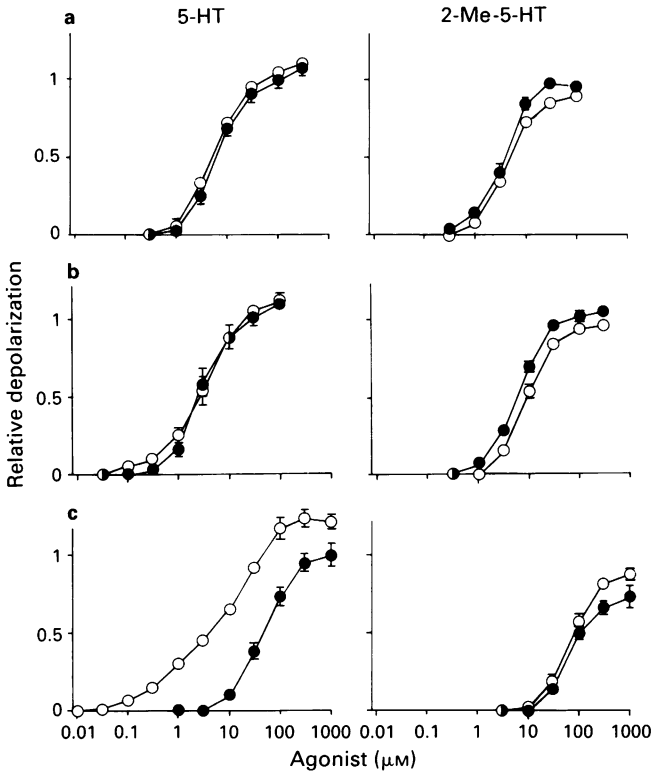

Figure 2 The effect of 1 μM ketanserin (1 h) on the dose-response curves to 5-hydroxytryptamine (5-HT) and 2-methyl-5-HT (2-Me-5-HT) on the superior cervical ganglia (SCG) from (a) rat, (b) mouse and (c) guinea-pig. Control (○); ketanserin (●). Since the agonist dose-response curves were reproducible (Table 1), the dose-response curves before (controls) and after ketanserin were determined on the same rat and mouse SCG. However, for reasons highlighted in the Methods, the control dose-response curves for the guinea-pig SCG were obtained from different animals. All responses are expressed as the relative depolarization compared to the depolarization of the last application of 100 μM 5-HT at the beginning of each experiment (see Methods). Relative depolarization data are the geometric means with s.e.mean shown by vertical bars. The number of determinations was the same for both curves in each graph: (a) left 8, (a) right 4; (b) left 4, (b) right 4; (c) left 6 and (c) right 7. Ketanserin did not significantly reduce the response to 10 μM–1 mM 2-Me-5-HT on the guinea-pig SCG (with the exception of the response to 300 μM). Ketanserin significantly reduced the response to 0.3 μM 5-HT on the mouse SCG (by 50–80%). Unfortunately, the magnitude of this effect is masked by the size of the symbols and the scale of the y axis, but further experiments are necessary before any clear conclusions can be reached.

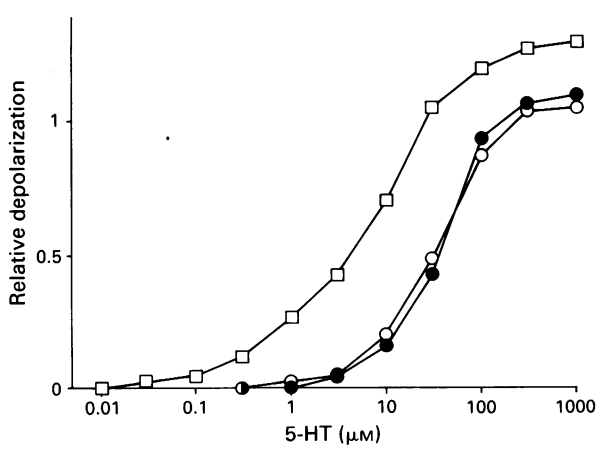

Figure 3 The effect of spiperone (0.03 μM, ●) and ketanserin (0.1 μM, ○), both for 1 h, on the 5-hydroxytryptamine (5-HT) dose-response curve of the guinea-pig superior cervical ganglion. Data from individual ganglia: those treated with spiperone or ketanserin were from the same guinea-pig, the untreated ganglion (□) from another.

Table 2 The pA_2 values of ICS 205-930 and (+)-tubocurarine against 2-methyl-5-hydroxytryptamine (2-Me-5-HT) on the superior cervical ganglion from three species

	Antagonist pharmacology of ganglionic depolarization to 2-methyl-5-HT			
	ICS 205-930		*(+)-Tubocurarine*	
	Concn range	*pA_2*	*Concn range*	*pA_2*
	(low, high) *n*	Mean (± s.d.) *n*	(low, high) *n*	Mean (± s.d.) *n*
Rat	0.3 nM	9.4	300 nM	7.1
	1.0 nM	0.2	3 μM	0.1
	7	5	6	6
Mouse	1 nM	8.7	3 nM	8.1
	10 nM	0.2	100 nM	0.1
	7	6	7	6
Guinea-pig	100 nM	7.2	1 μM	4.8
	300 nM	0.1	100 μM	0.2
	6	6	9	7

The pA_2 values were calculated from the Schild equation (1947, see Methods). The pA_2 values of each compound are significantly different between all species. The concentration-range often includes a concentration which produced no significant shift of the dose-response curve i.e. no greater than the repeat dose-ratios from Table 1. Consequently, the *n* value for this column is larger than that for the number of times that a pA_2 was calculated. The pA_2 values have been rounded to one decimal place but they have not been corrected for the small sensitivity shifts indicated in Table 1.

reduced by ketanserin. In the presence of 1 μM ketanserin, however, the mean pA_2 value for (+)-tubocurarine against 2-Me-5-HT was 4.7 ± 0.2 ($n = 4$), which was not significantly different from that determined in the absence of ketanserin (see Table 2).

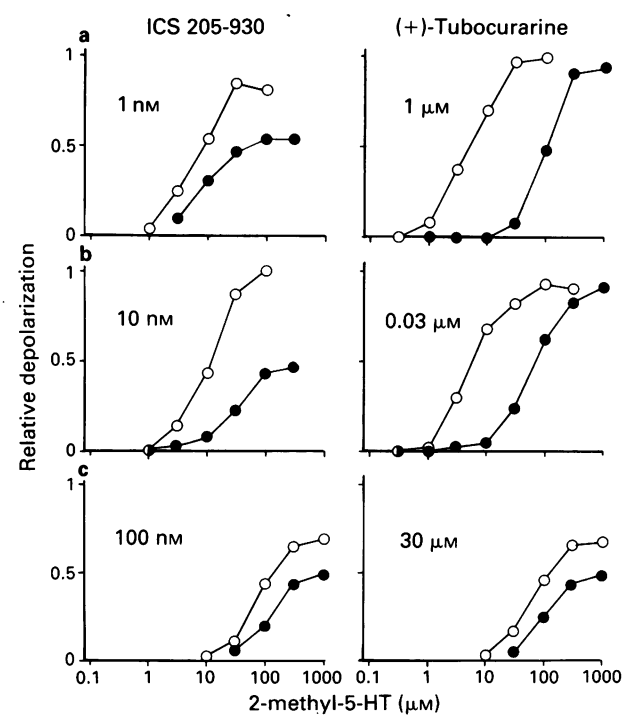

Figure 4 Graphs from individual experiments showing the effect of the indicated concentrations of the two $5\text{-}HT_3$ receptor antagonists (1 h, ●) on the dose-response curve to 2-methyl-5-hydroxytryptamine (2-Me-5-HT, ○) on the superior cervical ganglion from (a) rat, (b) mouse and (c) guinea-pig.

At the concentrations tested, none of the 5-HT_2 or 5-HT_3 receptor antagonists evoked a noticeable change in the ganglionic potential.

Discussion

The principal finding of this study is that the 5-HT_3 receptors of the rat, mouse and guinea-pig SCG appear to be pharmacologically distinguishable with the antagonists ICS 205-930 and (+)-tubocurarine. For these experiments, we used 2-Me-5-HT as the agonist of choice since there was a ketanserin-sensitive response to 5-HT on the guinea-pig SCG.

The 5-HT induced depolarization of the mouse SCG was followed by two other potentials, as seen on the rat SCG (Newberry & Gilbert, 1989). By analogy, therefore, it is possible that 5-HT can evoke on the mouse SCG a 5-HT_3 receptor-mediated fast depolarization with an underlying 5-HT_{1A} receptor-mediated hyperpolarization, followed by a slow depolarization that is mediated by an unidentified receptor. In contrast, the 5-HT depolarization of the guinea-pig SCG was not followed by a hyperpolarization.

2-Me-5-HT (Richardson *et al.*, 1985; Bradley *et al.*, 1986) evoked a smaller depolarization than 5-HT in all three species, particularly in the guinea-pig. A smaller maximum response to this agonist has also been seen on the rat isolated vagus nerve (Ireland & Tyers, 1987). Phenylbiguanide (100 μM) depolarized the SCG from the rat and mouse, but not the guinea-pig. This compound has been reported to be a partial agonist of 5-HT_3 receptors on the rat vagus (Ireland & Tyers, 1987), but it failed to depolarize the guinea-pig vagus (Burridge *et al.*, 1989). A species, rather than a preparation, difference in 5-HT_3 receptors was therefore suspected. It is possible, however, that marked variations in the substrate specificity of the 5-HT uptake systems of the SCG of these species (cf. Ireland *et al.*, 1987) could contribute to the different potencies of these agonists.

The depolarization of mammalian sympathetic ganglia by 5-HT is generally considered to be mediated by 5-HT_3 receptors because of its sensitivity to antagonists such as MDL 72222 and ICS 205-930 (Fozard, 1984b; Azami *et al.*, 1985; Richardson *et al.*, 1985; Round & Wallis, 1986; 1987). Moreover, the 5-HT-induced depolarization of the rabbit superior cervical ganglion (Nash *et al.*, 1984) and its preganglionic axons (Elliot & Wallis, 1988) is resistant to the 5-HT_2 receptor antagonists, ketanserin (Leysen *et al.*, 1981) and cyproheptadine. However, ketanserin shifted the dose-response curve to 5-HT on the guinea-pig SCG to the right (see Figures 2 and 3). Since ketanserin also has affinity for 5-HT_{1C} binding sites (Hoyer *et al.*, 1985), we investigated the action of spiperone (spiroperidol) which has a high affinity for 5-HT_2 receptors, but a relatively low affinity for 5-HT_{1C} receptors (Hoyer *et al.*, 1985). The similar and highly potent action of spiperone and ketanserin on the 5-HT dose-response curve of the guinea-pig ganglion (Figure 3) indicates that a large part of the 5-HT dose-response curve on this preparation may be mediated by 5-HT_2, rather than 5-HT_{1C}, receptors. This conclusion is not unreasonable given that other workers have suggested that 5-HT_2 receptors mediate neuronal depolarization and/or excitation (Davies *et al.*, 1987; 1988; North & Uchimura, 1989). The possible presence of 5-HT_2 and 5-HT_3 receptors on the guinea-pig SCG may correspond to the fast and slow depolarizations evoked by 5-HT on another sympathetic ganglion from this species, the coeliac ganglion (Wallis & Dun, 1988).

The effects of the 5-HT_3 receptor antagonists, ICS 205-930 and (+)-tubocurarine, were determined with 2-Me-5-HT as the agonist, given that the ganglionic depolarization to this agonist was demonstrated to be resistant to ketanserin in all three species. There were differences in the pA_2 values obtained with ICS 205-930 and (+)-tubocurarine between these species, although there was not a common trend i.e. the antagonist potency of ICS 205-930 ranked rat > mouse > guinea-pig, whereas for (+)-tubocurarine it ranked mouse > rat > guinea-pig.

Our data add to other reports showing a marked similarity of pA_2 values for ICS 205-930 as an antagonist of 5-HT_3 receptor-mediated responses on different tissues from the guinea-pig, e.g. it has a pA_2 of between 7 and 8 on the superior cervical ganglion (this report), vagus (Lattimer *et al.*, 1989; Burridge *et al.*, 1989) and ileum (Donatsch *et al.*, 1984; Lattimer *et al.*, 1989). Other antagonists, e.g. MDL 72222 and GR38032F, also have similar pA_2 values on various tissues from the guinea-pig (Burridge *et al.*, 1989; Grossman *et al.*, 1989; Lattimer *et al.*, 1989). It may therefore be concluded that the 5-HT_3 receptors at many sites in this species are similar. From the pA_2 values for ICS 205-930 on the vagus of the guinea-pig and the rat, it has recently been suggested that the 5-HT_3 receptors in these species are different (Burridge *et al.*, 1989; Lattimer *et al.*, 1989). Our work with ICS 205-930 on the SCG from those species would support such a suggestion.

Tubocurarine provided more conclusive evidence for species differences in 5-HT_3 receptors. The separation of its antagonist potencies between the species was considerably greater than for ICS 205-930. In contrast to ICS 205-930, it produced a parallel shift of the dose-response curve to 2-Me-5-HT on the rat and mouse (but not the guinea-pig) SCG, indicating that it may act competitively. Evidence supporting this type of action comes from work on neuroblastoma cells where its antagonism of the 5-HT_3 receptor-induced fast inward current was not voltage- or agonist-dependent (Peters *et al.*, 1990). In addition to a competitive action, (+)-tubocurarine may antagonize non-competitively since on rabbit nodose ganglion cells (Higashi & Nishi, 1982) it produced a parallel shift of the 5-HT dose-response curve, followed at higher concentrations, by a depression of the maximum response.

It should be noted that on guinea-pig submucous plexus neurones (Surprenant & Crist, 1988) 5-HT and 2-Me-5-HT evoked a ketanserin-resistant depolarization, associated with an increase in membrane resistance. This response was unaffected by (or indeed better observed in the presence of) ICS 205-930 (2 μM) or tubocurarine (200 μM). The pharmacology of this response would appear to be similar to that evoked by 2-Me-5-HT on the guinea-pig SCG. However, we do not believe that these responses are mediated by the same receptor since 2 μM ICS 205-930 or 200 μM tubocurarine would have substantially antagonized the response to 2-Me-5-HT on the guinea-pig SCG.

Recent electrophysiological experiments have shown that different types of channel can be activated by 5-HT_3 receptors. Thus, the channels activated by 5-HT on guinea-pig submucous plexus neurones have conductances of 9 and 15 pS (Derkach *et al.*, 1989), whereas the 5-HT_3-induced channel conductance on N1E-115 cells is 0.3 pS (Lambert *et al.*, 1989). Such work provides additional evidence for heterogeneity among 5-HT_3-receptor ion channel complexes.

In conclusion, superior cervical ganglia from rat, mouse and guinea-pig respond differently to agonists and antagonists of 5-HT receptors. The ganglionic depolarization to 5-HT cannot be assumed to be mediated solely by 5-HT_3 receptors since a 5-HT_2 receptor mediated depolarization may dominate the response to low concentrations of 5-HT on the guinea-pig SCG. The action of the selective 5-HT_3 receptor agonist, 2-Me-5-HT, showed that 5-HT_3 receptors do appear to mediate sympathetic ganglionic depolarization in each of these species. The affinities of the 5-HT_3 receptor antagonists, ICS 205-930 and (+)-tubocurarine, varied dramatically and independently among these species. These results suggest that the 5-HT_3 receptors in the rat, mouse and guinea-pig superior cervical ganglion may be pharmacologically distinct.

Note added in proof

Our conclusion that 5-HT_3 receptors may differ between species is further supported by recent data from two other research groups. The pharmacology of the 5-HT_3 receptor on the guinea-pig vagus clearly differs from that of the rat vagus (Butler *et al.*, 1990; *Br. J. Pharmacol.*, **101**, 591–598). In addition, the 5-HT_3 receptors mediating an inward current in rabbit and guinea-pig dissociated nodose ganglion cells are pharmacologically distinguishable (Peters, Malone & Lambert, personal communication). Like us, these groups detected differences in the potencies of 5-HT_3 antagonists on the same preparation taken from separate species. Interestingly, Peters *et al.* noted that (+)-tubocurarine was 90 times more potent on cells taken from rabbits as compared to guinea-pigs, its pIC_{50} on guinea-pig cells (5.0) being comparable to our pA_2 (4.8).

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References

AZAMI, J., FOZARD, J.R., ROUND, A.A. & WALLIS, D.I. (1985). The depolarising action of 5-hydroxytryptamine on rabbit vagal primary afferent and sympathetic neurones and its selective blockade by MDL 72222. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **328**, 423–429.

BRADLEY, P.B., ENGEL, G., FENIUK, W., FOZARD, J.R., HUMPHREY, P.P.A., MIDDLEMISS, D.N., MYLECHARANE, E.J., RICHARDSON, B.P. & SAXENA, P.R. (1986). Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. *Neuropharmacology*, **25**, 563–576.

BURRIDGE, J., BUTLER, A. & KILPATRICK, G.J. (1989). 5-HT_3 receptors mediate depolarisation of the guinea-pig isolated vagus nerve. *Br. J. Pharmacol.*, **96**, 269P.

DAVIES, M.F., DIESZ, R.A., PRINCE, D.A. & PEROUTKA, S.J. (1987). Two distinct effects of 5-hydroxytryptamine on single cortical neurones. *Brain Res.*, **423**, 347–352.

DAVIES, M., WILKINSON, L.S. & ROBERTS, M.H.T. (1988). Evidence for excitatory 5-HT_2-receptors on rat brainstem neurones. *Br. J. Pharmacol.*, **94**, 483–491.

DERKACH, V., SURPRENANT, A. & NORTH, R.A. (1989). 5-HT_3 receptors are membrane ion channels. *Nature*, **339**, 706–709.

DONATSCH, P., ENGEL, G., RICHARDSON, B.P. & STADLER, P. (1984). ICS 205-930: a highly selective and potent antagonist at peripheral neuronal 5-hydroxytryptamine (5-HT) receptors. *Br. J. Pharmacol.*, **81**, 34P.

ELLIOTT, P. & WALLIS, D.I. (1988). The depolarizing action of 5-hydroxytryptamine on rabbit isolated preganglionic cervical sympathetic nerves. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **338**, 608–615.

FORTUNE, D.H., IRELAND, S.J. & TYERS, M.B. (1983). Phenylbiguanide mimics the effects of 5-hydroxytryptamine on the rat isolated vagus nerve and superior cervical ganglion. *Br. J. Pharmacol.*, **79**, 298P.

FOZARD, J.R. (1984a). Neuronal 5-HT receptors in the periphery. *Neuropharmacology*, **23**, 1473–1486.

FOZARD, J.R. (1984b). MDL 72222: a potent and highly selective antagonist at neuronal 5-hydroxytryptamine receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **326**, 36–44.

GERSCHENFELD, H.M. & PAUPARDIN-TRITSCH, D. (1974). On the transmitter function of 5-hydroxytryptamine at excitatory and inhibitory monosynaptic junctions. *J. Physiol.*, **243**, 457–481.

GROSSMAN, C.J., BUNCE, K.T. & HUMPHREY, P.P.A. (1989). Investigation of the 5-HT_3 receptors in guinea-pig descending colon. *Br. J. Pharmacol.*, **97**, 451P.

HIGASHI, H. & NISHI, S. (1982). 5-Hydroxytryptamine receptors of visceral primary afferent neurones on rabbit nodose ganglia. *J. Physiol.*, **323**, 543–567.

HOYER, D., ENGEL, G. & KALKMAN, H.O. (1985). Molecular pharmacology of 5-HT_1 and 5-HT_2 recognition sites in rat and pig brain membranes: radioligand binding studies with [^{3}H]5-HT, [^{3}H]8-OH-DPAT, (−)[^{125}I]Iodocyanopindolol, [^{3}H]mesulergine and [^{3}H]ketanserin. *Eur. J. Pharamcol.*, **118**, 13–23.

IRELAND, S.J., STRAUGHAN, D.W. & TYERS, M.B. (1987). Influence of 5-hydroxytryptamine uptake on the apparent 5-hydroxytryptamine antagonist potency of metoclopramide in the rat isolated superior cervical ganglion. *Br. J. Pharmacol.*, **90**, 151–160.

IRELAND, S.J. & TYERS, M.B. (1987). Pharmacological characterisation of 5-hydroxytryptamine-induced depolarisation of the rat isolated vagus nerve. *Br. J. Pharmacol.*, **90**, 229–238.

KILPATRICK, G.J., JONES, B.J. & TYERS, M.B. (1987). Identification and distribution of 5-HT_3 receptors in rat brain using radioligand binding. *Nature*, **330**, 746–748.

LAMBERT, J.J., PETERS, J.A., HALES, T.G. & DEMPSTER, J. (1989). The properties of 5-HT_3 receptors in clonal cell lines studied by patch-clamp techniques. *Br. J. Pharmacol.*, **97**, 27–40.

LATTIMER, N., RHODES, K.F. & SAVILLE, V.L. (1989). Possible differences in 5-HT_3-like receptors in the rat and the guinea-pig. *Br. J. Pharmacol.*, **96**, 270P.

LEYSEN, J.E., AWOUTERS, F., KENNIS, L., LADURON, P.M., VANDENBERK, J. & JANSSEN, P.A.J. (1981). Receptor binding profile of R 41 468, a novel antagonist at 5-HT_2 receptors. *Life Sci.*, **28**, 1015–1022.

NASH, H.L., WALLIS, D.I. & ASH, G. (1984). 5-HT antagonists and blockade of neuronal (5-HT) receptors on ganglion cells. *Gen. Pharmacol.*, **15**, 339–344.

NEWBERRY, N.R. (1988). M1 and M2 receptors mediate different effects on synaptically evoked potentials of the rat superior cervical ganglion. *Neurosci. Lett.*, **88**, 100–106.

NEWBERRY, N.R. & GILBERT, M.J. (1989). 5-Hydroxytryptamine evokes three distinct responses on the rat superior cervical ganglion *in vitro*. *Eur. J. Pharmacol.*, **162**, 197–205.

NEWBERRY, N.R. & PRIESTLEY, T. (1987). Pharmacological differences between two muscarinic responses of the rat superior cervical ganglion *in vitro*. *Br. J. Pharmacol.*, **92**, 817–826.

NORTH, R.A. & UCHIMURA, N. (1989). 5-Hydroxytryptamine acts at 5-HT_2 receptors to decrease potassium conductance in rat nucleus accumbens neurones. *J. Physiol.*, **417**, 1–12.

PETERS, J.A., MALONE, H.M. & LAMBERT, J.J. (1990). Antagonism of 5-HT_3 receptor mediated currents in murine N1E-115 neuroblastoma cells by (+)-tubocurarine. *Neurosci. Lett.*, **110**, 107–112.

RICHARDSON, B.P. & ENGEL, G. (1986). The pharmacology and function of 5-HT_3 receptors. *Trends Neurosci.*, **9**, 424–428.

RICHARDSON, B.P., ENGEL, G., DONATSCH, P. & STADLER, P.A. (1985). Identification of serotonin M-receptor subtypes and their specific blockade by a new class of drugs. *Nature*, **316**, 126–131.

ROUND, A. & WALLIS, D.I. (1986). The depolarising action of 5-hydroxytryptamine on rabbit vagal afferents and sympathetic neurones *in vitro* and its selective blockade by ICS 205-930. *Br. J. Pharmacol.*, **88**, 485–494.

ROUND, A. & WALLIS, D.I. (1987). Further studies on the blockade of 5-HT depolarisations of rabbit vagal afferent and sympathetic ganglion cells by MDL 72222 and other antagonists. *Neuropharmacology*, **26**, 39–48.

SCHILD, H.O. (1947). pA, a new scale for the measurement of drug antagonism. *Br. J. Pharmacol. Chemother.*, **2**, 189–206.

SURPRENANT, A. & CRIST, J. (1988). Electrophysiological characterisation of functionally distinct 5-hydroxytryptamine receptors on guinea-pig submucous plexus. *Neuroscience*, **24**, 283–295.

TRENDELENBURG, U. (1956). The action of 5-hydroxytryptamine on the nictitating membrane and on the superior cervical ganglion of the cat. *Br. J. Pharmacol. Chemother.*, **11**, 74–80.

WALLIS, D.I. & DUN, N.J. (1988). A comparison of fast and slow depolarizations evoked by 5-HT in guinea-pig coeliac ganglion cells *in vitro*. *Br. J. Pharmacol.*, **93**, 110–120.

WALLIS, D.I. & NORTH, R.A. (1978). The action of 5-hydroxytryptamine on single neurones of the rabbit superior cervical ganglion. *Neuropharmacology*, **17**, 1023–1028.

WALLIS, D.I. & WOODWARD, B. (1975). Membrane potential changes induced by 5-hydroxytryptamine in the rabbit superior cervical ganglion. *Br. J. Pharmacol.*, **55**, 199–212.

WATLING, K.J. (1988). Radioligand binding studies identify 5-HT_3 recognition sites in neuroblastoma cell lines and mammalian CNS. *Trends Pharmacol. Sci.*, **9**, 227–229.

YAKEL, J.L. & JACKSON, M.B. (1988). 5-HT_3 receptors mediate rapid responses in cultured hippocampus and a clonal cell line. *Neuron*, **1**, 615–621.

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Effects of vasoactive intestinal peptide (VIP) on contractile responses of smooth muscle in rat stomach

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1 The effects of vasoactive intestinal peptide (VIP) on contractile responses to carbachol (CCh), KCl and caffeine of the circular smooth muscle in rat stomach were examined by the isometric tension recording method and by measurement of the intracellular Ca level, $[Ca]_i$, with fura 2.

2 Removal of extracellular Ca or nifedipine (0.1 μM) inhibited contractions induced by KCl (40 mM) and a low concentration (1 μM) of CCh but not that induced by caffeine (3 mM). After these treatments, the contraction induced by a high concentration of CCh (100 μM) changed to a phasic response.

3 VIP dose-dependently inhibited the contraction induced by 1 μM CCh, but not those caused by 40 mM KCl or 3 mM caffeine.

4 In Ca-free solution containing 2 mM EGTA, VIP inhibited the phasic contraction induced by 100 μM CCh, but not that induced by 30 mM caffeine.

5 CCh caused dose-dependent tension development concomitant with the increase in $[Ca]_i$. VIP reduced both responses and thus did not affect the $[Ca]_i$-force relation for CCh. In the chemically skinned muscle fibres, VIP had no effect on the pCa-tension relation.

6 It is suggested that the inhibitory effects of VIP on CCh-induced contractions are due to the inhibition of the processes of signal transduction from muscarinic receptors to voltage-dependent Ca channels and to intracellular Ca stores.

Introduction

Vasoactive intestinal peptide (VIP) produces relaxation of various smooth muscles (Piper *et al.*, 1970; Said & Mutt, 1970). VIP has been suggested as a neurotransmitter candidate in non-adrenergic, non-cholinergic (NANC) inhibitory nerves in several tissues, including rat stomach (De Beurma & Lefebvre, 1988; Ito *et al.*, 1988; 1990; Kamata *et al.*, 1988). In the rat stomach, we have found that there is a good correlation between the ontogenesis of the functional innervation of NANC inhibitory nerves and that of VIP immunoreactive fibres (Ito *et al.*, 1988), and that the electrical and cyclic nucleotide responses to activation of these nerves are similar to those to VIP (Ito *et al.*, 1990).

The contractions of various smooth muscles depend on the increase in the intracellular free-Ca concentration produced by an influx of extracellular Ca through voltage-dependent or receptor-operated Ca channels and by the release of Ca from intracellular Ca stores (Bolton, 1979; Kuriyama *et al.*, 1982). VIP has been shown to produce a decoupling of electrical and mechanical activities of smooth muscle (Morgan *et al.*, 1978; Ito & Takeda, 1982). However, the effect of VIP on the regulatory function of Ca is not well understood in visceral smooth muscles, though it has been described in vascular smooth muscles (Itoh *et al.*, 1985). KCl, carbachol (CCh) and caffeine have been reported to produce contractions through different sources of Ca in many tissues (Bolton, 1979). Therefore, the present study attempts to examine the effects of VIP on contractile responses to these agents, on intracellular Ca levels in intact smooth muscles and on the pCa-tension relation in skinned muscles of rat stomach.

Methods

Wistar rats of either sex, weighing 200–300 g, were stunned and exsanguinated. Their stomachs were excised and cut in the longitudinal direction along the greater curvature. With the help of a stereomicroscope, the mucosal, submucosal and longitudinal muscle layers were carefully removed from the circular muscle layer of the antrum in a dissection chamber filled with HEPES-Krebs solution. Then antral circular muscle strips (about 0.1 mm in width and 0.7–1 mm in length) were prepared with fine forceps and small knives made from razor blades.

Recording of mechanical activity

Mechanical activities of intact and skinned muscle fibres were measured isometrically by attaching a circular strip to a strain gauge transducer (AE801; AME, Norway). Each end of the strip was knotted by a single silk thread and tied to two tungsten needles. One of the needles was connected to the strain gauge transducer and the other to a manipulator. Experimental solutions were poured into small wells (volume about 0.5 ml) engraved on an aluminium plate in such a way that the surface of the solution was raised by surface tension from the plate surface. The muscle strip was placed horizontally into the meniscus of the solution, so that by moving the plate horizontally it could be transferred from one well into another rapidly (Ohta *et al.*, 1989). After a 30 min equilibration period, the strip was repeatedly exposed to isotonic KCl (150 mM) for 1 min until responses become stable, after which the experiments were started. The temperature of the plate was controlled by circulating water or exothermic transistors underneath the plate. The experiments were carried out at 32–34°C for intact muscle fibres and at 20°C for skinned muscle fibres.

Chemically skinned muscle fibres were obtained by exposing the fibres to saponin (50 $\mu g\,ml^{-1}$) in relaxing solution for 30 min at 20°C. To prevent the deterioration of the contractile response of skinned muscle fibres, calmodulin (1 μM) was added throughout the experiments. Calmodulin was prepared from bovine brain according to the method of Gopalakrishna & Anderson (1982).

Measurement of intracellular Ca level

The intracellular Ca level was measured with a fluorescent indicator, fura 2 (Grynkiewicz *et al.*, 1985). Antral circular

muscle strips were exposed to 10 μM fura 2 acetoxymethyl ester (fura 2-AM) in HEPES-Krebs solution for 2–3 h at 32–34°C with constant mixing. Pluronic F127 (0.005%), a noncytotoxic detergent, was also added in order to increase the solubility of fura 2-AM. After the fura 2 loading, muscle strips were washed with fura 2-AM-free HEPES-Krebs solution for more than 30 min and then used for experiments. Experiments were performed with a fluorometer using dual wavelength excitation (CAM-200, Japan Spectroscopic, Tokyo, Japan) as described by Yanagisawa *et al.* (1989). In brief, the muscle strip loaded with fura 2 was positioned horizontally in a plastic chamber (volume about 0.5 ml) on an inverted microscope stage for simultaneous measurement of tension and the fluorescent signal. Experimental solutions were rapidly injected by a syringe from one end of the organ bath and overflowing solution was aspirated from the other end. The tension of the muscle strip was recorded with a strain gauge transducer as described above. The excitation light was obtained from a Xenon high-pressure lamp (150 W) equipped with a monochrometer and the excitation light alternating between 340 and 380 nm (10 nm-band path) was obtained with a chopping wheel (100 Hz). The ratio of the fluorescence due to excitation at 340 nm to that of 380 nm was calculated from successive illumination periods and is referred to as the F340/F380. The fluorescent signal was obtained by using a Nikon CF UV (Fluor) lens and the light emitted from the muscle fibres was collected by a photomultiplier through a 500 nm (20 nm-band path) filter. The present results were obtained from the preparations in which the fluorescent signals excited by F340 and F380 became mirror images. In the present experiments, the absolute intracellular Ca concentration was not determined, since intracellular fura 2 binds to some soluble proteins and the binding constant of fura 2 to Ca in cytoplasm is not known. Therefore, F340/F380 was used as an indicator of relative $[Ca^{2+}]_i$ (Himpens & Somlyo, 1988; Ozaki *et al.*, 1988).

Solutions

The ionic composition of the HEPES-Krebs solution was as follows (mM): NaCl 144, KCl 5.8, $MgCl_2$ 1.2, $CaCl_2$ 2.5, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonate (HEPES) 5, glucose 11.1 (pH 7.4). High-KCl solutions were prepared by replacing NaCl with KCl isosmotically. In Ca-free solution, $CaCl_2$ was omitted and 2 mM ethyleneglycol-*bis*-(β-aminoethylether)N,N'-tetraacetic acid (EGTA) was always added. The pH was adjusted with NaOH or KOH. In the majority of the experiments, the bathing solutions were not pre-gassed with O_2. The contractile responses in the solutions pre-gassed with O_2 were not different from those in the solutions without gassing.

All solutions used to soak the skinned muscle fibres contained 20 mM piperazine-N,N'-*bis*(ethylenesulphonate) (PIPES) as a pH buffer (pH 7.0 with KOH) and 3.5 mM MgATP, and had an ionic strength of 0.2 M, adjusted with K methanesulphonate (Ms). Solutions activating the skinned muscle fibres contained various concentrations of free Ca buffered by 10 mM EGTA. The free-Ca concentration was changed by adding appropriate amounts of $CaMs_2$ to EGTA. The apparent binding constant of EGTA for Ca was calculated by the formula given by Harafuji & Ogawa (1980). The relaxing solution was composed of (mM): $MgMs_2$ 5.1, $ATPNa_2$ 4.2, KMs 126, EGTA 2 and PIPES 20.

Drugs

The chemicals used were: $ATPNa_2$ (Boehringer), caffeine (Wako), carbachol and verapamil (Sigma), methoxyverapamil (D600, Ezai), EGTA, fura 2-AM, HEPES, PIPES and pluronic F127 (Dojin), Ms (Tokyo Kasei), nifedipine (Bayer), saponin (ICN) and VIP (Protein Inc.).

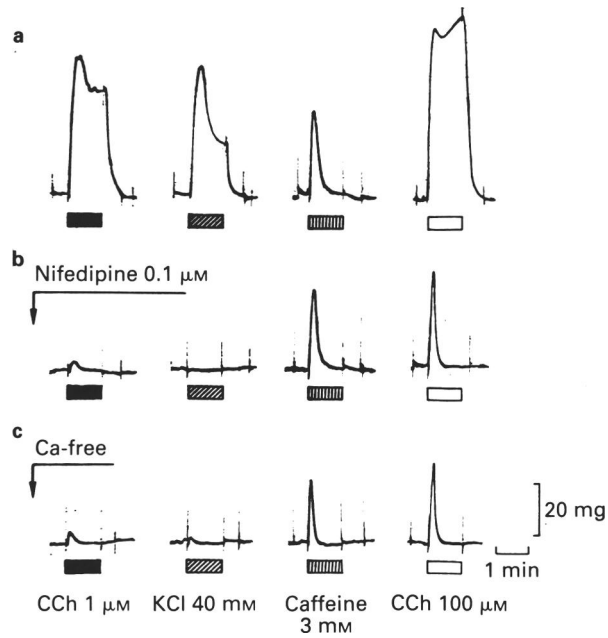

Figure 1 The effects of nifedipine (b) and extracellular Ca removal (c) on contractile responses to KCl, carbachol (CCh) and caffeine. (a) Control. CCh (1 μM; ■, 100 μM; □), KCl (40 mM; ▨) and caffeine (3 mM; ▥) were applied for 1 min. Nifedipine (0.1 μM) was applied more than 10 min before application of these agents. Extracellular Ca removal was performed by treatment with Ca-free solution containing 2 mM EGTA for 2 min.

Results

Effects of extracellular Ca removal and nifedipine on carbachol-, KCl- and caffeine-induced contractions

In order to determine the source of Ca contributing to contractions evoked by CCh, KCl and caffeine, the effects of extracellular Ca removal and nifedipine, a specific inhibitor of dihydropyridine (DHP)-sensitive voltage-dependent Ca channels, were examined. Typical records of contractions evoked by CCh, KCl and caffeine before and after these treatments are shown in Figure 1 and their relative amplitudes are summarized in Table 1. Application of these three agents for 1 min evoked dose-dependent contractions (CCh, 0.1–100 μM; KCl, 10–150 mM; caffeine; 0.5–30 mM) and the 50–75% effective concentration was 1 μM for CCh, 40 mM for KCl and 3 mM for caffeine. As shown in Figure 1a, both KCl (40 mM) and CCh (1 μM and 100 μM) produced a phasic contraction followed by a tonic one. In some cases, CCh at 1 μM caused oscillatory contractions superimposed on the tonic component. In contrast, 3 mM caffeine elicited a phasic contraction only.

After treatment with 0.1 μM nifedipine (Figure 1b) or Ca-free solution containing 2 mM EGTA (Figure 1c), 40 mM KCl evoked neither a phasic nor a tonic response and the 1 μM CCh-induced contraction was also greatly inhibited, while the

Table 1 The effects of extracellular Ca removal and nifedipine on carbachol (CCh)-, KCl- and caffeine-induced contractions

		Ca-free	*Nifedipine*
CCh	1 μM	4.2 ± 0.8 (n = 7)	3.3 ± 1.0 (n – 6)
	100 μM	38.5 ± 5.2 (n = 6)	34.6 ± 4.1 (n = 8)
KCl	40 mM	0 (n = 7)	0 (n = 8)
Caffeine	3 mM	86.6 ± 6.9 (n = 5)	91.2 ± 3.8 (n = 8)

Each value indicates the % of the peak contractile response to each drug before Ca removal or treatment with nifedipine (0.1 μM). For further explanation, see the legend to Figure 1.

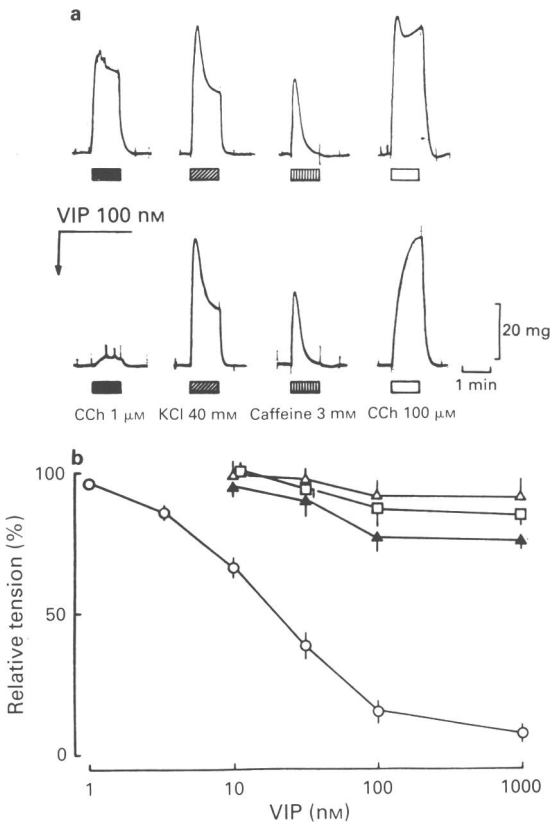

Figure 2 Effects of vasoactive intestinal peptide (VIP) on contractions evoked by carbachol (CCh), KCl and caffeine. (a) Typical records of effects of VIP (100 nM) on CCh- (1 μM; ■, 100 μM; □), KCl- (40 mM; ▨) and caffeine- (3 mM; ▥) induced contractions. (b) Concentration-response curves for VIP on contractions evoked by CCh (1 μM; ○, $n = 8$) and caffeine (3 mM; □, $n = 6$) and on the phasic (△, $n = 8$) and tonic (▲, $n = 8$) responses of KCl- (40 mM) induced contractions. The amplitude of contraction evoked in the absence of VIP was normalized as 100%. Vertical lines indicate s.e.mean.

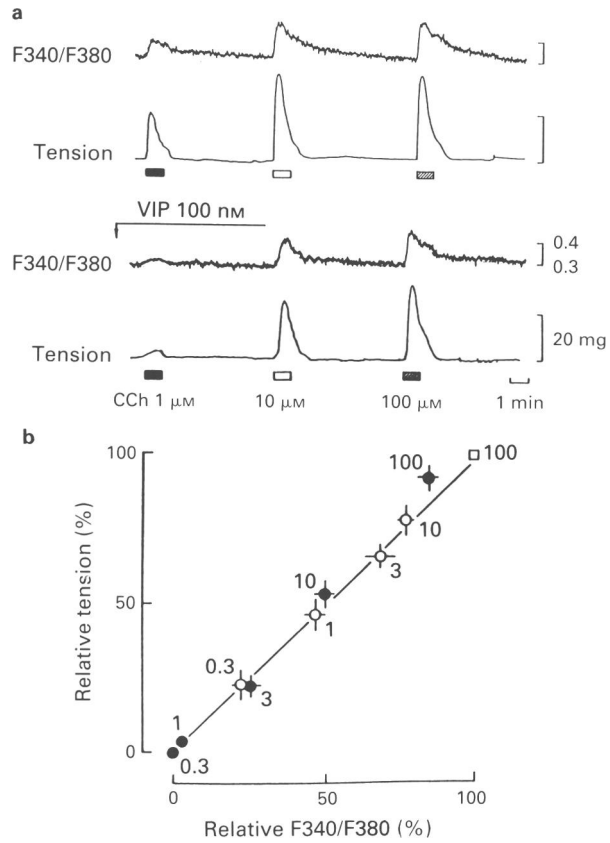

Figure 3 Effects of vasoactive intestinal peptide (VIP) on increases in $[Ca]_i$ and contractions in response to carbachol (CCh). (a) Typical chart recording. The intracellular Ca level, $[Ca]_i$, is indicated by F340/F380. (b) Relation between the increase in $[Ca]_i$ and tension in response to CCh in the presence (●; $n = 5$) and absence (○; $n = 6$) of VIP (100 nM). Numbers below symbols indicate CCh concentrations (μM). The amplitude of tension and increase in $[Ca]_i$ in response to CCh (100 μM) in the absence of VIP were normalized as 100%. Vertical and horizontal lines indicate s.e.mean.

phasic contraction evoked by 3 mM caffeine was only slightly reduced. The tonic contraction but not the phasic one induced by a high concentration of CCh (100 μM) was inhibited by these treatments. Other Ca channel blockers, such as D600 and verapamil, had effects similar to that of nifedipine.

Effect of vasoactive intestinal peptide on the contractile responses to carbachol-, KCl- and caffeine

The inhibitory effect of VIP on CCh-induced contraction developed gradually. When CCh (1 μM) and VIP (30 nM) were applied simultaneously, the peak amplitude of the CCh-induced contraction was 97.3 ± 2.4% of the control. The contractile response to CCh was reduced to 74.8 ± 4.1% for 1 min pretreatment with VIP, 55.3 ± 2.6% for 2 min, 40.4 ± 5.0% for 4 min and 41.7 ± 1.9% for 8 min ($n = 4$). Therefore, the contractile responses to the agents were examined more than 4 min after treatment with VIP. Figure 2a shows typical contractile responses to CCh, KCl and caffeine in the presence and absence of VIP (100 nM). VIP greatly inhibited the 1 μM CCh-induced contraction. Although VIP markedly slowed the rising phase of the contraction induced by CCh at 100 μM, it did not affect its peak amplitude of contraction. KCl and caffeine-induced contractions were hardly affected by VIP. Figure 2b shows the effects of various concentrations of VIP on the 1 μM CCh-, 40 mM KCl- and 3 mM caffeine-induced contractions. VIP inhibited the contraction evoked by 1 μM CCh in a dose-dependent manner and its 50% inhibitory dose was about 20 nM. In contrast, contractions evoked by KCl and caffeine were only slightly affected even after much longer pretreatment with VIP.

Effects of vasoactive intestinal peptide on the intracellular Ca level increase induced by carbachol

Figure 3a shows the effects of VIP (100 nM) on the rise in $[Ca]_i$ and tension development in response to various concentrations of CCh. The increase in $[Ca]_i$ in response to CCh preceded the development of tension. Although VIP did not affect the resting $[Ca]_i$ level, it reduced the increase in the $[Ca]_i$ level and contraction in response to CCh simultaneously. Figure 3b shows the relationship between the increase in the $[Ca]_i$ level and the amplitude of contraction in response to CCh in the presence and absence of VIP (100 nM). This relation, obtained in the presence of VIP, was not different from that in the absence of VIP. This result indicates that the inhibition of CCh-induced contraction by VIP is accompanied by a decrease in intracellular Ca.

Effects of vasoactive intestinal peptide on contractions utilizing intracellular stored Ca

In gastric circular smooth muscles, CCh at a high concentration and caffeine evoked monophasic contractions in the Ca-free solution as shown in Figure 1c. We examined the effects of VIP on these contractions in detail. After the Ca stored in muscle cells was completely depleted by repetitive applications of caffeine (30 mM) in Ca-free solution containing 2 mM EGTA, the muscle was exposed to 2.5 mM Ca together with isotonic KCl (150 mM) for 2 min to load the Ca in intracellular Ca stores (Ca-loading). After the tissue was washed for 4 min with repeatedly changing Ca-free solutions containing

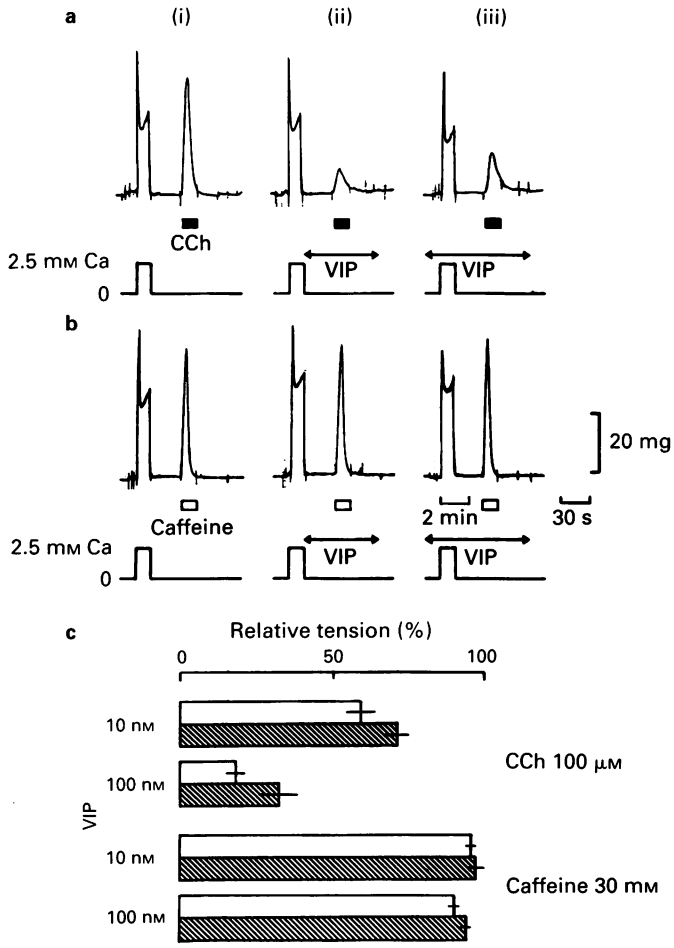

Figure 4 Effects of vasoactive intestinal peptide (VIP) on carbachol (CCh)- and caffeine-induced contractions in Ca-free solution containing 2 mM EGTA. After 2.5 mM Ca was applied together with isotonic KCl (150 mM) for 2 min (Ca-loading), the muscle fibre was washed with Ca-free solution containing 2 mM EGTA for 4 min (Ca-washing). Thereafter, (a) CCh (100 μM; ■) or (b) caffeine (30 mM; □) was applied for 30 s in Ca-free solution containing 2 mM EGTA. Chart speed is 4 times slower in the Ca-loading and Ca-washing steps than in the next steps. (i) Control; (ii) VIP (100 nM) was applied after Ca-loading; (iii) VIP was applied before Ca-loading. (c) The % amplitude of contractions evoked by CCh and caffeine in Ca-free solutions containing 2 mM EGTA. Open columns show the data obtained when VIP is introduced after Ca-loading and hatched columns before Ca-loading (n = 5). Horizontal lines indicate s.e.mean.

2 mM EGTA (Ca-washing), application of CCh (100 μM) or caffeine (30 mM) for 30 s produced a transient contraction in the Ca-free solution containing 2 mM EGTA (Figures 4a(i) and 4b(i)). Contractions no longer occurred when KCl (40 mM) was applied instead of CCh or caffeine, indicating that external Ca was sufficiently removed during the Ca-washing period (data not shown). VIP (100 nM) was applied during the periods of Ca-washing and subsequent CCh or caffeine stimulation. VIP significantly inhibited the contraction evoked by CCh in a dose-dependent manner, but not that caused by caffeine (Figures 4a(ii), 4b(ii) and open columns in 4c). When VIP was applied from the time of Ca-loading, the inhibitory effect of VIP was less pronounced (Figure 4a(iii) and hatched columns in 4c).

In order to see whether this inhibitory effect of VIP was due to the inhibition of Ca release from intracellular Ca stores or to an enhancement of Ca extrusion through the plasma membrane, the contractions induced in Ca-free solution containing 2 mM EGTA by successive applications of CCh and caffeine were examined in the presence or absence of 100 nM VIP (Figure 5). After CCh had evoked a contraction, a small contraction could be evoked by the application of caffeine.

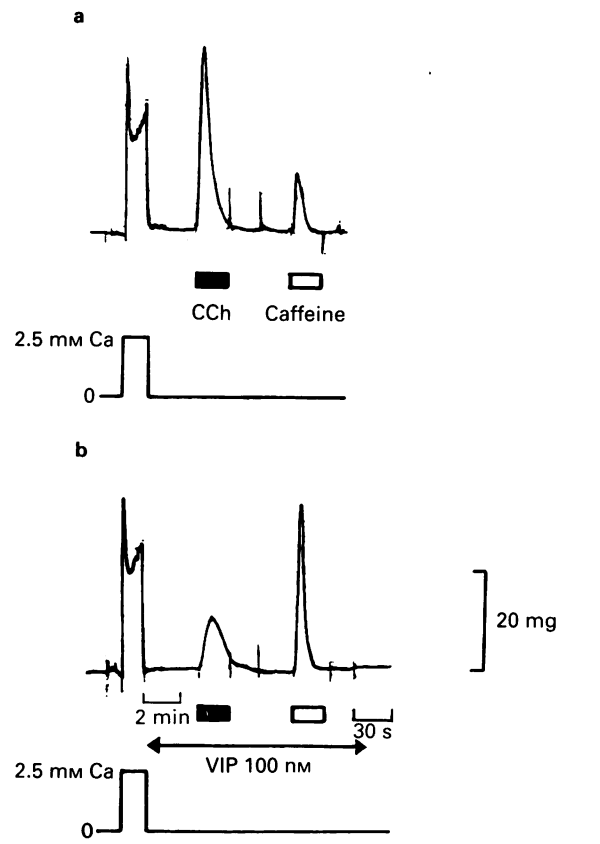

Figure 5 Effects of vasoactive intestinal peptide (VIP) on successive carbachol (CCh)- and caffeine-induced contractions in Ca-free solution containing 2 mM EGTA. After Ca-loading, CCh (100 μM; ■) and caffeine (30 mM; □) were applied successively for 30 s with a 1 min interval. (a) Control; (b) VIP (100 nM) was applied after Ca-loading. For further explanations, see the legend to Figure 4.

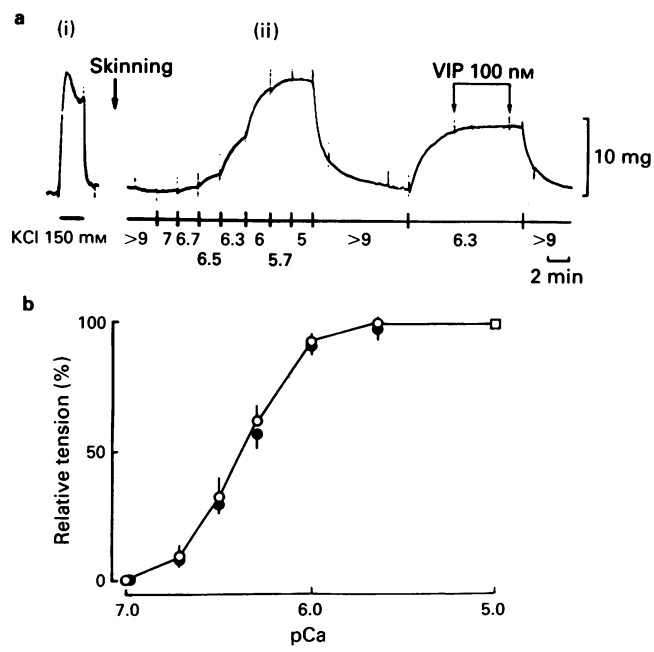

Figure 6 Effects of vasoactive intestinal peptide (VIP) on the Ca-induced contraction in skinned fibres. (a) (i) Isotonic KCl (150 mM)-induced contraction in intact muscles. (ii) After skinning with saponin, various concentrations of Ca were applied cumulatively. After the pCa 6.3-induced contraction reached a steady level, VIP (100 nM) was applied. (b) pCa-tension relation observed in the presence (●; n = 4) or absence (○; n = 5) of VIP (100 nM). The amplitude of contraction evoked by pCa 5.0 in the absence of VIP was normalized as 100%. Vertical lines indicate s.e.mean.

However, after caffeine evoked a contraction, CCh or caffeine did not (data not shown). When the contraction induced by a preceding application of CCh was inhibited by VIP, the caffeine-induced contraction was enhanced (3.7 ± 0.3 times the control, $n = 10$)

Effects of vasoactive intestinal peptide on the contractile machinery in skinned fibres

Figure 6a shows a typical record of contraction induced by cumulative application of Ca and the effect of VIP (100 μM) on the contraction evoked by pCa 6.3 in skinned muscle fibres. VIP did not modify the amplitude of contraction induced by Ca. The effect of VIP (100 nM) on the cumulative pCa-tension relation is shown in Figure 6b. The minimal concentration of Ca required to produce the contraction of skinned muscle fibres was pCa 6.7 and the amplitude of contraction was increased with increasing concentrations of Ca up to pCa 5.7. VIP had no effect on the pCa-tension relation.

Discussion

The present results indicate that VIP inhibits the contraction induced by CCh but not those caused by KCl and caffeine in the gastric circular smooth muscle of the rat. VIP inhibited the increase in $[Ca]_i$ together with tension development in response to CCh and thus did not affect the $[Ca]_i$-tension relation for CCh. Therefore, it seems likely that the change in the Ca sensitivity of the contractile system is not responsible for the inhibitory action of VIP on CCh-induced contraction. Furthermore, VIP did not influence the pCa-tension relation in skinned fibres of gastric smooth muscles, as was the case of vascular smooth muscles (Itoh *et al.*, 1985).

The contraction evoked by CCh at a low concentration was inhibited by the removal of extracellular Ca or by treatment with the Ca channel blocker, nifedipine. As VIP was effective in inhibition of the contractions and the rise in $[Ca]_i$ in response to CCh, VIP probably inhibits the CCh-induced Ca influx across the cell membrane through DHP-sensitive voltage-dependent Ca channels. However, the KCl-induced contraction was resistant to VIP. Therefore, it seems unlikely that the action of VIP on CCh-induced contractions is due to a direct inhibition of voltage-dependent Ca channels. VIP could, however, interefere with the processes of signal transduction from muscarinic receptors to voltage-dependent Ca channels.

The phasic contractions evoked by CCh and caffeine in Ca-free solution are known to be due to Ca release from intracellular Ca stores (Endo *et al.*, 1980; Itoh *et al.*, 1981). Similar phasic contractions induced by these agents were observed in gastric circular smooth muscles. In Ca-free solution containing 2 mM EGTA, VIP inhibited the contraction induced by CCh but not that induced by caffeine. After VIP inhibited the CCh-induced contraction, the contraction induced by a subsequent application of caffeine was significantly enlarged in the presence of VIP. These results indicate that the Ca stores in this tissue are probably activated by both drugs and that the inhibitory action of VIP on the CCh-induced contraction in Ca-free solution may result from an inhibition of the CCh-induced Ca release from intracellular Ca stores rather than from an increase in Ca extrusion through the cell membrane. The difference between the effects of VIP on CCh- and caffeine-induced contractions could be attributable to the fact that caffeine acts directly on intracellular Ca stores (Endo *et al.*, 1980; Itoh *et al.*, 1981) but CCh does not. It seems likely that VIP inhibits some steps between muscarinic receptor activation and the Ca release from intracellular Ca stores.

It has been shown that muscarinic receptors couple to guanine nucleotide binding proteins (Haga *et al.*, 1987) and stimulation of these receptors leads to the activation of phospholipase C, which causes inositol breakdown and the production of inositol 1,4,5-trisphosphate (IP_3; Gardner *et al.*, 1988; Secrest *et al.*, 1989), a putative messenger for the mobilization of Ca from non-mitochondrial stores (Berridge & Irvine, 1984). Therefore, the contraction induced by CCh in the absence of extracellular Ca may result from IP_3 formation in the rat gastric smooth muscle.

More recently, we have found that VIP increases intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) concentrations and that the time course was comparable to that of relaxation (Ito *et al.*, 1990). The time-dependence of the inhibitory effect of VIP on CCh-induced contraction may imply the involvement of second messenger systems, such as cyclic AMP. Furthermore, Ca sequestering action into the Ca stores has been reported to be increased by cyclic AMP (Bhalla *et al.*, 1978; Itoh *et al.*, 1982). The inhibitory effect of VIP on CCh-induced contractions in Ca-free solution containing 2 mM EGTA was less pronounced with application of VIP before the Ca-loading period than after it, suggesting that VIP has a Ca-sequestering action on Ca storage sites in addition to the inhibition of CCh-induced Ca release.

In conclusion, VIP inhibits CCh-induced contraction in the presence and absence of extracellular Ca. VIP seems not to have direct inhibitory effects on DHP-sensitive voltage-dependent Ca channels, the Ca-release mechanism from intracellular Ca stores and the contractile system. The site of the inhibitory action of VIP may be the processes of signal transduction from muscarinic receptors to DHP-sensitive voltage-dependent Ca channels and to intracellular Ca stores.

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References

BERRIDGE, M.J. & IRVINE, R.F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature*, **312**, 315–321.

BHALLA, R.C., WEBB, R.C., SINGH, D. & BROCK, T. (1978). Role of cyclic AMP in rat aortic microsomal phosphorylation and calcium uptake. *Am. J. Physiol.*, **234**, H508–H514.

BOLTON, T.B. (1979). Mechanism of action of transmitters and other substances on smooth muscle. *Physiol. Rev.*, **59**, 606–718.

DE BEURMA, F.A. & LEFEBVRE, R.A. (1988). Vasoactive intestinal polypeptide as possible mediator of relaxation in the rat gastric fundus. *J. Pharm. Pharmacol.*, **40**, 711–715.

ENDO, M., KITAZAWA, T. & YAGI, S. (1980). Different features of responses of the sarcoplasmic reticulum in cardiac and smooth muscles. In *Muscle Contraction: Its Regulatory Mechanisms*. ed. Ebashi, S., Maruyama, K. & Endo, M. pp. 447–493. Tokyo: Japan Sci. Soc. Press.

GARDNER, A.L., CHOO, L.K. & MITCHELSON, F. (1988). Comparison of the effect of some muscarinic agonists on smooth muscle function and phosphatidylinositol turnover in the guinea-pig taenia coli. *Br. J. Pharmacol.*, **94**, 199–211.

GOPALAKRISHNA, R. & ANDERSON, W.B. (1982). Ca^{2+}-induced hydrophobic site of calmodulin: application for purification of calmodulin by phenyl-sepharose affinity chromatography. *Biochem. Biophys. Res. Commun.*, **104**, 830–836.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

HAGA, T., HAGA, K., BERSTEIN, G., NISHIYAMA, T., UCHIYAMA, H. & ICHIYAMA, A. (1987). Molecular properties of muscarinic receptors. *Trends Pharmacol. Sci.*, **8** (Suppl. Subtypes Muscarinic Receptor III), 12–18.

HARAFUJI, H. & OGAWA, Y. (1980). Re-examination of the apparent binding constant of ethylene glycol *bis*(β-aminoethyl ether)-N,N,N',N'- tetraacetic acid with calcium around neutral pH. *J. Biochem.*, **87**, 1305–1312.

HIMPENS, B. & SOMLYO, A.P. (1988). Free-calcium and force tran-

sients during depolarization and pharmacomechanical coupling in guinea-pig smooth muscle. *J. Physiol.*, **395**, 507–530.

ITO, S., KROKAWA, A., OHGA, A., OHTA, T. & SAWABE, K. (1990). Mechanical, electrical and cyclic nucleotide responses to peptide VIP and inhibitory nerves stimulation in rat stomach. *J. Physiol.*, **430**, 337–353.

ITO, S., OHTA, T., KIMURA, A. & OHGA, A. (1988). Development of substance P- and vasoactive intestinal polypeptide-containing neurones in the rat stomach. *Q. J. Exp. Physiol.*, **73**, 729–736.

ITO, Y. & TAKEDA, K. (1982). Non-adrenergic inhibitory nerves and putative transmitters in the smooth muscle of the cat trachea. *J. Physiol.*, **330**, 497–511.

ITOH, T., IZUMI, H. & KURIYAMA, H. (1982). Mechanisms of relaxation induced by activation of β-adrenoceptors in smooth muscle cells of the guinea-pig mesenteric artery. *J. Physiol.*, **326**, 475–493.

ITOH, T., KURIYAMA, H. & SUZUKI, H. (1981). Excitation-contraction coupling in smooth muscle cells of the guinea-pig mesenteric artery. *J. Physiol.*, **321**, 513–538.

ITOH, T., SASAGURI, T., MAKITA, Y., KANMURA, Y. & KURIYAMA, H. (1985). Mechanisms of vasodilation induced by vasoactive intestinal polypeptide in rabbit mesenteric artery. *Am. J. Physiol.*, **249**, H231–H240.

KAMATA, K., SAKAMOTO, A. & KASUYA, Y. (1988). Similarities between the relaxations induced by vasoactive intestinal peptide and by stimulation of the non-adrenergic non-cholinergic neurons in the rat stomach. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **338**, 401–406.

KURIYAMA, H., ITO, Y., SUZUKI, H., KITAMURA, K. & ITOH, T. (1982). Factors modifying contraction-relaxation cycle in vascular smooth muscles. *Am. J. Physiol.*, **243**, H641–H662.

MORGAN, K.G., SCHMALZ, P.F. & SZURSZEWSKI, J.H. (1978). The inhibitory effects of vasoactive intestinal polypeptide on the mechanical and electrical activity of canine antral smooth muscle. *J. Physiol.*, **282**, 437–450.

OHTA, T., ENDO, M., NAKANO, T., MOROHOSHI, Y., WANIKAWA, K. & OHGA, A. (1989). Ca-induced Ca release in malignant hyperthermia-susceptible pig skeletal muscle. *Am. J. Physiol.*, **256**, C358–C367.

OZAKI, H., SATOH, T., KARAKI, H. & ISHIDA, Y. (1988). Regulation of metabolism and contraction by cytoplasmic calcium in the intestinal smooth muscle. *J. Biol. Chem.*, **263**, 14074–14079.

PIPER, R.J., SAID, S.I. & VANE, J.R. (1970). Effects on smooth muscle preparations of unidentified vasoactive peptide from intestinal and lung. *Nature*, **225**, 1144–1146.

SAID, S.I. & MUTT, V. (1970). Potent peripheral and splanchnic vasodilator peptide from normal gut. *Nature*, **225**, 863–864.

SECREST, R.A., SCHOEPP, D.D. & COHEN, M.L. (1989). Comparison of contractions to serotonin, carbamylcholine and prostaglandin $F_{2\alpha}$ in rat stomach fundus. *J. Pharmacol. Exp. Ther.*, **250**, 971–978.

YANAGISAWA, T., KAWADA, M. & TAIRA, N. (1989). Nitroglycerin relaxes canine coronary arterial smooth muscle without reducing intracellular Ca^{2+} concentrations measured with fura-2. *Br. J. Pharmacol.*, **98**, 469–482.

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Adenosine-5′-O-(2-thiodiphosphate) is a potent agonist at P_2 purinoceptors mediating insulin secretion from perfused rat pancreas

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1 The effects of a P_2 purinoceptor agonist, adenosine 5′-O-(2-thiodiphosphate) (ADP-β-S) have been studied on insulin secretion and flow rate of the isolated perfused pancreas of the rat.

2 In the presence of a moderately stimulating glucose concentration (8.3 mM), ADP-β-S (4.95–495 nM) evoked a biphasic insulin response in a concentration-dependent manner. A comparison of relative potency between ADP-β-S and adenosine 5′-triphosphate (ATP) showed that ADP-β-S was 100 times more potent than ATP. On the other hand, in the presence of a non stimulatory glucose concentration (4.2 mM), ADP-β-S (165 nM) did not modify the basal insulin secretion.

3 ADP-β-S, at concentrations effective on insulin secretion and also at higher concentrations (1.65 and 16.5 μM), provoked an increase of the pancreatic flow rate in a concentration-dependent manner.

4 Our results show that ADP-β-S is a potent insulin secretory P_2 purinoceptor agonist. As it is resistant to hydrolysis it might be useful in studying the effect of activation of the P_2 purinoceptor of β cells on insulin secretion *in vivo*.

Introduction

Adenine nucleotides can influence numerous biological processes by acting on specific receptors on the cellular surface (Burnstock & Brown, 1981; Gordon, 1986). Previously we have shown that exogenous adenosine triphosphate and diphosphate (ATP and ADP) stimulated insulin release from rat pancreatic β cells, an effect mediated by P_2 purinoceptor activation (Loubatières-Mariani *et al.*, 1979; Chapal & Loubatières-Mariani, 1981).

P_2 purinoceptors have been subdivided into P_{2x} and P_{2y} largely on the basis of the rank order of agonist potency of structural analogues of ATP (Burnstock & Kennedy, 1985). The agonist potency order at P_{2x} purinoceptors is α,β-methylene ATP > ATP ≈ 2-methylthio ATP whereas at P_{2y} purinoceptors the order is 2-methylthio ATP ≫ ATP ≥ α,β-methylene ATP. According to this classification we have characterized the P_2 purinoceptor present on rat β cells as a P_{2y} subtype, 2-methylthio ATP being more potent than ATP in stimulating insulin secretion (Bertrand *et al.*, 1987).

ADP analogues with modification on the terminal phosphate have been reported to be more effective (Burnstock *et al.*, 1984) or selective (Hourani *et al.*, 1988) P_{2y} receptor agonists. Furthermore, in contrast to 2-methylthio ATP, these ADP analogues are more resistant to ectonucleotidases than natural agonists (Welford *et al.*, 1986).

In the present study, we have investigated the effects of adenosine-5′-O-(2-thiodiphosphate) (ADP-β-S), a phosphorothioate analogue of ADP, on insulin secretion from the isolated perfused pancreas of the rat. The insulin secretory effect of ADP-β-S was compared with that of ATP, taken as reference in previous experiments. The present work shows that ADP-β-S is a very potent agonist at P_{2y} purinoceptors mediating insulin release.

Methods

Our experiments were performed on male Wistar rats fed *ad libitum* and weighing 330 to 350 g. The surgical procedure for the rat isolated perfused pancreas has been described previously (Loubatières *et al.*, 1969; Bertrand *et al.*, 1986). The pancreas was perfused at 37.5°C with a Krebs Ringer bicarbonate buffer containing pure bovine serum albumin (fraction V) 2 g l^{-1} and glucose 8.3 or 4.2 mM; the pH was about 7.4. Each organ was perfused at a constant pressure (Bertrand *et al.*, 1987).

ADP-β-S or ATP, was infused for 30 min from time 45 min. Samples were taken every min during the first 6 min, then at 8, 10, 15, 20 and 30 min. Insulin in the pancreatic effluent was assayed by the method of Herbert *et al.* (1965) using the antibody supplied by Miles Laboratories (Paris). [^{125}I]-insulin was obtained from International CIS (Gif-Sur-Yvette, France). Pure rat insulin (Novo, Copenhagen, Denmark) was used as the reference standard, the biological activity of which was 22.3 μu ng^{-1}. The intra- and inter-assay variations were respectively 9 and 13.5%.

Analysis of results

For the kinetics of insulin output rate and flow rate the results for each point are expressed as changes in relation to the value at time 45 min taken as 100%. Data are expressed as mean ± standard error of the mean (s.e.mean).

In order to establish the concentration-response curves for insulin secretion and flow rate, we determined respectively the increase of mean insulin output and flow rates over the 30 min of nucleotide infusion as follows: (nucleotide AUC − control AUC)/30 (AUC = area under the curve). The values obtained were plotted as a function of the logarithm of ADP-β-S or ATP concentrations.

For statistical studies of changes in pancreatic flow rate, the AUC were submitted to analysis of variance followed by the multiple comparison test (Zar, 1974).

Drugs

Adenosine-5′-triphosphate (ATP) and adenosine-5′-O-(2-thiodiphosphate) (ADP-β-S) were obtained from Boehringer Mannheim. ATP was the sodium salt and ADP-β-S was the lithium salt.

Results

Effect of adenosine-5′-O-(2-thiodiphosphate) on insulin secretion

In the presence of 8.3 mM glucose (controls), insulin release increased slightly with time (Figure 1).

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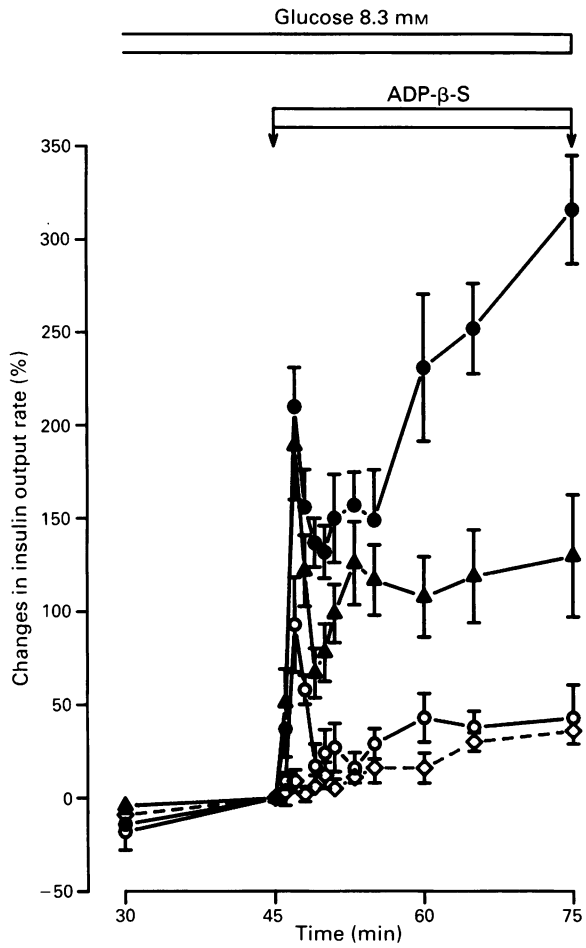

Figure 1 Effects of increasing concentrations of adenosine-5'-O-(2-thiodiphosphate) (ADP-β-S) on insulin secretion from the isolated perfused pancreas of the rat: (○) 49.5 nM (n = 4); (▲) 165 nM (n = 7); (●) 495 nM (n = 7), controls (◇) (n = 6). The insulin output rate (ng min^{-1}) at 45 min for each set of experiments was: 35.25 ± 4.32; 30.20 ± 5.45; 31.47 ± 4.20; 32.91 ± 8.09 respectively. Each point represents the mean with s.e.mean shown by vertical lines.

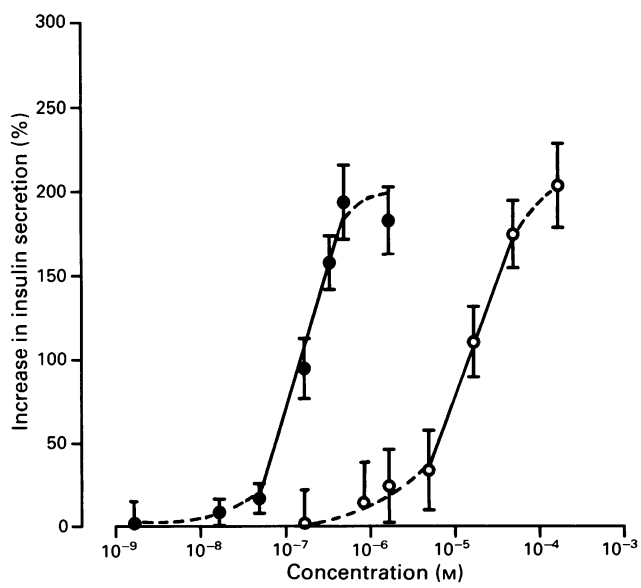

Figure 2 Concentration-response curves for adenosine-5'-O-(2-thiodiphosphate) (ADP-β-S, ●)- and adenosine 5'-triphosphate (ATP, ○)-induced insulin secretion. Each point represents the mean of 4–7 experiments and vertical lines indicate the s.e.mean. Solid lines represent the calculated regression lines.

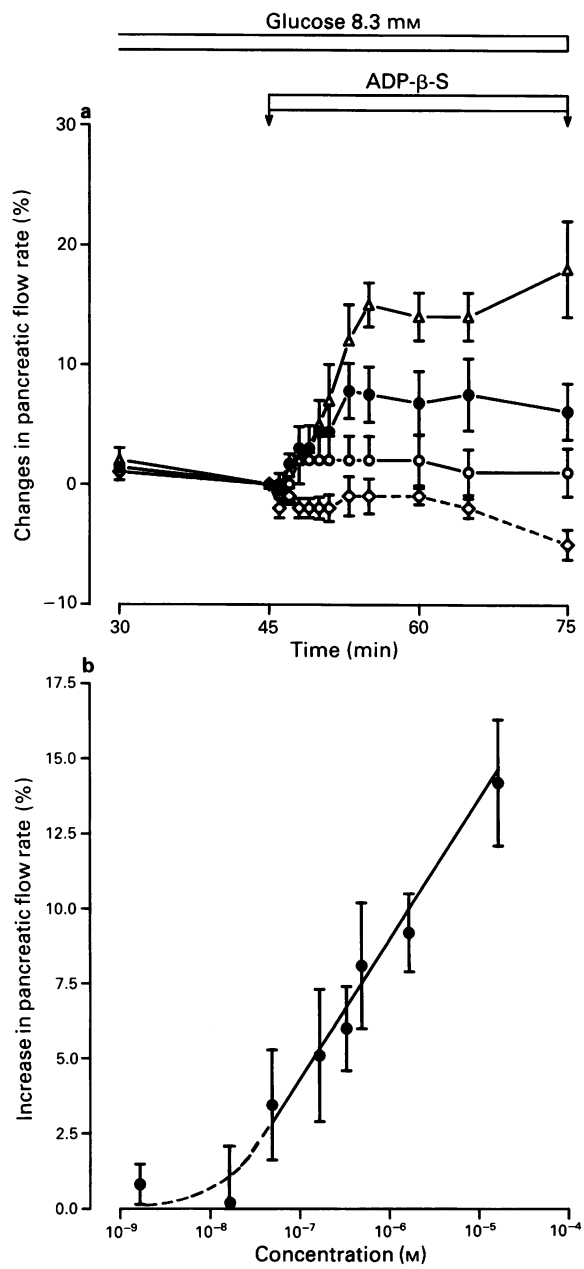

Figure 3 Effects of adenosine-5'-O-(2-thiodiphosphate) (ADP-β-S) on pancreatic flow rate of the rat: (a) (○) 49.5 nM (n = 4); (●) 495 nM (n = 7); (△) 16.5 μM (n = 6); controls (◇) (n = 6). (b) Concentration-response curve. Each point represents the mean with vertical lines showing s.e.mean.

The effects of ADP-β-S were studied at concentrations ranging from 1.65×10^{-9} to 1.65×10^{-5} M. At 1.65 and 16.5 nM ADP-β-S did not significantly affect insulin release. In the range 49.5–495 nM, ADP-β-S evoked a biphasic insulin response which was concentration-dependent (Figure 1). The kinetics of the insulin response were qualitatively similar to those of the response elicited by ATP (Loubatières-Mariani *et al.*, 1979). When it was used at 1.65 or 16.5 μM, insulin responses were not significantly different from those observed with 495 nM.

The concentration-response curves for ADP-β-S and ATP are shown in Figure 2. For each compound we carried out a linear regression analysis from the values of increase of mean insulin output rate. A comparison of potency was performed by the parallel line assay method (Armitage, 1980). The two concentration-response curves did not deviate significantly from parallelism; ADP-β-S was 100 fold more potent than ATP with [65–150] for 95% confidence limits.

In the presence of a non stimulatory concentration of glucose (4.2 mM), ADP-β-S at 165 nM, (the concentration inducing the half-maximum effect in the presence of glucose 8.3 mM) did not influence basal insulin output rate. The insulin output during the 30 min of ADP-β-S infusion was 10.0 ± 1.9 ng versus 9.8 ± 1.2 ng in the controls.

Effect of adenosine-5'-O-(2-thiodiphosphate) on pancreatic flow rate

In the absence of ADP-β-S the pancreatic flow rate decreased slightly with time (Figure 3a).

ADP-β-S at 1.65 and 16.5 nM did not significantly modify the flow rate. At concentrations effective on insulin secretion (49.5–495 nM), it induced a progressive and slight increase of the pancreatic flow rate (Figure 3a) which reached a maximum 8–10 min after the start of the infusion and remained sustained. ADP-β-S at the highest concentration tested (16.5 μM) induced a vasodilator effect which was more pronounced. With ADP-β-S 49.5 nM, 165 nM, 495 nM and 16.5 μM the AUC were, respectively, 3044 ± 54 (NS), 3118 ± 71 ($P < 0.05$), 3183 ± 63 ($P < 0.01$), and 3364 ± 63 ($P < 0.001$) versus controls (2945 ± 17). The effect of ADP-β-S on the mean flow rate was concentration-dependent at the tested concentrations in the range 49.5 nM–16.5 μM (Figure 3b).

The effects of ATP on flow rate were studied at concentrations ranging from 0.165 to 165 μM. With ATP 0.165 μM the flow rate was not modified. At the higher concentrations (1.65–49.5 μM) the flow rate responded differently according to the pancreas; it was either not changed or slightly increased. At 1.65, 4.95 and 49.5 μM the AUC were respectively 3089 ± 85 (NS), 3110 ± 102 (NS) and 3215 ± 107 (NS) versus 2940 ± 16 in controls. Only the concentration of 16.5 μM induced a significant increase of the flow rate: the area under the curve was 3241 ± 110 ($P < 0.05$) versus controls. This effect was not observed at the highest concentration tested (165 μM): the AUC was 2941 ± 84.

Discussion

This study shows that ADP-β-S, like ATP, induces a biphasic insulin release when rat isolated pancreas is perfused in the presence of a moderately stimulatory concentration of glucose (8.3 mM). On the other hand, ADP-β-S itself is ineffective on basal insulin secretion in the presence of a non stimulatory glucose concentration (4.2 mM). Thus, this agent is a potentiator of glucose-induced insulin release. Previous results have demonstrated that ATP or ADP, which were ineffective with glucose at a concentration of 4.2 mM (Bertrand *et al.*, 1989) stimulated insulin release in the presence of glucose 8.3 mM, via P_2 purinoceptors (Loubatières-Mariani *et al.*, 1979); the P_2 purinoceptor involved was characterized as a P_{2y} subtype, since 2-methylthio ATP was more potent than ATP (Bertrand *et al.*, 1987). The present work shows that an ADP analogue, ADP-β-S, is a potent insulin secretory agent in the presence of a stimulatory glucose concentration. ADP-β-S, at concentrations ranging from 4.95×10^{-8} M to 4.95×10^{-7} M induced a concentration-dependent response and was 100 times more potent than ATP. Like 2-methylthio ATP, ADP-β-S exhibits a much higher potency than ATP at P_{2y} purinoceptors of the β cell. Whereas 2-methylthio ATP is degraded at the same rate as ATP, ADP-β-S has been reported to be resistant to degradation (Welford *et al.*, 1986). However, the high potency of ADP-β-S on β cells appears not to be related to its stability, since another stable analogue of ADP, α,β-methylene ADP, has been shown to display nearly the same activity as ATP on insulin secretion (Chapal & Loubatières-Mariani, 1981). Furthermore no correlation has been reported between the rate of breakdown of an agonist and its pharmacological potency at P_{2y} receptors (Welford *et al.*, 1986). ADP-β-S has been shown to be more potent than ATP or ADP at P_{2y} purinoceptors involved in relaxation of the guinea-pig isolated taenia coli (Burnstock *et al.*, 1984) and in activation of the turkey erythrocyte phospholipase C (Boyer *et al.*, 1989). On the other hand, ADP-β-S was equipotent with ATP or ADP at P_{2x} purinoceptors mediating contraction of the guinea-pig urinary bladder and vas deferens (Burnstock *et al.*, 1984; Fedan *et al.*, 1986). Since ADP-β-S appears to be a more potent agonist at the P_{2y} purinoceptor, our results provide further pharmacological evidence for a P_{2y} purinoceptor on rat β cells.

We have previously shown that the pancreatic vascular bed was transiently constricted by ATP or ADP at very high concentrations (> 165 μM) (Chapal & Loubatières-Mariani, 1983). The agonist potency order of ATP and its structural analogues on the decrease of flow rate: α,β-methylene ATP > β,γ-methylene ATP > 2-methylthio ATP > ATP indicated the involvement of a P_{2x} purinoceptor (Chapal & Loubatières-Mariani, 1983; Bertrand *et al.*, 1987). In our study, ADP-β-S, even at the highest concentrations, elicited in contrast, an increase in pancreatic flow rate which was concentration-dependent from 4.95×10^{-8} M. Nevertheless, it should be noted that ADP-β-S at the concentration (495 nM) inducing a maximum effect on insulin secretion, did not induce a maximum vasodilator effect, since a much more pronounced vasodilatation was observed at the highest concentration tested (16.5 μM). A slight but significant vasodilator effect occurred with ATP only at 16.5 μM but could not be observed at the high concentration of 165 μM. The disappearance of the vasodilator effect could be explained by the opposing vasoconstrictor effect of ATP, via P_{2x} purinoceptors, which becomes apparent at high concentrations, as previously shown (Chapal & Loubatières-Mariani, 1983); such a mechanism has also been proposed in the mesenteric bed of the rat in which tone has been increased by noradrenaline (Ravelic & Burnstock, 1988). In fact, ATP can mediate vessel relaxation or constriction through endothelium P_{2y} purinoceptors and smooth muscle P_{2x} purinoceptors respectively (Burnstock, 1987; Pearson & Gordon, 1989) but it could also act indirectly, after hydrolysis into adenosine, on P_1 purinoceptors mediating vasodilatation. Concerning the pancreatic vascular bed, adenosine has been reported to increase the flow rate by acting on P_1 purinoceptors (Soulaymani *et al.*, 1985). However, it seems unlikely that vasodilatation elicited by ADP-β-S involves an action via adenosine, since this ADP analogue is resistant to hydrolysis. So ADP-β-S, a stable and more specific P_{2y} agonist evoked, at the concentrations used, only a vasodilator effect, whereas ATP exhibited more complex effects.

We have previously suggested that ATP and ADP, via P_2 purinoceptor activation, could exert an important role in the control of the β cell by potentiating acetylcholine (Bertrand *et al.*, 1986) and glucose (Bertrand *et al.*, 1989) stimulations. Furthermore we have shown that 2-methylthio ATP infused directly into the dog pancreatico-duodenal artery (this procedure was used because of the rapid metabolism of this analogue) induced an insulin secretion sufficient to provoke a reduction of glycaemia (Ribes *et al.*, 1988). Since ADP-β-S is a more stable and potent insulin secretory P_{2y} purinoceptor agonist, it might therefore be a useful agent to stimulate insulin secretion and decrease glycaemia *in vivo*.

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References

ARMITAGE, P. (1980). Biological assay. In *Statistical Methods in Medical Research*. ed. Armitage, P. pp. 442–456. Oxford: Blackwell.

BERTRAND, G., CHAPAL, J. & LOUBATIERES-MARIANI, M.M. (1986). Potentiating synergism between adenosine diphosphate or triphosphate and acetylcholine on insulin secretion. *Am. J. Physiol.*, **251**, E416–E421.

BERTRAND, G., CHAPAL, J., LOUBATIERES-MARIANI, M.M. & ROYE, M. (1987). Evidence for two different P_2-purinoceptors on β cell and pancreatic vascular bed. *Br. J. Pharmacol.*, **91**, 783–787.

BERTRAND, G., GROSS, R., CHAPAL, J. & LOUBATIERES-MARIANI, M.M. (1989). Difference in the potentiating effect of adenosine triphosphate and α,β-methylene ATP on the biphasic insulin response to glucose. *Br. J. Pharmacol.*, **98**, 998–1004.

BOYER, J.L., DOWNES, C.P. & HARDEN, T.K. (1989). Kinetics of activation of phospholipase C by P_{2y} purinergic receptor agonists and guanine nucleotides. *J. Biol. Chem.*, **264**, 884–890.

BURNSTOCK, G. (1987). Local control of blood pressure by purines. *Blood Vessels*, **24**, 156–160.

BURNSTOCK, G. & BROWN, C.M. (1981). An introduction to purinergic receptors. In *Purinergic Receptors*. ed. Burnstock, G.W. pp. 1–45. London: Chapman and Hall.

BURNSTOCK, G., CUSAK, N.J. & MELDRUM, L.A. (1984). Effects of phosphorothioate analogues of ATP, ADP and AMP on guinea-pig taenia coli and urinary bladder. *Br. J. Pharmacol.*, **82**, 369–374.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

CHAPAL, J. & LOUBATIERES-MARIANI, M.M. (1981). Effects of phosphate-modified adenine nucleotide analogues on insulin secretion from perfused rat pancreas. *Br. J. Pharmacol.*, **73**, 105–110.

CHAPAL, J. & LOUBATIERES-MARIANI, M.M. (1983). Evidence for purinergic receptors on vascular smooth muscle in the pancreas. *Eur. J. Pharmacol.*, **87**, 423–430.

FEDAN, J.S., HOGABOOM, G.K. & O'DONNELL, J.P. (1986). Further comparison of contractions of the smooth muscle of the guinea-pig isolated vas deferens induced by ATP and related analogs. *Eur. J. Pharmacol.*, **129**, 279–291.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

HERBERT, V., LAW, K.S., GOTTLIEB, C.W. & BLEICHER, S.J. (1965). Coated charcoal immunoassay of insulin. *J. Clin. Endocr.*, **25**, 1375–1384.

HOURANI, S.M.O., WELFORD, L.A., LOIZOU, G.D. & CUSAK, N.J. (1988). Adenosine 5'-(2-fluorodiphosphate) is a selective agonist at P_2-purinoceptors mediating relaxation of smooth muscle. *Eur. J. Pharmacol.*, **147**, 131–136.

LOUBATIERES, A.L., MARIANI, M.M., DE MALBOSC, H., RIBES, G. & CHAPAL, J. (1969). Etude expérimentale d'un nouveau sulfamide hypoglycémiant particulièrement actif, le HB 419 ou glibenclamide. I. Action bêtacytotrope et insulino-sécrétrice. *Diabetologia*, **5**, 1–10.

LOUBATIERES-MARIANI, M.M., CHAPAL, J., LIGNON, F. & VALETTE, G. (1979). Structural specificity of nucleotides for insulin secretory action from the isolated perfused rat pancreas. *Eur. J. Pharmacol.*, **59**, 277–286.

PEARSON, J.D. & GORDON, J.L. (1989). P_2 purinoceptors in the blood vessel wall. *Biochem. Pharmacol.*, **38**, 4157–4163.

RALEVIC, V. & BURNSTOCK, G. (1988). Actions mediated by P_2-purinoceptorsubtypes in the isolated perfused mesenteric bed of the rat. *Br. J. Pharmacol.*, **95**, 637–645.

RIBES, G., BERTRAND, G., PETIT, P. & LOUBATIERES-MARIANI, M.M. (1988). Effects of 2-methylthio ATP on insulin secretion in the dog *in vivo*. *Eur. J. Pharmacol.*, **155**, 171–174.

SOULAYMANI, R., GROSS, R., CHAPAL, J. & LOUBATIERES-MARIANI, M.M. (1985). Effet dilatateur de l'adénosine sur les vaisseaux du pancréas de rat. *C.R. Soc. Biol.*, **179**, 371–374.

WELFORD, L.A., CUSAK, L.N. & HOURANI, S.M.O. (1986). ATP analogues and the guinea-pig taenia coli: a comparison of the structure-activity relationships of ectonucleotidases with those of the P_2-purinoceptor. *Eur. J. Pharmacol.*, **129**, 217–224.

ZAR, J.H. (1974). *Biostatistical Analysis*. Englewood Cliffs, N.J.: Prentice-Hall.

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A comparison of the relative activities of a number of $GABA_B$ antagonists in the isolated vas deferens of the rat

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1 A series of $GABA_B$ receptor antagonists were tested against (±)-baclofen for activity on the presynaptic $GABA_B$ receptor in the rat vas deferens.

2 All the antagonists tested caused a rightward shift in the concentration-response curve to (±)-baclofen.

3 pA_2 values calculated from full Schild analysis were as follows: phaclofen, $pA_2 = 4.3$; δ-amino valeric acid, $pA_2 = 4.4$; 3-aminopropyl(diethoxymethyl)phosphinic acid (CGP 35348), $pA_2 = 5.0$; 3-aminopropyl(n-hexyl)phosphinic acid (3-APHPA), $pA_2 = 4.5$.

4 These results show that none of the above compounds possess potent antagonist activity at the $GABA_B$ receptor (i.e. $pA_2 > 6$) in this peripheral tissue. In addition, the more recently available phosphinic acid antagonists, appear to offer no great advance over the $GABA_B$ antagonists previously available.

Introduction

$GABA_B$ receptors are known to be located on peripheral autonomic and enteric nerve terminals (Bowery *et al.*, 1981; Ong & Kerr, 1983). Their pharmacological function appears to be the modulation of excitatory neurotransmitter release in adrenergic systems such as field stimulated guinea-pig atria, rat vas deferens and anococcygeus (Bowery *et al.*, 1981; Muhyaddin *et al.*, 1982) and cholinergic systems such as the guinea-pig ileum (Kaplita *et al.*, 1982; Ong & Kerr, 1983). There is evidence for the existence of intrinsic GABAergic neurones in the enteric nervous system (for review see Jessen *et al.*, 1987; Hills & Jessen, 1991) although their precise role in intestinal function is still uncertain. Recent reports suggest that GABAergic neurones may additionally be present in prevertebral sympathetic ganglia (Hills *et al.*, 1988), and in superior cervical ganglia (Kasa *et al.*, 1988; Wolff *et al.*, 1989). There is to date however, little evidence for a GABAergic innervation in the other autonomically innervated tissues such as the heart, and reproductive organs, studied here.

Limited success in investigations of the physiological significance of the $GABA_B$ receptor subtype has been obtained with the agonist, baclofen. However, few definitive studies have been undertaken because of the lack of an effective $GABA_B$ antagonist.

Recently, a number of investigators have used phaclofen to delineate the function of $GABA_B$ receptors in central systems (Dutar & Nicoll, 1988). This and other $GABA_B$ antagonists such as saclofen and 2-hydroxy saclofen have been considered to be more potent and more selective than previously used compounds such as δ-amino valeric acid (DAVA) and 4-aminobutylphosphonic acid (4-ABPA) (Kerr *et al.*, 1988; 1989). In addition to these compounds, Ciba-Geigy have published a patent (Baylis *et al.*, 1989) claiming $GABA_B$ antagonist activity for a series of phosphinic acids.

Here, using baclofen as agonist, we have examined the relative antagonist potencies of a range of $GABA_B$ antagonists (Figure 1), including two phosphinic acid analogues, 3-aminopropyl(diethoxymethyl)phosphinic acid (CGP 35348, Bittiger *et al.*, 1989) and 3-aminopropyl(n-hexyl)phosphinic acid (3-APHPA) (Ciba-Geigy), on the isolated vas deferens of the rat as an *in vitro* functional pharmacological assay system.

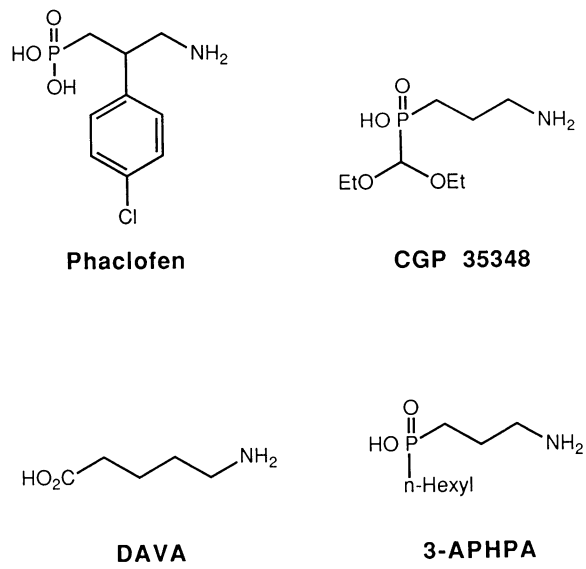

Figure 1 Chemical structures of the $GABA_B$ antagonists phaclofen, δ-amino valeric acid (DAVA), 3-aminopropyl(diethyloxymethyl)-phosphinic acid (CGP 35348) and 3-aminopropyl-(n-hexyl)phosphinic acid (3-APHPA) used in this study.

Methods

Male wistar rats (200–250 g) were killed by a blow to the head and their vasa deferentia removed. Vas deferens preparations were suspended between two platinum ring electrodes in organ baths containing modified Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.3, $NaHCO_3$ 16.3, $MgSO_4$ 0.6, $CaCl_2$ 2.5 and glucose 7.7, which was continually gassed with $95\%O_2/5\%CO_2$. A resting tension of 1 g was applied and the preparations were field stimulated from Grass SD11 stimulators with the following stimulus parameters: 1 ms pulses of supramaximal voltage at 50 Hz for 100 ms every 10 s. Isometric muscle responses were measured with a strain gauge transducer (Biomed) and displayed on an Ormed multitrace pen recorder. Preparations were allowed to equilibrate for 1 h before the addition of a compound to the organ bath.

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Baclofen concentration-response curves were constructed sequentially and the inhibitory response calculated as % maximum response to baclofen. Antagonists were tested by adding the appropriate concentration to the organ bath 10 min prior to baclofen. At least four concentrations of each antagonist was tested in the range 10–1000 μM, and each was tested on at least four vas deferens preparations from separate animals. pA_2 values were derived from full Schild analysis. Statistical evaluation has been carried out by linear regression analysis and 95% confidence intervals (CI) calculated for the pA_2 values and slopes obtained from the Schild analysis.

Drugs used

(±)-Baclofen (Research Biochemicals Inc.); δ-amino valeric acid (DAVA), (Sigma); phaclofen, (Tocris Neuramin); 3-aminopropyl(diethoxymethyl)phosphinic acid (CGP 35348) (W. Howson, SB) and 3-aminopropyl(n-hexyl)phosphinic acid (3-APHPA) (W. Howson, SB), were used. All compounds were dissolved in distilled water, subsequent dilutions being made in distilled water, and compounds were added to the organ bath in volumes no greater than 1% total volume.

Results

Baclofen inhibits the field stimulation-induced twitch response in the rat vas deferens (IC_{50} = 5.9 ± 1.4 μM, n = 8). Such an effect has been previously documented for the mouse vas deferens (Bowery *et al.*, 1981). Baclofen is thought to reduce the release of the excitatory neurotransmitter from the sympathetic nerve endings via $GABA_B$ receptors located presynaptically (Hill & Bowery, 1986).

Table 1 Antagonism of baclofen concentration-response curves in the isolated vas deferens by phaclofen, δ-amino valeric acid (DAVA), 3-aminopropyl(diethyloxymethyl)-phosphinic acid (CGP 35348) and 3-aminopropyl(n-hexyl)-phosphinic acid (3-APHPA)

Antagonist	pA_2	*Slope*
Phaclofen	4.25 (4.07, 4.55)	1.02 (0.66, 1.37)
DAVA	4.42 (4.16, 4.89)	0.86 (0.54, 1.18)
CGP 35348	5.03 (4.61, 5.98)	0.64 (0.35, 0.93)*
3-APHPA	4.55 (4.29, 4.96)	1.01 (0.66, 1.36)

The data are derived from Schild plots with at least 4 points obtained from at least 4 tissues from separate animals.
* Value is significantly different from one, * $P < 0.05$

All the antagonists tested produced a rightward shift in the concentration-response curve to (±)-baclofen. For each antagonist, increasing concentrations produced progressive near parallel shifts in the concentration-response curves and no reduction in the maximum response to (±)-baclofen was observed (Figure 2). pA_2 values for each antagonist and the slope of the plot derived from Schild analysis (Figure 3) are given in Table 1.

As may be seen from Table 1 and Figure 4a, the numerical differences in the pA_2 values obtained for the various antagonists tested were not great, the new Ciba-Geigy compounds (CGP 35348 and 3-APHPA) being only marginally more potent than for example, phaclofen or DAVA. Statistical analysis of the slopes of the plots (see Figure 4b), shows pha-

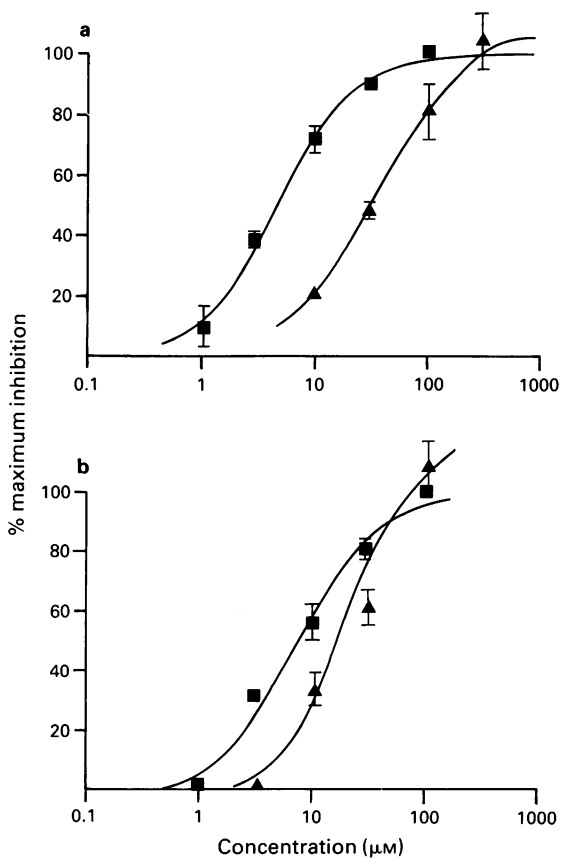

Figure 2 Concentration-response curves for the (±)-baclofen-mediated inhibition of the field stimulation induced twitch response in the rat vas deferens: (a) control curve (■) and curve in the presence of 3-aminopropyl(diethyloxymethyl)phosphinic acid (CGP 35348, 100 μM) (▲); (b) control curve (■) and curve in the presence of 3-aminopropyl(n-hexyl)phosphinic acid (3-APHPA, 30 μM) (▲) where $n > 4$.

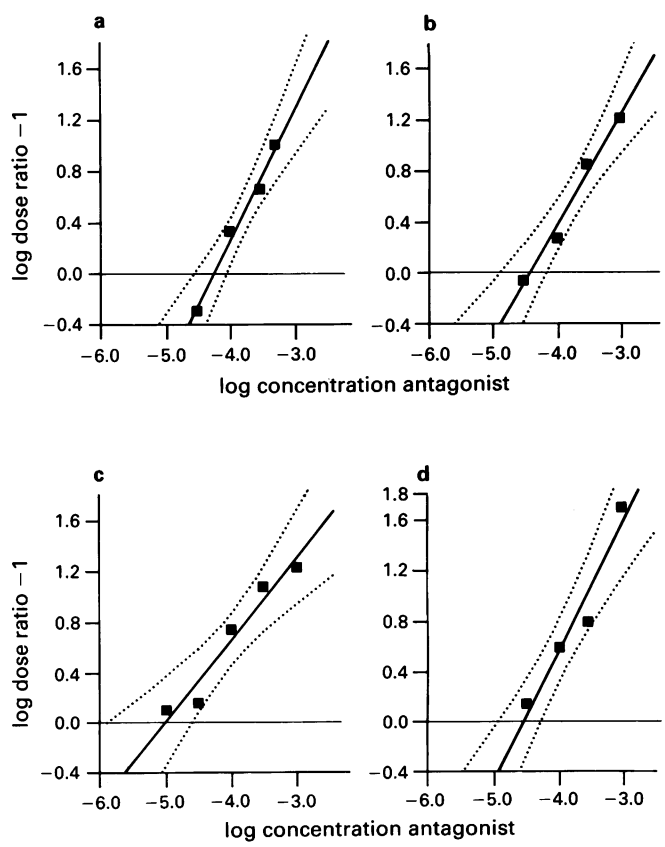

Figure 3 Schild plots (log dose ratio − 1 versus log antagonist concentration) for phaclofen (a), δ-amino valeric acid (DAVA) (b), 3-aminopropyl(diethyloxymethyl)phosphinic acid (CGP 35348) (c) and 3-aminopropyl(n-hexyl)phosphinic acid (3-APHPA) (d), tested against (±)-baclofen in the rat vas deferens. The graphs show the best line fit with 95% confidence intervals. The pA_2 may be obtained from the negative logarithm of the intercept on the x axis.

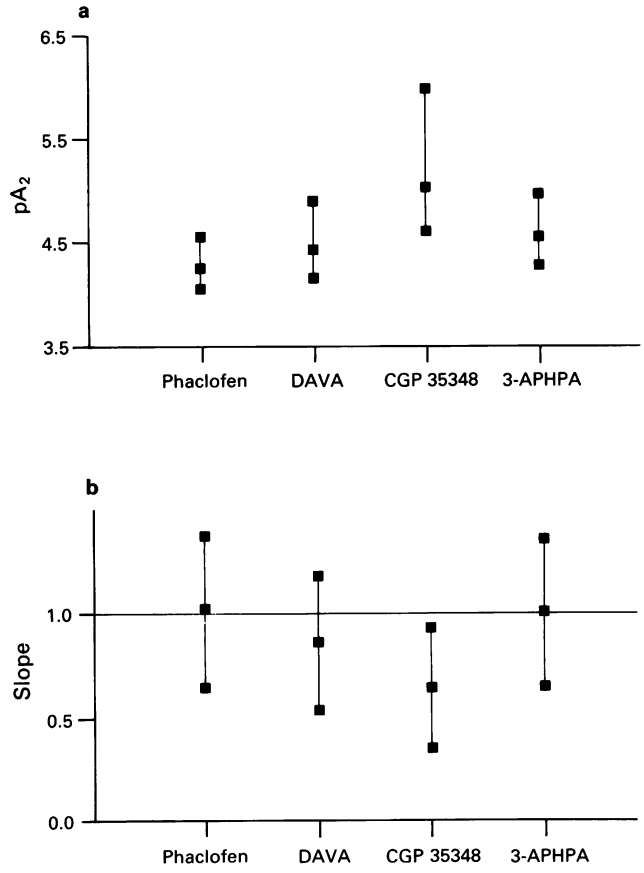

Figure 4 A graphical representation of calculated pA_2 values (a), and slopes (b) obtained from Schild analysis for phaclofen, δ-amino valeric acid (DAVA), 3-aminopropyl(diethyloxymethyl)phosphinic acid (CGP 35348) and 3-aminopropyl(n-hexyl)phosphinic acid (3-APHPA). The bars show the 95% Cl for each value.

clofen, DAVA and 3-APHPA to have slopes close to one, and it can therefore be assumed that these compounds are true competitive $GABA_B$ antagonists. Although no reduction in the maximal agonist response was observed with CGP 35348, the Schild plot gave a slope significantly less than one, (see 95% Cl for CGP 35348 on Table 1). A slope of less than one obtained from Schild analysis of antagonist data is normally taken to indicate non-competitive antagonism and thus to invalidate the pA_2 value so derived.

None of the antagonists tested showed agonist activity at high concentrations, indicating that they were not partial agonists. In the presence of the $GABA_B$ antagonists, high baclofen concentrations (100–300 μM) sometimes caused a greater inhibition of the twitch response than was observed with baclofen in the absence of antagonist (see Figure 2b). This phenomenon which caused a slight steepening of the concentration-response curves, occurred with all four of the antagonists tested in about 50% of experiments with each compound. There was a tendency towards the potentiation occurring at low antagonist concentrations for phaclofen and DAVA and at high antagonist concentrations for CGP 35348 and 3-APHPA, showing no clear association with compounds having a slope less than one in the Schild analysis.

Discussion

The results of studies using a variety of experimental systems indicate that compounds such as phaclofen and DAVA are antagonists of $GABA_B$ receptor-mediated pharmacological responses (Curtis *et al.*, 1988; Kerr *et al.*, 1988; 1989). Phaclofen has been claimed to be selective for $GABA_B$-mediated events (Dutar & Nicoll, 1988; Soltesz *et al.*, 1988; Karlsson & Olpe, 1989) although its binding shows only weak affinity for the $GABA_B$ receptor (phaclofen $IC_{50} = 118\,\mu M$) (Bowery, 1989). DAVA has been used as a $GABA_B$ antagonist since the early eighties (Muhyaddin *et al.*, 1982); however, results with this compound are often complicated by its $GABA_A$ agonist activity (Allan *et al.*, 1986; Bowery, 1989). The limited data available on CGP 35348 suggests that it is selective for the $GABA_B$ receptor, is active *in vitro* against baclofen at concentrations between 100–300 μM, and inhibits agonist binding in the rat cortex with an IC_{50} of 35 μM (Bittiger *et al.*, 1989). No data specifically relating to 3-APHPA have been published.

In agreement with previous reports, the pA_2 values obtained in our experiments are low and the antagonist phaclofen, which has a structure containing a chlorophenyl substituted ring, has similar activity in terms of antagonist potency, to the unsubstituted compounds, DAVA and the phosphinic acids (CGP 35348 and 3-APHPA). These relatively poor pA_2 values are borne out by the excessive concentrations often employed in pharmacological studies required to antagonize baclofen- or GABA-mediated effects (Kerr *et al.*, 1987; Dutar & Nicoll, 1988; Hunter *et al.*, 1989). The experiments with CGP 35348, suggest that this compound is not a true competitive antagonist. It is possible that there is allosteric modulation or non-receptor interaction which accounts for some of the observed antagonism seen with this compound. Recent reports have claimed that CGP 35348 is an effective antagonist against baclofen *in vivo* at doses of 30–100 $mg\,kg^{-1}$ (Bittiger *et al.*, 1989).

The observation that potentiation of high baclofen mediated inhibition of twitch responses occurred in the presence of antagonist, is not readily explicable. One possible explanation for this effect may be an increase in tissue sensitivity to the agonist caused by the presence of the antagonist. This phenomenon was not further investigated in these experiments, although it would be of interest to see if the effect were peculiar to baclofen or occurred either with other $GABA_B$ agonists such as 3-aminopropylphosphinic acid or in other tissues where a postsynaptic rather than a presynaptic $GABA_B$ receptor was present.

In conclusion these results confirm that phaclofen, DAVA, CGP 35348 and 3-APHPA are weak but effective $GABA_B$ antagonists. Although the most potent antagonist, CGP 35348 (pA_2, 5.03), offers some improvement in antagonist activity, it would appear not to be a true competitive pharmacological antagonist at the $GABA_B$ receptor.

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References

ALLAN, R.D. & DICKENSON, H.W. (1986). Evidence that antagonism by δ-aminovaleric acid of $GABA_B$ receptors in the guinea-pig ileum may be due to an interaction between $GABA_A$ and $GABA_B$ receptors. *Eur. J. Pharmacol.*, **120**, 119–122.

BAYLIS, E.K., BITTIGER, H., FROSTL, W., HALL, R.G., MAIER, L., MICKEL, S.J. & OLPE, H.R. (1989). Substituted propane phosphinic acid compounds. EP 319479.

BITTIGER, H., FROSTL, W., HAUSER, K., KARLSSON, G., KLEBS, K., OLPE, H.R., POZZA, M., RADEKE, E., STEINMANN, M., VAN REISEN, H. & VASSOUT, A. (1990). Biochemistry, electrophysiology and pharmacology of a new $GABA_B$ antagonist. In *$GABA_B$ Receptors in Mammalian Function*. ed. Bowery, N.G., Bittiger, H. & Olpe, H.R. Chichester: John Wiley. (in press).

BOWERY, N.G. (1989). $GABA_B$ receptors and their significance in mammalian pharmacology. *Trends Pharmacol. Sci.*, **10**, 401–407.

BOWERY, N.G., DOBLE, A., HILL, D.R., HUDSON, A.L., SHAW, T.S., TURNBULL, M.J. & WARRINGTON, R. (1981). Bicuculline insensitive GABA receptors on peripheral autonomic nerve terminals. *Eur. J. Pharmacol.*, **71**, 53–70.

CURTIS, D.R., GYNTHER, B.D., BEATTIE, D.T., KERR, D.I.B. & PRAGER,

R.H. (1988). Baclofen antagonism by 2-hydroxy saclofen in the cat spinal cord. *Neurosci. Letts.*, **92,** 97–101.

DUTAR, P. & NICOLL, R.A. (1988). Physiological role for $GABA_B$ receptors in the central nervous system. *Nature*, **332,** 156–158.

HILL, D.R. & BOWERY, N.G. (1986). In *GABA-ergic Mechanisms in the Mammalian Periphery*. ed. Erdo, S.L. & Bowery, N.G. pp. 87–97. New York: Raven Press.

HILLS, J.M. & JESSEN, K.R. (1991). Transmission: GABA, 5-HT and Dopamine. In *The Autonomic Nervous System. Volume 1, Autonomic Neuroeffector Mechanisms*. ed. Burnstock, G. & Hoyle, C.H.V. Series ed. Burnstock, G. London: Harwood Academic Publishers: (in press).

HILLS, J.M., KING, B.F., MIRSKY, R. & JESSEN, K.R. (1988). Immunohistochemical localisation and electrophysiological actions of GABA in prevertebral ganglia in guinea-pig. *J. Auton. Nerv. Syst.*, **22,** 129–140.

HUNTER, A.J., MURRAY, T.K., TOCZEK, J.M., BOAR, B.R. & GREEN, A.R. (1989). The effects of the putative $GABA_B$ antagonists phaclofen and δ-aminovaleric acid on $GABA_B$ function *in vivo*. *Br. J. Pharmacol.*, **95,** 763P.

JESSEN, K.R., MIRSKY, R. & HILLS, J.M. (1988). GABA as an autonomic neurotransmitter: studies on intrinsic GABAergic neurons in the myenteric plexus of the gut. *Trends Neurosci.*, **10,** 255–262.

KAPLITA, P.V., WALTERS, D.H. & TRIGGLE, D.J. (1982). γ-Aminobutyric acid action on the guinea-pig ileal myenteric plexus. *Eur. J. Pharmacol.*, **79,** 43–51.

KARLSSON, G. & OLPE, H.R. (1989). Late inhibitory postsynaptic potentials in rat pre-frontal cortex may be mediated by $GABA_B$ receptors. *Experientia*, **45,** 157–158.

KASA, P., JOO, F., DOBO, E., WENTHOLD, R.J., OTTERSEN, D.P., STORM-MATHISEN, J. & WOLFFE, J.R. (1988). Heterogenous distribution of GABA-immunoreactive nerve fibers and axon terminals in the superior cervical ganglion of the adult rat. *Neuroscience*, **26,** 635–644.

KERR, D.I.B., ONG, J., JOHNSTON, G.A.R., ABBENANTE, J. & PRAGER, R.H. (1988). 2-Hydroxy saclofen: an improved antagonist at central and peripheral $GABA_B$ receptors. *Neuroscience Letts.*, **92,** 92–96.

KERR, D.I.B., ONG, J., JOHNSTON, G.A.R. & PRAGER, R.H. (1989). $GABA_B$ receptor mediated actions of baclofen in rat isolated neocortical slice preparations: antagonism by phosphonic analogues of GABA. *Brain Research*, **480,** 312–316.

KERR, D.I.B., ONG, J., PRAGER, R.H., GYNTHER, B.D. & CURTIS, D. (1987). Phaclofen: a peripheral and central baclofen antagonist. *Brain Research*, **405,** 150–154.

MUYHADDIN, M., ROBERTS, P.J. & WOODRUFF, G.N. (1982). Presynaptic γ-aminobutyric acid receptors in the rat anococcygeus muscle and the antagonism by 5-aminovaleric acid. *Br. J. Pharmacol.*, **77,** 163–168.

ONG, J. & KERR, D.I.B. (1983). $GABA_A$ and $GABA_B$ receptor mediated modification of intestinal motility. *Eur. J. Pharmacol.*, **86,** 9–17.

SOLTESZ, L., HABY, M., LERESCHE, N. & CRUNELLI, V. (1988). The $GABA_B$ antagonist phaclofen inhibits the late potassium dependent IPSP in cat and rat thalamic and hippocampal neurons. *Brain Research*, **448,** 351–354.

WOLFFE, J.R., KASA, P., DOBO, E., WENTHOLD, R.J. & JOO, F. (1989). Quantitative analysis of the number and distribution of neurons richly innervated by GABA-immunoreactive axons in the rat superior cervical ganglion. *J. Comp. Neurol.*, **282,** 264–273.

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Pharmacology of a cholecystokinin receptor on 5-hydroxytryptamine neurones in the dorsal raphe of the rat brain

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1 The effect of bath application of sulphated cholecystokinin octapeptide (CCK-8) was studied on neurones in slices containing rat raphe nucleus.

2 Intracellular recordings were made from neurones in the dorsal raphe nucleus. Some of the neurones with the characteristics of 5-hydroxytryptamine (5-HT)-containing cells which were inhibited by 5-HT and excited by noradrenaline were excited by cholecystokinin. The response to cholecystokinin was dose-dependent over the range 10 to 1000 nM.

3 The response to CCK-8 persisted in the presence of tetrodotoxin. Either reduction of extracellular calcium or addition of 25 mM magnesium did not block the CCK response, suggesting it was mediated by receptors located on the membrane of the raphe neurones.

4 The agonist and antagonist specificity of the CCK response was determined. The CCK_B selective agonist, pentagastrin, was inactive when applied at concentrations up to 10 μM. the CCK_A receptor antagonist L-364,718 (1 to 100 nM) blocked the response to cholecystokinin. Much higher (1–10 μM) concentrations of the CCK_B receptor antagonist L-365,260 were required for inhibition of the CCK response.

5 These data support the existence of a CCK receptor, located on raphe neurones in the rat, with a pharmacological profile very similar to that described for the CCK_A type.

Introduction

The dorsal raphe nucleus is the largest of the raphe nuclei and contains the highest density of 5-hyroxytryptamine (5-HT) containing neurones in the rat brain. It is found at the rostral portion of the tegmentum pontis at the border of the pons and mesencephalon, in the midline between the medial longitudinal fasciculi and the aqueduct. 5-HT receptors are present on the soma and dendrites of raphe 5-HT neurones and activation of these receptors by stimulating 5-HT containing nerve terminals or bath application of 5-HT agonists causes an increase in an inwardly rectifying potassium conductance (Williams *et al.*, 1988; Pan *et al.*, 1989) leading to hyperpolarization of the neuronal membrane and a reduction in action potential firing. The same neurones receive a direct synaptic noradrenergic input (Baraban & Aghajanian, 1981) and are excited by noradrenaline and phenylephrine (Aghajanian, 1985).

Although the mechanism of action of monoamines on dorsal raphe neurones appears well understood, nothing is known of the possible modulation of brain stem raphe neurones by peptides, such as cholecystokinin (CCK). No data are available concerning the distribution of CCK receptors within the rat raphe nuclei but CCK-8-like immunoreactive neurones are present in rat dorsal raphe (Vanderhaegen *et al.*, 1980; Van Der Koy *et al.*, 1981). Autoradiographic and binding methods have shown that the majority of CCK receptors in the brain are the B subtype (Hill *et al.*, 1987), thus determination of the receptor type involved in any functional response monitored *in vitro* is essential. Previous electrophysiological studies have relied heavily on the agonist selectivity difference between the two types of CCK receptor identified so far, to confirm that functional CCK_B receptors exist in rat hippocampus (Dodd & Kelly, 1981; Boden & Hill, 1986) and rat ventromedial hypothalamus (VMH) (Boden & Hill, 1988). However, the availability of potent, selective antagonists for the two types of CCK receptor makes it possible now to identify clearly the CCK receptor type. In the present study the effects of sulphated cholecystokinin octapeptide (CCK-8) were examined on neurones which responded to 5-HT and phenylephrine in an *in vitro* slice preparation of the raphe and the selective CCK antagonists, L-364,718 and L-365,260 were used as tools to characterize the receptor involved.

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Methods

Preparation of slices and recording from neurones

Intracellular recordings were made with electrodes fabricated from fibre filled glass (Clark GC120F-10) and filled with 1 M potassium acetate. The resistance of such electrodes measured in physiological saline varied between 80 and 140 MΩ. Current-clamp recordings from dorsal raphe neurones in the slice preparation from rat pons-mesencephalon were made with an Axoclamp-2a amplifier in bridge mode. Current pulses were of sufficient length (200 ms) to ensure full saturation of membrane capacitance. Male rats (50–100 g) were killed by cervical dislocation and the brain rapidly removed. Coronal slices (350 μm) were cut with a Vibratome in cold (4°C) physiological saline. A single slice was placed in a tissue bath through which flowed physiological artificial cerebrospinal fluid (ACSF) (2 ml min^{-1}) at 35°C. The content of the ACSF was as follows (concentrations mM): NaCl 126, KCl 5.0, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.4, glucose 11 and $NaHCO_3$ 25; the solution was gassed with 95%O_2, 5%CO_2 at 35°C. Magnesium chloride (10–20 mM) was substituted for calcium chloride to obtain calcium-free ACSF. Drugs dissolved in ACSF were applied by superfusion. L-364,718 (3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-1H-indole-2-carboxamide) and L-365,260 (3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-N'-(3-methylphenyl)urea) were synthesized in Medicinal Chemistry, Parke-Davis, Cambridge, U.K. CCK-8 was obtained from either Peninsula Laboratories or Cambridge Research Biochemicals. All other drugs were obtained from either Sigma or Aldrich.

Analysis of data

Dose-response curves were constructed from firing rates or the depolarization of the membrane potential of the neurones in the presence of tetrodotoxin (TTX). The number of action potentials occurring during a CCK response was analyzed on-line through a CED 1401 interface and IBM AT with a specially designed suite of software (MRATE, Cambridge Electronic Design, Cambridge, UK). Best-fit curves for dose-response curves were obtained by fitting a simple hyperbolic function ($f\{x\} = (B.x)/(x + a)$) using RS1 version 12.10E software (BBN Software, Cambridge, Massachusetts, U.S.A.). The equilibrium constant was then calculated from the equation, Dose-ratio $- 1 =$ [antagonist]/equilibrium constant. These data were expressed as mean $\pm$ s.e.mean. Analyses of significance was by Student's t test, $P = 0.05$ being taken as significant.

Results

Neuronal types in the raphe

In agreement with previous studies (Williams *et al.*, 1988), two populations of neurones were observed in the raphe. One population of neurones did not respond to either 5-HT, or phenylephrine. Neurones which did not respond to 5-HT and phenylephrine did not respond to CCK-8 ($n = 21$). The second group of neurones ($n = 56$) were characterized by the presence of large, long-lasting after-hyperpolarizations and broad action potentials. These neurones were hyperpolarized by a 1 min application of 5-HT (1–10 μM), in agreement with previously reported data (Aghajanian & Lakoski, 1984; Yoshimura & Higashi, 1985), identifying them as 5-hydroxytryptaminergic raphe neurones. They were used subsequently in studies of the action of CCK-8 on raphe neurones.

CCK-8 effects on raphe neurones

Of the 41 neurones which responded to CCK-8 (10–1000 nM), most were excited ($n = 32$), while a few were inhibited ($n = 9$). Fifteen neurones did not respond to CCK-8 application. On neurones which were excited by CCK-8, a 60 s application of CCK-8 produced a fully reversible membrane depolarization which lasted for between 2 and 5 min. The amplitude of the excitatory CCK-8 response was not diminished when a subsequent application of the peptide was made some 10 min after the first, indicating that little or no desensitization had occurred. The effect of CCK-8 was dose-dependent (Figure 1). Depolarizing responses to CCK-8 were not significantly different from control when recorded from neurones in slices treated with TTX (1 μM, 10 min, Figure 2a) which blocked both spontaneous action potentials and electrically evoked potentials, or from neurones in slices perfused with calcium-free ACSF containing 10 to 25 mM magnesium (Figure 2b), indicating a direct action of the peptide on the neuronal membrane.

CCK-agonist studies

The experiments were repeated with pentagastrin (Boc-B-alanine CCK-4), a selective agonist for CCK_B receptors in the rat VMH (Boden & Hill, 1988). Pentagastrin (1 to 10 μM) had no effect on CCK-8-sensitive neurones ($n = 3$).

CCK-antagonist studies

L-364,718 is a selective antagonist of CCK at the CCK_A receptor (Chang & Lotti, 1986). Bath application of L-364,718 (1 to 100 nM on 9 neurones) produced a rapid and potent antagonism of the CCK-8-induced excitation. Figure 3 shows the effect of L-364,718 on responses to CCK-8 recorded from

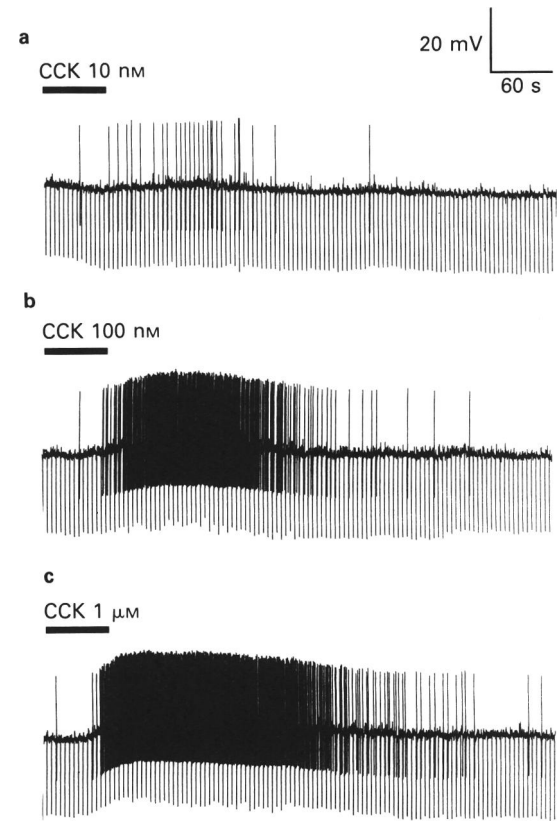

Figure 1 Pen recorder trace from a single neurone in a dorsal raphe nucleus *in vitro* showing dose-dependent excitation by cholecystokinin octapeptide (CCK-8). Resting potential of the neurone was -54 mV. No spontaneous activity was present prior to application of the peptide. Top trace, when CCK-8 (10 nM) was added for a period of 60 s indicated by the black bar, the neurone depolarized and action potentials were elicited. Full recovery from the effects of the peptide were seen on returning the slice to normal, drug-free saline. The depolarizing nature of the response can be seen more clearly when a concentration of 100 nM CCK-8 was applied (b). Finally when 1 μM CCK-8 was applied a much longer lasting effect was seen (c) and some 5 min elapsed before full recovery. Downward deflections are electrotonic voltage pulses resulting from current injection via the microelectrode. The action potentials are somewhat truncated as a result of the limited frequency response of the pen recorder.

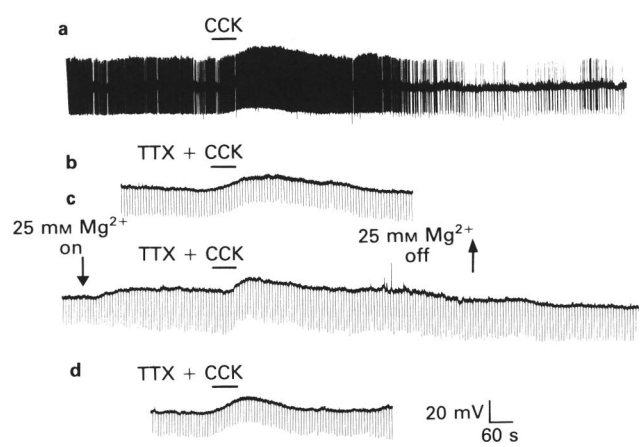

Figure 2 The response to cholecystokinin octapeptide (CCK-8) 100 nM persists in the presence of 25 mM magnesium ions: (a) shows the response to CCK-8 in control conditions; (b) shows that the CCK-8 response is still present during exposure to tetrodotoxin (TTX) 1 μM which abolished the sodium-dependent action potentials. Addition of 25 mM $MgCl_2$ shown in (c) led to a decrease in membrane conductance. The CCK-8 response still occurred in these conditions. The membrane conductance returned to control values on returning to ACSF containing 1.2 mM magnesium. Trace (d) shows that the response to CCK-8 was still present after returning to control ACSF.

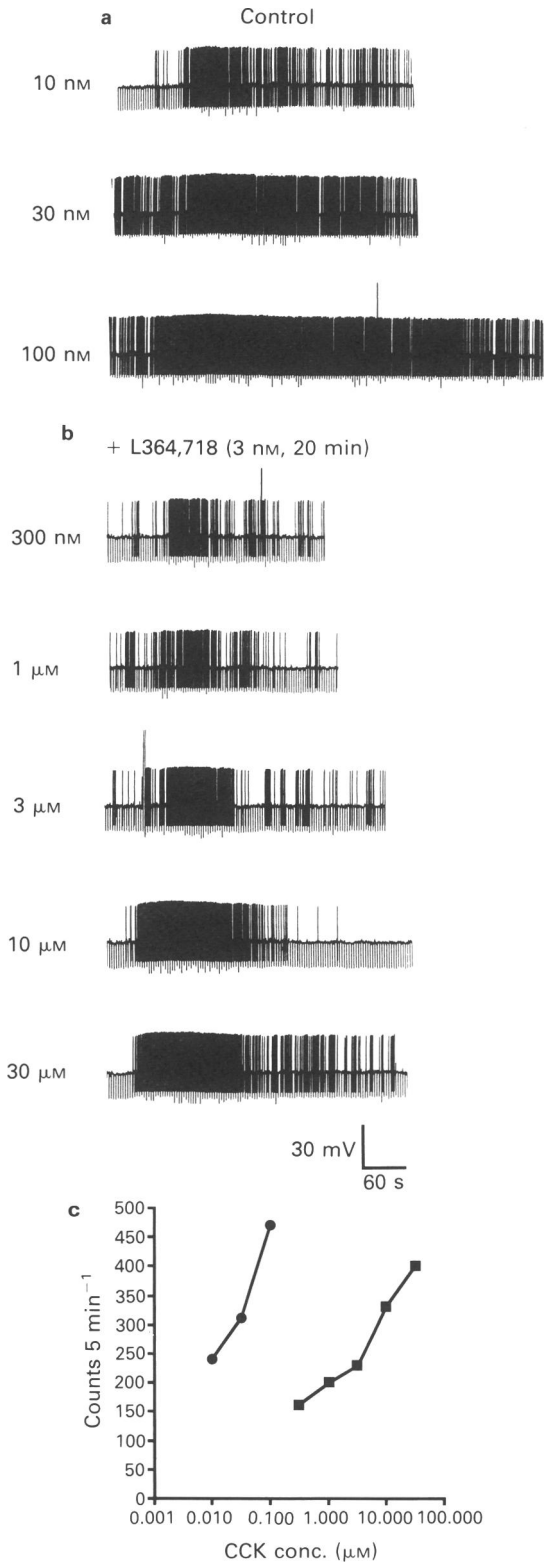

Figure 3 The potent CCK_A antagonist L-364,718 blocks the response to cholecystokinin octapeptide (CCK-8). Traces in column (a) were obtained from a dorsal raphe neurone and show dose-dependent effects of CCK-8 (1 min applications) from 10–100 nM. Pen recorder traces in column (b) were taken from the same neurone after a 20 min incubation in L-364,718 (3 nM) and show that the response to CCK was considerably reduced by the antagonist. The data were used to produce a dose-response curve (c) of the number of action potentials occurring in a 5 min period immediately following peptide application as a function of concentration of CCK-8. The control dose-response curve to CCK (●) was shifted to the right in a parallel fashion in the presence of L-364,718 (3 nM) (■), indicative of a competitive antagonism. The equilibrium constant for L-364,718 was 31 pM in this example.

a rat dorsal raphe neurone. On neurones which were excited by CCK-8, the equilibrium constant for L-364,718 was calculated to be 0.127 ± 0.043 nM ($n = 4$). L-365,260 is a gastrin/CCK_B antagonist, possessing nanomolar affinity for the guinea-pig cortical CCK_B receptor but only micromolar affinity for the guinea-pig pancreatic CCK_A receptor (Chang & Lotti, 1986). Bath application of L-365,260 (10 μM for 15 to 20 min) had a weak antagonist action on the response to CCK-8 (Figure 4) and the equilibrium constant for L-365,260 was calculated to be 724.2 ± 93.8 nM ($n = 4$).

Antagonism of the CCK-8-induced depolarization with either L-364,718 or L-365,260 occurred in the presence of TTX; neither antagonist caused a change in resting membrane potential or resistance.

Actions of other antagonists on the response to CCK

Bath application of the α-adrenoceptor antagonist, prazosin (1 μM) or the 5-HT receptor antagonist spiperone (10 μM), which abolished responses to phenylephrine and 5-HT respectively, had no effect on the response to CCK-8 ($n = 4$). Carbachol (1–10 μM) had no effect on CCK-sensitive neurones ($n = 3$).

Discussion

We have shown for the first time that CCK excites a population of dorsal raphe neurones in a dose-dependent manner. Confirmation that CCK acted directly on the recorded neurone came from experiments using TTX-treated preparations where extracellular calcium had been omitted and replaced by high magnesium. Under these conditions, when sodium-dependent action potentials were blocked and no electrically evoked potential could be seen, we were unable to see any change in the magnitude of the response to CCK. The CCK response was dose-dependent over the range tested (10–1000 nM), similar to that seen in extracellular recordings of CCK_B responses from rat VMH neurones. One major difference in the CCK response recorded from raphe neurones however, was in the lack of effect of CCK_B agonist, pentagastrin.

Secondly, we demonstrated that this effect of CCK occurs via CCK_A type receptors located on the neuronal membrane. The equilibrium constant obtained with L-364,718 was similar to the equilibrium constant obtained in binding studies to CCK_A binding sites in the pancreas (Chang & Lotti, 1986). This suggests that CCK excites a subpopulation of neurones in the dorsal raphe by acting at a CCK_A receptor. This is supported, not only by the lack of effect seen with the CCK_B agonist, pentagastrin, but also by the relatively weak antagonist action of the potent CCK_B receptor antagonist, L-365,260 (Lotti & Chang, 1989). We found that the equilibrium constant for L-365,260 on raphe neurones was similar to the equilibrium constant observed for L-365,260 at CCK_A sites in binding studies on guinea-pig pancreatic membranes. The persistence of the CCK response in the presence of prazosin and spiperone, shows that the mechanism of action of CCK was unlikely to involve either noradrenaline or 5-HT.

Autoradiographic studies indicate that the CCK_B receptor is the predominant type in the rodent central nervous system (Hill *et al.*, 1987). Thus, in the VMH there is a good correlation between a high density of CCK_B binding sites and a biological response which is mediated via CCK_B receptors (Boden & Hill, 1988). Interestingly, in the raphe, carbachol (10 μM), an agonist on muscarinic receptors on ventromedial hypothalamic neurones where the CCK response is mediated via a CCK_B receptor (Boden & Hill, 1988), had no effect on neurones which responded to CCK-8.

Although the majority of CCK receptors in the central nervous system are of the B type, CCK_A receptors in the interpeduncular nucleus have been demonstrated both autoradiographically (Hill *et al.*, 1988) and electrophysiologically (Hill

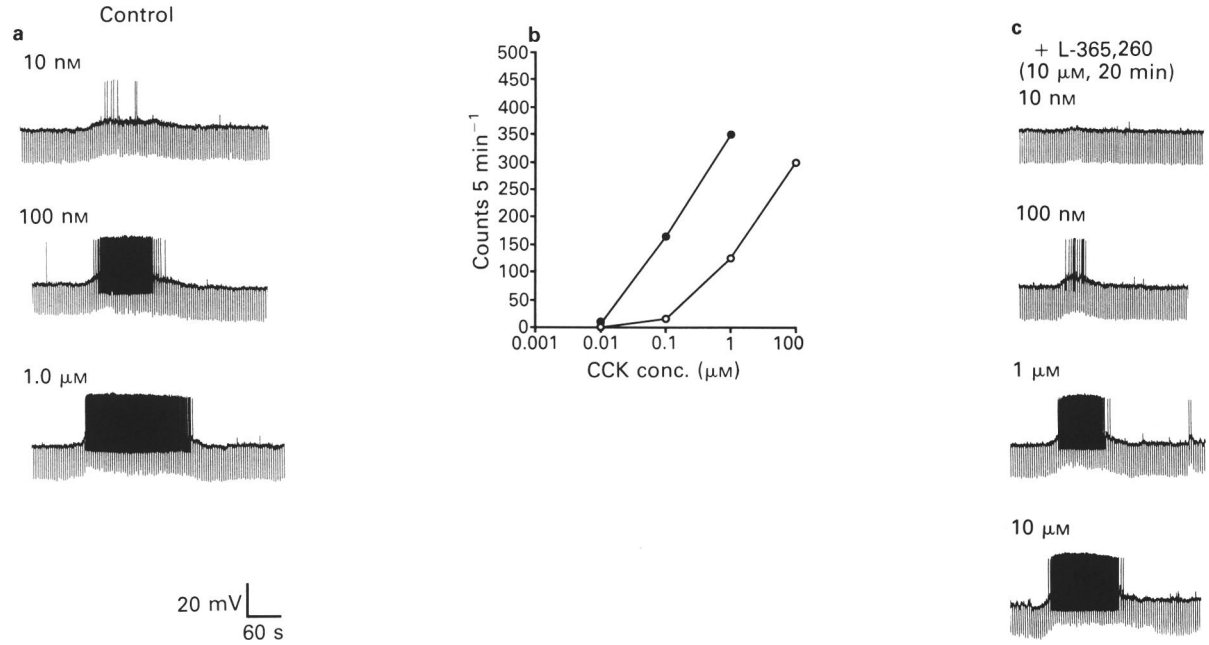

Figure 4 The CCK_B antagonist L-365,260 blocks the response to cholecystokinin octapeptide (CCK-8). Traces in column (a) were obtained from a dorsal raphe neurone and show dose-dependent effects of CCK-8 (1 min applications) from 10–1000 nM. Pen recorder traces from a silent neurone are used for clarity to show the excitatory action of CCK. The traces in column (a) show the effects of CCK before exposure to L-365,260. The prolonged exposure of L-365,260 had no effect on its own on either the membrane potential or activity of the neurone. Traces in column (c) were taken from the same neurone after a 20 min incubation in L-365,260 (10 μM) and show that the response to CCK was reduced by this antagonist. The data were used to produce a dose-response curve (b), with the number of action potentials occurring during the CCK-8 response on the y-axis and the dose of CCK-8 on the x-axis. The control dose-response curve to CCK (○) was shifted to the right in a parallel fashion in the presence of L-365,260 10 μM (●), indicative of a competitive antagonism. The equilibrium constant for L-365,260 was calculated to be 865.8 nM in this example.

& Boden, 1989). Evidence for the existence of CCK_A receptors in other rat brain regions may be hampered by their comparatively low density. For example, in spite of the fact that autoradiographic studies fail to reveal the presence of CCK_A binding sites in the rat nucleus accumbens, it has recently been shown that CCK_A receptors mediating dopamine release are present in this region (Marshall *et al.*, 1990). Our results presented in this paper show clear electrophysiological actions of CCK in the dorsal raphe nucleus and provide further evidence for a central role for CCK_A receptors in rat brain. Studies are in progress to determine the mechanism of action of CCK on raphe neurones.

References

AGHAJANIAN, G.K. (1985). Modulation of a transient outward current in serotonergic neurones by alpha 1-adrenoceptors. *Nature*, **315**, 501–503.

AGHAJANIAN, G.K. & LAKOSKI, J.M. (1984). Hyperpolarisation of serotonergic neurons by serotonin and LSD: studies in brain slices showing increased K^+ conductance. *Brain Res.*, **305**, 181–185.

BARABAN, J.H. & AGHAJANIAN, G.K. (1981). Noradrenergic innervation of serotonergic neurones in the dorsal raphe: demonstration by electron microscope autoradiography. *Brain Res.*, **204**, 1–11.

BODEN, P. & HILL, R.G. (1986). Evidence for the cholecystokinin (CCK) fragments CCK-8S, CCK-4 and pentagastrin sharing a common site of action on rat hippocampal neurones *in vitro*. *J. Physiol.*, **381**, 48P.

BODEN, P. & HILL, R.G. (1988). Effects of cholecystokinin and related peptides on rat ventromedial neurones *in vitro*. *Br. J. Pharmacol.*, **94**, 246–252.

CHANG, R.S.L. & LOTTI, V.J. (1986). Biochemical and pharmacological characterization of a new extremely potent and selective nonpeptide cholecystokinin antagonist. *Proc. Natl. Acad. Sci., U.S.A.*, **83**, 4923–4926.

DODD, J. & KELLY, J.S. (1981). The actions of cholecystokinin and related peptides on pyramidal neurones of the mammalian hippocampus. *Brain Res.*, **205**, 337–350.

HILL, D.R., CAMPBELL, N.J., SHAW, T.M. & WOODRUFF, G.N. (1987). Autoradiographic localization and biochemical characterization of peripheral type CCK receptors in rat CNS using highly selective nonpeptide CCK antagonists. *J. Neurochem.*, **7**, 2967–2976.

HILL, D.R., SHAW, T.M., DOURISH, C.T. & WOODRUFF, G.N. (1988). CCK-A receptors in the rat interpeduncular nucleus: evidence for a presynaptic location. *Brain Res.*, **454**, 101–105.

HILL, R.G. & BODEN, P.R. (1989). Electrophysiological actions of CCK in the mammalian central nervous system. In *The Neuropeptide Cholecystokinin (CCK). Anatomy and Biochemistry, Receptors, Pharmacology and Physiology*. ed. Hughes, J., Dockray, G. & Woodruff, G.N. pp. 186–194. Ellis Horwood: Chichester.

LOTTI, V.J. & CHANG, R.S.L. (1989). A new potent and selective nonpeptide gastrin antagonist and brain cholecystokinin receptor (CCK-B) ligand: L-365,260. *Eur. J. Pharmacol.*, **162**, 273–280.

MARSHALL, F., PINNOCK, R.D., BARNES, S. & HUGHES, J. (1990). Characterization of cholecystokinin octapeptide-stimulated dopamine release from rat nucleus accumbens *in vitro*. *Br. J. Pharmacol.*, **99**, 845–848.

PAN, Z.Z., COLMERS, W.F. & WILLIAMS, J.T. (1989). 5-HT-mediated synaptic potentials in the dorsal raphe nucleus: interactions with excitatory amino acid and GABA neurotransmission. *J. Neurophysiol.*, **62**, 481–486.

VANDERHAEGEN, J.J., LOSTRA, F., DEMAY, J. & GILLES, C. (1980). Immunohistochemical localisation of cholecystokinin and gastrin-like peptides in the brain and hypophysis of the rat. *Proc. Natl. Acad. Sci., U.S.A.*, **77**, 1190–1194.

VAN DER KOY, D., HUNT, S., STEINBUSCH, H.W.M. & VERHOFSTAD, A.A.J. (1981). Separate populations of cholecystokinin and 5-HT containing cells in the rat dorsal raphe and their contribution to its ascending projections. *Neurosci. Lett.*, **26**, 25–31.

WILLIAMS, J.T., COLMERS, W.F. & PAN, Z.Z. (1988). Voltage- and ligand-activated inwardly rectifying currents in dorsal raphe neurones *in vitro*. *J. Neurosci.*, **8**, 3499–3506.

YOSHIMURA, M. & HIGASHI, H. (1985). 5-Hydroxytryptamine mediates inhibitory postsynaptic potentials in rat dorsal raphe neurons. *Neurosci. Lett.*, **53**, 69–74.

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The pressor response to central administration of β-endorphin results from a centrally mediated increase in noradrenaline release and adrenaline secretion

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1 The effects of intracerebroventricular (i.c.v.) and intracisternal (i.c.) administration of β-endorphin (0.01, 0.1 and 1.0 nmol kg^{-1}) were examined in conscious rabbits.

2 After i.c.v. β-endorphin, mean arterial pressure (MAP) increased, heart rate (HR) fell, plasma noradrenaline, adrenaline and glucose increased and there was a rise in $Paco_2$ and fall in Pao_2; these effects were reversed by intravenous (i.v.) naloxone (300 nmol kg^{-1}).

3 A combination of prazosin (2 mg kg^{-1}) and yohimbine (1 mg kg^{-1}), given i.v., prevented the rise in MAP induced by i.c.v. β-endorphin.

4 After i.c. β-endorphin, MAP, HR and plasma catecholamines were not significantly altered but there was a similar degree of respiratory depression.

5 Clonidine (1.0 μg kg^{-1}, i.c.) reduced MAP and HR; these effects were not blocked by i.v. naloxone (6 μmol kg^{-1}).

6 These results demonstrate that β-endorphin acts centrally, probably mainly on periventricular μ-opioid receptors, to increase adrenaline secretion and sympathetic nerve activity leading to α-adrenoceptor-mediated vasoconstriction. The respiratory depression is probably mediated by brainstem μ-receptors.

7 A role for β-endorphin in the central hypotensive action of α_2-adrenoceptor agonists was opposed by finding that opioid receptor antagonism with naloxone did not block the effects of clonidine.

Introduction

β-Endorphin is a 31 amino acid peptide corresponding to residues 61–91 of β-lipotrophin, which is derived from proopiomelanocortin. It has been located in sites involved in central cardiovascular control such as the hypothalamus and nucleus of the tractus solitarius (NTS) (Akil *et al.*, 1984), and administration of β-endorphin either intravenously (i.v.), intracisternally (i.c.) or by microinjection into the NTS has been shown to reduce blood pressure in the rat (Lemaire *et al.*, 1978; Bolme *et al.*, 1978; Petty *et al.*, 1982). However, these studies were conducted on anaesthetized animals in which the pressor effects of opiates are reversed or abolished (Holaday 1982; May *et al.*, 1988). Central administration of selective μ- and δ-opioid agonists increases blood pressure in conscious rabbits (May *et al.*, 1989a), and therefore β-endorphin may be expected to raise blood pressure in conscious animals as it binds with equal affinity to μ- and δ-receptors (Paterson *et al.*, 1983).

We have studied the cardiovascular and respiratory effects of β-endorphin injected intracerebroventricularly (i.c.v.) or i.c. in conscious rabbits. The opioid receptor antagonist naloxone was used to confirm that these effects depended on stimulation of opioid receptors. The role of the nervous system was evaluated by measuring plasma catecholamines and by examining the effect of α-adrenoceptor blockade. There is evidence that β-endorphin mediates the hypotensive effect of clonidine (Farsang *et al.*, 1980; Ramirez-Gonzalez *et al.*, 1983) but this is controversial and has not been supported by other reports (Conway *et al.*, 1984; Head & de Jong, 1985). We have, therefore, examined the effect of opioid receptor antagonism with naloxone on the hypotensive effect of i.c. clonidine in conscious rabbits.

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Methods

Surgical procedures

Experiments were carried out on normotensive, male Sandy Half-Lop rabbits (National Institute for Medical Research, Mill Hill, London) weighing between 2.6 and 3.4 kg, and allowed free access to food and water.

Injections into the right lateral ventricle were made via cannulae (Harvard Apparatus) implanted aseptically under general anaesthesia using alphaxalone 0.9%/alphadolone acetate 0.3% (Saffan, Glaxovet) (1 ml i.v. followed by 0.25 ml i.v. as required). Coordinates were 1.0 mm caudal to bregma, 3.0 lateral to midline and 8.5 mm below the dura (May *et al.*, 1989c). β-Endorphin was injected i.c.v. in a volume of 25 μl from a sterilised Hamilton syringe with a needle that protruded 1 mm from the cannula tip. The injection was flushed in with 25 μl of saline from a needle that reached half way down the cannula shaft. For injection into the cisterna magna the catheter was inserted under Saffan general anaesthesia as previously described (May *et al.*, 1989b). Injections were given in 25 μl and flushed in with 20 μl of saline; the dead volume of the catheter was 10 μl. The injection sites were confirmed at the end of a series of experiments by injection of bromophenol blue (1%) either i.c.v. or i.c. followed by the appropriate volume of saline.

Experimental protocol

Animals were not used for at least 7 days post operation. Before each experiment cannulae were inserted into the central artery and marginal vein of the ear under 1% lignocaine local anaesthesia. The arterial cannula was connected to a pressure transducer (Bell and Howell) and BP and HR were recorded on a Devices polygraph.

Before the experiment the patency of the i.c.v. cannula was checked by allowing saline to flow into it under gravity. The

experiment was continued only if saline flowed freely into the lateral ventricle and animals which showed any cardiovascular effects (usually bradycardia) were not used. This occurred rarely (<4%), when the animal had been used several times, and was associated with an infection around the cannula tip. No such problems were encountered with the i.c. cannulae. Different groups of rabbits were used for each experiment, except for one rabbit which was used in two groups and in this case the two experiments were 7 days apart.

β-Endorphin (human) (Cambridge Research Biochemicals) was dissolved in sterile, non-pyrogenic, isotonic saline and filtered through a sterile 0.2 μm filter before injection. In the dose-response studies, two groups of six rabbits with either an i.c.v. or an i.c. cannula were studied while conscious. After a 30 min control period increasing doses of β-endorphin (0.01, 0.1 and 1.0 nmol kg^{-1}) were given at hourly intervals. In previous studies we demonstrated that neither i.c.v. nor i.c. saline has any effect on MAP, HR, plasma catecholamines, respiration or behaviour in conscious rabbits (May *et al.*, 1989b).

The effect of naloxone hydrochloride (Sigma) on the responses to β-endorphin was examined in six conscious rabbits. β-Endorphin (1.0 nmol kg^{-1}) was given i.c.v., followed after 15 min by an i.v. dose of naloxone of 30 nmol kg^{-1} and after a further 20 min by a higher dose of naloxone (300 nmol kg^{-1}). Naloxone was dissolved in saline and given in a volume of 100 μl kg^{-1}. We have demonstrated that i.v. naloxone has no cardiovascular effects in conscious rabbits (May *et al.*, 1988).

Blockade of α_1- and α_2-adrenoceptors was produced with a combination of prazosin hydrochloride (Sigma) and yohimbine hydrochloride (Sigma). Prazosin hydrochloride (2 mg kg^{-1} in 400 μl kg^{-1} of 0.04 M lactic acid) and yohimbine hydrochloride (1 mg kg^{-1} in 200 μl kg^{-1} of distilled water) were given i.v. 10 min before i.c.v. administration of β-endorphin (1.0 nmol kg^{-1}). These doses of prazosin and yohimbine blocked the pressor effect of an i.v. dose (5 μg kg^{-1}) of noradrenaline bitartrate (Sigma). After these doses the pressor effect of noradrenaline remained blocked for at least 1 h. A combination of α_1- and α_2-adrenoceptor antagonists was used as prazosin, given alone in doses from 2 to 5 mg kg^{-1}, only reduced the pressor effect of noradrenaline by a maximum of 80%.

The ability of endogenous opioid peptides to mediate the cardiovascular effects of clonidine was studied in six rabbits each with an indwelling i.c. cannula. The effect of a near maximal dose of i.c. clonidine (1 μg kg^{-1}) was examined after pretreatment with either i.v. saline (333 μl kg^{-1}) or i.v. naloxone (6 μmol kg^{-1}) given 10 min before clonidine; the treatments were randomised.

Blood collection and analysis

Catecholamines were measured in plasma from blood (2.5 ml) collected from the arterial cannula into cooled tubes containing 50 μl EGTA (0.095%)/glutathione (0.06%) and kept on ice until centrifugation at 4°C and then storage at −70°C. Catecholamines were separated by high performance liquid chromatography (h.p.l.c.) and detected by electrochemical detection (May *et al.*, 1988). Plasma glucose was measured by a glucose oxidase method on plasma from arterial blood (2.0 ml) collected into fluoride oxalate tubes. Immediately following blood collection an equal volume of isotonic saline was given i.v. Arterial blood (100 μl) was collected into a heparinised capillary tube for the measurement of blood gases (Instrument Laboratory 230 blood gas analyser).

Statistical analysis

Data are presented as means ± s.e.mean. Analysis was performed on the differences from the control values by the SAS statistical programme. The control values for MAP and HR consist of the mean of the six readings taken during the control period. A logarithmic transformation was performed on the values for plasma noradrenaline, adrenaline and glucose before analysis. All the cardiovascular and biochemical data were subjected to analysis of variance and where the null hypothesis was rejected Bonferroni's comparisons were performed on the means; $P < 0.05$ was considered significant.

Results

Effects of i.c.v. and i.c. β-endorphin

β-Endorphin given i.c.v. caused a dose-related increase in MAP in conscious rabbits (Figure 1). The lowest dose (0.01 nmol kg^{-1}) had no effect but after the intermediate dose (0.1 nmol kg^{-1}) MAP reached 11 ± 5 mmHg above control. After the highest i.c.v. dose (1.0 nmol kg^{-1}), MAP was significantly elevated at all times ($P < 0.05$) and reached 26 ± 6 mmHg above control after 30 min. There was an initial fall in HR but by 60 min it had increased above control. After i.c. β-endorphin there were no changes after the two lowest doses. After the highest dose (1.0 nmol kg^{-1}) MAP reached a maximum of 12 ± 6 mmHg above control after 60 min but this was not significant.

Samples for plasma catecholamines were taken at the end of the control period, immediately before the highest dose (60 min after the middle dose), and at 5, 30 and 60 min after the highest dose. Following i.c.v. administration of the highest

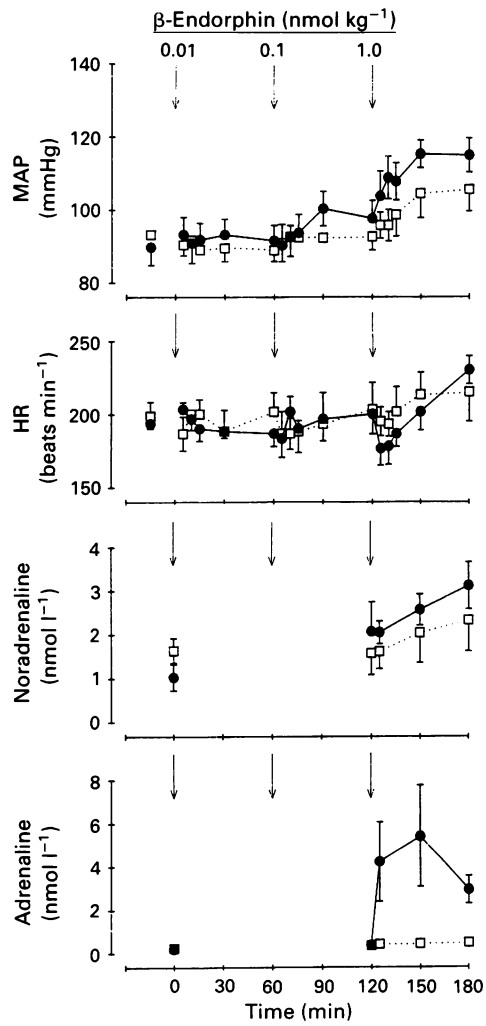

Figure 1 Effect of increasing doses of β-endorphin (0.01, 0.1 and 1.0 nmol kg^{-1}) given at 60 min intervals either i.c.v. (●) or i.c. (□) on mean arterial pressure (MAP), heart rate (HR), plasma noradrenaline and adrenaline in conscious rabbits. The control values for MAP and HR are the mean of six readings taken during the control period. Each point represents the mean ($n = 6$), with s.e.mean shown by vertical bars.

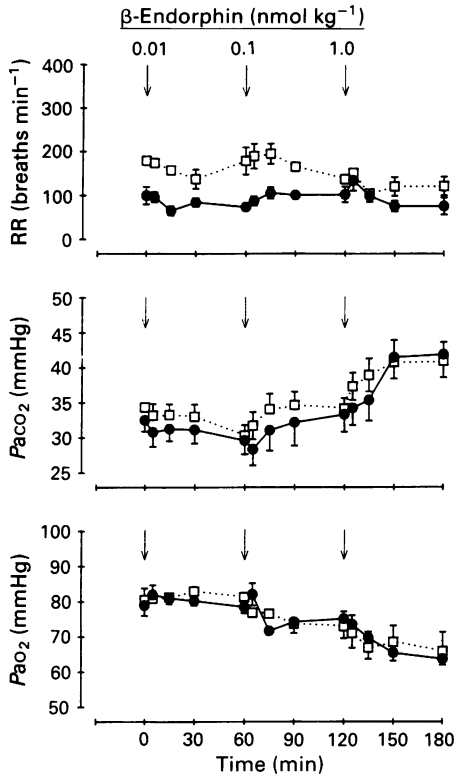

Figure 2 Effect of increasing doses of β-endorphin (0.01, 0.1 and 1.0 nmol kg^{-1}) given at 60 min intervals either i.c.v. (●) or i.c. (□) on respiration rate (RR), $Paco_2$ and Pao_2 in conscious rabbits. Each point represents the mean ($n = 6$), with s.e.mean shown by vertical bars.

dose of β-endorphin, plasma noradrenaline was not increased after 5 min but was significantly elevated after 30 and 60 min ($P < 0.05$) (Figure 1). This dose of β-endorphin caused an immediate, significant increase in plasma adrenaline after 5 min and it remained elevated after 30 and 60 min ($P < 0.05$) (Figure 1). After i.c. β-endorphin (1.0 nmol kg^{-1}) there was a smaller rise in noradrenaline than after i.c.v. administration and no change in adrenaline (Figure 1). Plasma glucose significantly increased from 7.25 ± 0.17 mmol l^{-1} to 11.78 ± 0.94 and 10.56 ± 0.73 mmol l^{-1} at 30 and 60 min respectively after i.c.v. β-endorphin (1.0 nmol kg^{-1}). After i.c. administration the increases were smaller but remained significant; plasma glucose increased from 7.09 ± 0.15 mmol l^{-1} to 8.72 ± 0.43 and 8.91 ± 0.33 mmol l^{-1} at 30 and 60 min respectively.

Blood gases were not significantly changed after the two lowest doses of β-endorphin given either i.c.v. or i.c. After the highest i.c.v. dose there was a gradual increase in $Paco_2$ and a fall in Pao_2, and these changes were significant after 30 and 60 min ($P < 0.05$) (Figure 2). Following i.c. administration there were similar, changes in $Paco_2$ and Pao_2 (Figure 2). There were no changes in respiration rate after either i.c.v. or i.c. β-endorphin.

β-Endorphin also caused sedation after i.c.v. administration. There was no effect after the lowest dose but after 0.1 nmol kg^{-1} the rabbits were less active and after 1.0 nmol kg^{-1} they became heavily sedated. No behavioural changes were observed after i.c. administration.

Effect of i.v. naloxone on the responses to i.c.v. β-endorphin

In six rabbits i.c.v. β-endorphin (1.0 nmol kg^{-1}) increased MAP ($P < 0.05$) and reduced HR after 15 min (Figure 3). After 10 min plasma noradrenaline and adrenaline had significantly increased ($P < 0.05$), $Paco_2$ had increased by 6.5 ± 1.8 mmHg ($P < 0.05$) and Pao_2 had fallen by 12.8 ± 2.6 mmHg ($P < 0.05$). Naloxone (30 nmol kg^{-1}) given i.v. 15 min after β-

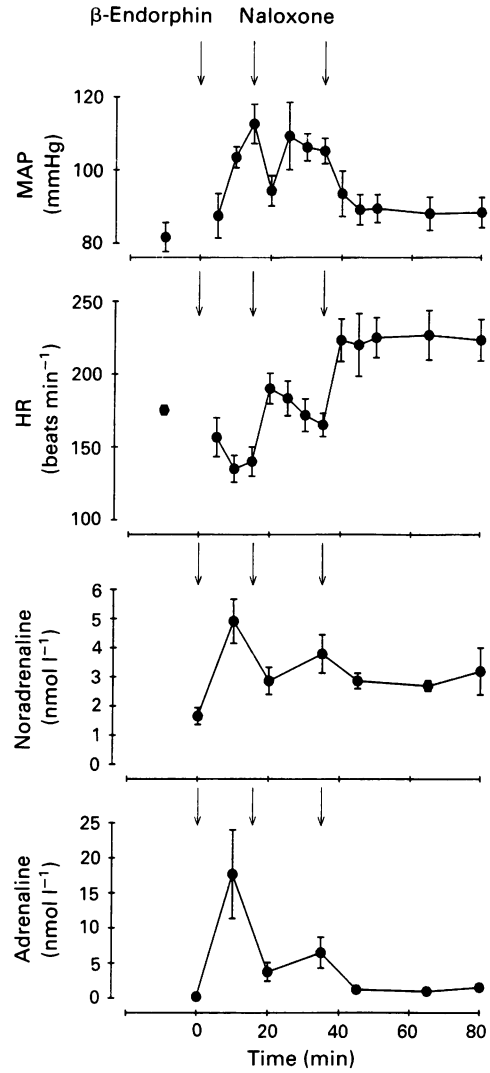

Figure 3 Effect of i.v. naloxone on the changes in mean arterial pressure (MAP), heart rate (HR) and plasma catecholamines in response to i.c.v. β-endorphin (1.0 nmol kg^{-1}) in conscious rabbits. Naloxone (30 and 300 nmol kg^{-1}) was given 15 and 35 min respectively after β-endorphin. The control values for MAP and HR are the mean of six readings taken during the control period. Each point represents the mean ($n = 6$), with s.e.mean shown by vertical bars.

endorphin caused a transient reduction in MAP, returned HR to control and reduced plasma catecholamine levels (Figure 3). However, $Paco_2$ continued to increase to reach 12.2 ± 1.1 mmHg above control and Pao_2 to fall to reach 18.9 ± 3.0 mmHg below control ($P < 0.05$). Subsequent administration of a higher dose of naloxone (300 nmol kg^{-1}), given 35 min after β-endorphin (20 min after the lower dose of naloxone), caused MAP to return to and remain at control levels, and HR to increase to 48 ± 14 beats min^{-1} above control. After this dose of naloxone plasma catecholamines returned towards control (Figure 3). The higher dose of naloxone abolished the β-endorphin-induced respiratory depression and reversed its sedative effect.

Effect of α-adrenoceptor blockade on the responses to i.c.v. β-endorphin

A combination of prazosin (2 mg kg^{-1}) and yohimbine (1 mg kg^{-1}), given i.v., significantly reduced MAP and increased HR within 5 min in six rabbits (Figure 4). After 10 min there were significant increases in plasma noradrenaline and plasma adrenaline ($P < 0.05$). Subsequent administration of β-endorphin (1.0 nmol kg^{-1}) caused no increase in MAP or fall in HR, although there were further increases

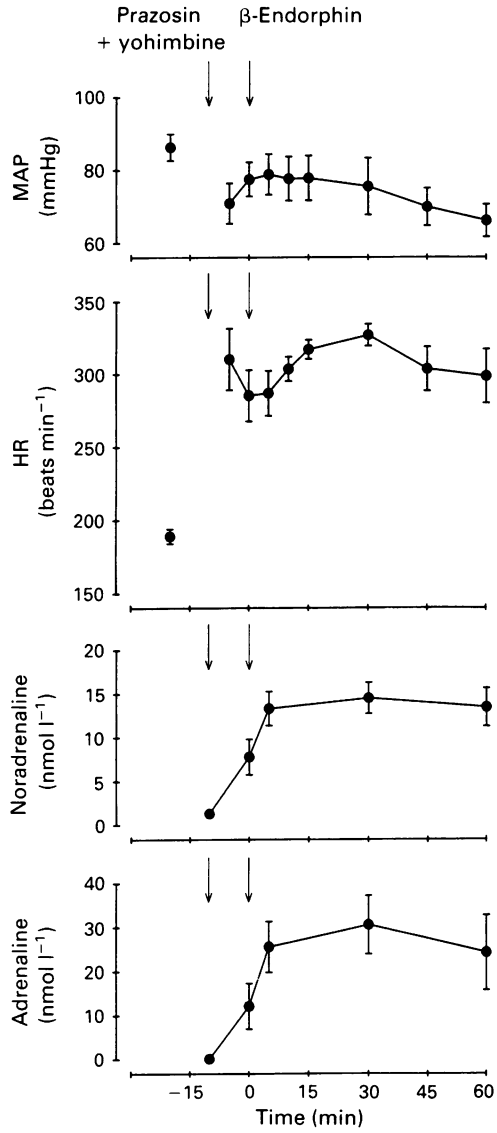

Figure 4 Effect of i.v. prazosin ($2\,mg\,kg^{-1}$) and yohimbine ($1\,mg\,kg^{-1}$) on the changes in mean arterial pressure (MAP), heart rate (HR) and plasma catecholamines in response to i.c.v. β-endorphin ($1.0\,nmol\,kg^{-1}$) in conscious rabbits. Prazosin and yohimbine were given together 10 min before β-endorphin. The control values for MAP and HR are the mean of six readings taken during the control period. Each point represents the mean ($n = 6$), with s.e.mean shown by vertical bars.

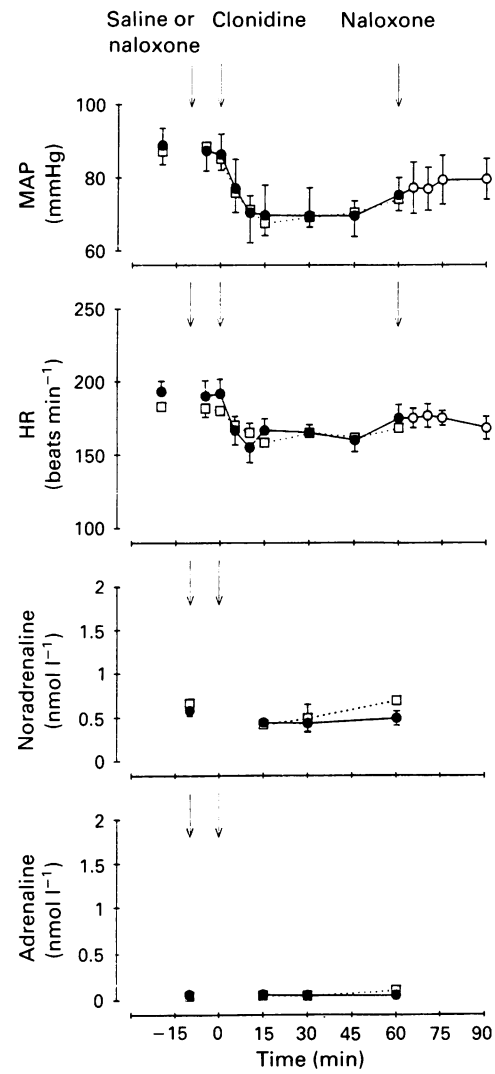

Figure 5 Effect of i.c. clonidine ($1.0\,\mu g\,kg^{-1}$) on mean arterial pressure (MAP), heart rate (HR) and plasma catecholamines after pretreatment with i.v. saline ($333\,\mu l\,kg^{-1}$) (●) or i.v. naloxone ($6\,\mu mol\,kg^{-1}$) (□). In the saline treated group i.v. naloxone ($6\,\mu mol\,kg^{-1}$) was given 60 min after clonidine (○). The control values for MAP and HR are the mean of six readings taken during the control period. Each point represents the mean ($n = 6$), with s.e.mean shown by vertical bars.

above control in noradrenaline and plasma adrenaline within 5 min. Catecholamines remained elevated at this level for the remainder of the experiment (Figure 4).

α-Adrenoceptor blockade did not alter the β-endorphin-induced respiratory depression. At 60 min after β-endorphin respiration rate was unchanged, $Paco_2$ was increased by 19.1 ± 3.0 mmHg ($P < 0.05$) and Pao_2 was reduced by 16.0 ± 2.7 mmHg ($P < 0.05$). Administration of i.v. naloxone ($300\,nmol\,kg^{-1}$) returned blood gases to control levels within 5 min.

Effect of opioid receptor antagonism on the response to i.c. clonidine

Clonidine ($1.0\,\mu g\,kg^{-1}$) given i.c., 10 min after i.v. saline ($333\,\mu l\,kg^{-1}$), reduced MAP by 19 ± 5 mmHg ($P < 0.05$) after 15 min and HR by 38 ± 10 beats min^{-1} ($P < 0.05$) after 10 min (Figure 5). There was a tendency for plasma noradrenaline to fall after clonidine but this did not reach significance and there was no effect on plasma adrenaline. At 60 min after clonidine, administration of i.v. naloxone ($6\,\mu mol\,kg^{-1}$) caused no change in MAP or HR. When i.c. clonidine was given 10 min after i.v. naloxone ($6\,\mu mol\,kg^{-1}$), the clonidine-induced changes in MAP, HR and plasma noradrenaline were not significantly different from those when it was given after saline (Figure 5).

Discussion

This study demonstrates that in conscious rabbits, β-endorphin acts centrally to cause a dose-related increase in MAP, respiratory depression and sedation. The cardiovascular effects do not result from leakage of β-endorphin to the periphery as i.v. β-endorphin ($29\,nmol\,kg^{-1}$) does not increase MAP in conscious rabbits of the same strain used in the present experiments (personal communication, Dr S.J. Potocnik). Following i.c.v. β-endorphin the rise in pressure was accompanied by a large increase in plasma adrenaline and a smaller rise in plasma noradrenaline. In the rabbit, noradrenaline is not found in the adrenal medulla (Hokfelt, 1952) and following splanchnic nerve stimulation only adrenaline is secreted (West, 1950), indicating that β-endorphin increases sympathetic outflow to both noradrenergic nerves and to the adrenal medulla. All the effects of β-endorphin were reversed

by naloxone demonstrating that they depended on stimulation of opioid receptors. This is not the result of an opposing hypotensive effect of naloxone as, in agreement with other authors (Petty & Reid, 1982; Rutter *et al.*, 1987), we have demonstrated that i.v. naloxone given alone has no effect on MAP or HR in conscious rabbits (May *et al.*, 1988). The lower dose of naloxone briefly returned MAP and HR to control levels, and then as MAP increased there was a corresponding fall in HR. After the higher dose of naloxone, when the rise in MAP was permanently reversed, HR increased above control. Similar increases in HR occurred when naloxone was given after the μ-receptor agonists DAMGO (Pfeiffer *et al.*, 1983; May *et al.*, 1989a) and morphine (May *et al.*, 1989b), and may partly result from antagonism by naloxone of opioid-induced vagal stimulation as the increase in HR is reduced by pretreatment with atropine methyl nitrate (Pfeiffer *et al.*, 1983).

The β-endorphin-induced rise in MAP was inhibited by a combination of the selective α_1- and α_2-adrenoceptor antagonists, prazosin and yohimbine respectively, demonstrating that the pressor effect was due to the increase in sympatho-adrenal outflow leading to α-adrenoceptor-mediated vasoconstriction. The requirement for both α-adrenoceptor antagonists to block the pressor effects of noradrenaline indicates that in the rabbit, noradrenaline-induced vasoconstriction is mediated by postsynaptic α_2- as well as α_1-adrenoceptors.

After i.c. β-endorphin the changes in MAP and noradrenaline were considerably less and there was no rise in adrenaline but the degree of respiratory depression was similar to that after i.c.v. administration. The responses to β-endorphin did not result from non-specific effects due to injection of the vehicle as in conscious rabbits, i.c.v. and i.c. saline have no effect on the measured variables (May *et al.*, 1989b).

In previous studies in conscious rabbits, using an identical protocol, we demonstrated that i.c.v. and i.c. injection of the selective μ-opioid agonist (D-Ala^2-, MePhe^4-,Gly^5-ol)enkephalin (DAMGO) caused a similar pattern of responses to β-endorphin (May *et al.*, 1989a). In the same study the selective δ-opioid agonist (D-Pen^{2,5})enkephalin (DPDPE) increased MAP, but in contrast to DAMGO, administration of DPDPE increased HR, did not increase adrenaline secretion or cause respiratory depression and induced behavioural activation rather than sedation. Thus, although β-endorphin binds equally to μ- and δ-opioid receptors (Paterson *et al.*, 1983), its effects following i.c.v. and i.c. injection are qualitatively the same as those of a selective μ-agonist suggesting that they result predominantly from an action on μ-receptors.

The opioid receptors leading to the increase in sympathetic outflow are probably located mainly in periventricular structures as the responses are greater after i.c.v. than i.c. administration. We have demonstrated the presence of μ-opioid receptors in hypothalamic nuclei in rabbit brain by *in vitro* autoradiography (May *et al.*, 1989a), and this may be a site at which β-endorphin acts to increase sympatho-adrenal outflow. This is supported by the finding that in conscious rats microinjection of the μ-agonist DAMGO into the anterior hypothalamus produced similar increases in sympatho-adrenal outflow and blood pressure to those seen after i.c.v. administration (Pfeiffer *et al.*, 1983). The tendency for MAP to rise after i.c. administration indicated that additional actions at brainstem sites, such as the dorsal motor nucleus of the vagus which contains μ-receptors (May *et al.*, 1989a), cannot be discounted. More discrete administration of β-endorphin will be required to localise accurately the site, or sites, at which β-endorphin acts to increase sympatho-adrenal outflow.

Opioid-induced respiratory depression results from an action on μ- but not δ- or κ-receptors (May *et al.*, 1989a) located in the ventral medulla (Florez *et al.*, 1968). This area will be reached by drugs given i.c.v. and i.c. and may be the site at which β-endorphin acts to produce respiratory depression as similar responses occurred after both routes of administration. The hyperglycaemia following i.c.v. β-endorphin is probably mainly due to increased adrenaline secretion, as has been shown following central administration of β-endorphin in conscious rats (Van Loon & Appel, 1981) and following i.v. administration of the μ-opioid agonist morphine in conscious rabbits (May *et al.*, 1988). The rise in plasma glucose after i.c. β-endorphin, when there was no increase in plasma adrenaline, could result from a mechanism mediated by central opioid receptors but independent of catecholamine release as has previously been demonstrated in conscious rabbits (Knudtzon, 1986).

The pressor response to β-endorphin demonstrated in this study is at variance with other reports in which i.c. β-endorphin produced a temporary rise followed by a fall in blood pressure in anaesthetized rats (Bolme *et al.*, 1978), a fall in blood pressure and HR in anaesthetized cats (Feldberg & Wei, 1978), and microinjection into the NTS of anaesthetized rats reduced blood pressure and HR (Petty *et al.*, 1982). The absence of any increase in pressure in these studies may result from the use of anaesthetics, which have been shown to block or reverse the pressor effects resulting from stimulation of opioid receptors (Kayaalp & Kaymakcalon, 1966; Holaday, 1982; May *et al.*, 1988).

β-Endorphin has been proposed as a mediator of the centrally-induced hypotensive action of α_2-adrenoceptor agonists (Farsang *et al.*, 1980; Ramirez-Gonzalez, 1983; Marmo *et al.*, 1984; Tackett & Laskey, 1987). However, this has not been confirmed by other reports (Conway *et al.*, 1984; Head & de Jong, 1985) and our finding that β-endorphin increases blood pressure does not support this hypothesis. In view of these differences we examined the effect of opioid receptor antagonism on the response to clonidine in conscious rabbits. Clonidine was given i.c. to avoid any peripheral effects; the dose of naloxone was the same as that used in the majority of studies and the animals were conscious to exclude the effects of anaesthetics. In our studies naloxone did not inhibit the clonidine-induced fall in blood pressure, heart rate and plasma catecholamines, or reverse the hypotension when given after clonidine. These results indicate that in conscious, normotensive rabbits endogenous opioid peptides are not involved in the central hypotensive effect of clonidine. This does not exclude a role for opioid peptides in the hypotensive action of clonidine in hypertensive animals, as has been suggested in rats (Farsang *et al.*, 1980). However, this finding has not been confirmed by other authors in conscious or anaesthetized, spontaneously hypertensive and normotensive rats (Conway *et al.*, 1984; Head & de Jong, 1985). It has been suggested that the inter-relationship described in some reports may result from the use of anaesthetics or the effect of stress induced by indirect methods of measuring blood pressure (Head & de Jong, 1985), and our present findings in conscious, unstressed animals support this hypothesis.

In conclusion, we have demonstrated that in conscious rabbits, β-endorphin acts on central opioid receptors, possibly in the hypothalamus, to increase sympatho-adrenal outflow and elevate blood pressure. Endogenous opioid peptides such as β-endorphin may mediate the cardiovascular responses to stress as opioid receptor antagonists prevent stress-induced increases in blood pressure (Naranjo *et al.*, 1986). The inability of naloxone to prevent the clonidine-induced fail in MAP and HR indicates that endogenous opioid peptides do not mediate the central hypotensive effects of α_2-adrenoceptor agonists.

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References

AKIL, H., WATSON, S.J., YOUNG, E., LEWIS, M.E., KHACHATURIAN, H. & WALKER, J.M. (1984). Endogenous opioids: biology and function. *Annu. Rev. Neurosci.*, **7**, 223–255.

BOLME, P., FUXE, K., AGNATI, L.F., BRADLEY, R. & SMYTHIES, J. (1978). Cardiovascular effects of morphine and opioid peptides following intracisternal administration in chloralose-anaesthetised rats. *Eur. J. Pharmacol.*, **48**, 319–324.

CONWAY, E.L., BROWN, M.J. & DOLLERY, C.T. (1984). No evidence for involvement of endogenous opioid peptides in effects of clonidine on blood pressure, heart rate and plasma norepinephrine in anaestehtised rats. *J. Pharmacol. Exp. Ther.*, **229**, 803–808.

FARSANG, C., RAMIREZ-GONZALEZ, M.D., MUCCI, L. & KUNOS, G. (1980). Possible role of an endogenous opiate in the cardiovascular effects of central alpha adrenoceptor stimulation in spontaneously hypertensive rats. *J. Pharmacol. Exp. Ther.*, **214**, 203–208

FELDBERG, W. & WEI, E. (1978). Central cardiovascular effects of enkephalins and C-fragment of liptrophin. *J. Physiol.*, **280**, 18P.

FLOREZ, J., McCARTHY, L.E. & BORISON, H.L. (1968). A comparative study in the cat of the respiratory effects of morphine injected intravenously and into cerebrospinal fluid. *J. Pharmacol. Exp. Ther.*, **163**, 448–455.

HEAD, G.A. & DE JONG, W. (1985). Cardiovascular responses to central clonidine, alpha-methyldopa and 6 hydroxydopamine in conscious normotensive and spontaneously hypertensive rats following naloxone. *J. Cardiovasc. Pharmacol.*, **7**, 321–326.

HOKFELT, B. (1952). Noradrenaline and adrenaline in mammalian tissues. *Acta Physiol. Scand.*, **25**, (Suppl. 92), 39–58.

HOLADAY, J.W. (1982). Cardiorespiratory effects of mu and delta opiate agonists following third or fourth ventricular injections. *Peptides*, **3**, 1023–1029.

KAYAALP, S.O. & KAYMAKCALON, S. (1966). A comparative study of the effects of morphine in unanaesthetised and anaesthetised cats. *Br. J. Pharmacol.*, **26**, 196–204.

KNUDTZON, J. (1986). Hypoinsulinaemic and hyperglycaemic effects of β-endorphin in rabbits. *Horm. Metabol. Res.*, **18**, 505–509.

LEMAIRE, I., TSENG, R. & LEMAIRE, S. (1978). Systemic administration of β-endorphin: potent hypotensive effect involving serotonergic pathway. *Proc. Natl. Acad. Sci., U.S.A.*, **75**, 6240–6242.

MARMO, E., ROSSI, F., LAMPA, E., BERRINO, L., RUSSO, S., MATERA, C. & GENTILE, B. (1984). Participation of opiate peptidergic central mechanism in the hypotension and bradycardia of alpha$_2$-adrenergic stimulating drugs: an experimental study in dogs with clonidine, flutonidine, guanaberz and guanfacine. *Curr. Ther. Res.*, **36**, 1145–1151.

MAY, C.N., DASHWOOD, M.R., WHITEHEAD, C.J. & MATHIAS, C.J. (1989a). Differential cardiovascular and respiratory responses to central administration of selective opioid agonists in conscious rabbits; correlation with receptor distribution. *Br. J. Pharmacol.*, **98**, 903–913.

MAY, C.N., HAM, I.W., HESLOP, K.E., STONE, F.A. & MATHIAS, C.J. (1988). Intravenous morphine causes hypertension, hyperglycaemia and increases sympatho-adrenal outflow in conscious rabbits. *Clin. Sci.*, **75**, 71–77.

MAY, C.N., WHITEHEAD, C.J., DASHWOOD, M.R. & MATHIAS, C.J. (1989b). Investigation of the central sites at which morphine acts to cause hypertension in conscious rabbits. *Br. J. Pharmacol.*, **97**, 873–881.

MAY, C.N., WHITEHEAD, C.J., HESLOP, K.E. & MATHIAS, C.J. (1989c). Evidence that intravenous morphine stimulates central opiate receptors to increase sympatho-adrenal outflow and cause hypertension in conscious rabbits. *Clin. Sci.*, **79**, 431–437.

NARANJO, J.R., URDIN, M.C., BORRELL, J. & FUENTES, J.A. (1986). Evidence for a central but not adrenal, opioid mediation in hypertension induced by brief isolation in the rat. *Life Sci.*, **38**, 1923–1930.

PATERSON, S.J., ROBSON, L.E. & KOSTERLITZ, H.W. (1983). Classification of opioid receptors. *Br. Med. Bull.*, **39**, 31–36.

PETTY, M.A., DE JONG, W. & DE WEID, D. (1982). An inhibitory role of β-endorphin in central cardiovascular regulation. *Life Sci.*, **30**, 1835–1840.

PETTY, M.A. & REID, J.L. (1982). The effect of opiates on arterial baroreflex function in the rabbit. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **319**, 206–211.

PFEIFFER, A., FEUERSTEIN, G., ZERBE, R.L., FADEN, A. & KOPIN, I.J. (1983). μ-Receptors mediate opioid cardiovascular effects at anterior medullary sites through sympatho-adrenomedullary and parasympathetic pathways. *Endocrinology*, **113**, 929–938.

RAMIREZ-GONZALEZ, M.D., TCHAKAROR, L., GARCIA, R.M. & KUNOS, G. (1983). β-endorphin acting on the brainstem is involved in the antihypertensive action of clonidine and alpha-methyldopa in rats. *Circ. Res.*, **53**, 150–157.

RUTTER, P.C., POTOCNIK, S.J. & LUDBROOK, J. (1987). Sympatho-adrenal mechanisms in cardiovascular responses to naloxone after haemorrhage. *Am. J. Physiol.*, **252**, H40–H46.

TACKETT, R.L. & LASKEY, R. (1987). Naloxone inhibits the centrally-mediated hypotensive actions of BHT-933. *Life Sci.*, **41**, 2063–2067.

VAN LOON, G.R. & APPEL, N.M. (1981). β-endorphin-induced hyperglycaemia is mediated by increased central sympathetic outflow to adrenal medulla. *Brain Res.*, **204**, 236–241.

WEST, G.B. (1950). Liberation of adrenaline from the suprarenal gland of the rabbit. *Br. J. Pharmacol. Chemother.*, **5**, 542–548.

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The effects of adenosine triphosphate (ATP) and related purines on human isolated subcutaneous and omental resistance arteries

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1 Human resistance arteries were obtained from specimens of omentum and subcutaneous fat removed at surgery. They were studied *in vitro* by use of a myograph technique to determine the effects of purines on the arteries.

2 In preparations where tone had been raised with noradrenaline, low concentrations (1 nM–1 μM) of adenosine triphosphate (ATP) and 2-methylthioATP, but not α,β-methyleneATP, produced concentration-dependent relaxation. There was a lack of relationship between the relaxation response to acetylcholine and that to ATP.

3 In preparations under basal tone, high concentrations (1 μM–1 mM) of ATP, 2-methylthioATP and α,β-methyleneATP produced concentration-dependent contractions.

4 The rank order of potency of the purine nucleotide analogues for the relaxation response was 2-methylthioATP > ATP > α,β-methyleneATP and for the contractile response it was α,β-methyleneATP > ATP = 2-methylthioATP.

5 Adenosine produced concentration-dependent relaxation in preparations under raised tone and was less potent than ATP but did not produce contraction in preparations at basal tone. Relaxation responses to adenosine, but not to ATP, were antagonized by 8-phenyltheophylline.

6 These results indicate the presence of vasodilator P_{2y}- and P_1-purinoceptors and vasoconstrictor P_{2x}-purinoceptors on human resistance arteries isolated from omental and subcutaneous sites.

Introduction

Purines have been shown to exert a variety of receptor-mediated effects in a number of tissues (Williams, 1987). The receptors through which these effects are manifest have been classified into P_1- and P_2-purinoceptors. These are distinguished on the basis of several criteria, including a different rank order agonist potency. At the P_1-purinoceptor, adenosine is more potent than adenosine 5'-triphosphate (ATP), whereas at the P_2-purinoceptor the reverse is true. Furthermore, the P_1-purinoceptor is susceptible to blockade by methylxanthines such as 8-phenyltheophylline whilst the P_2-purinoceptor is resistant to these antagonists (Burnstock, 1978). More recently it has become apparent that the P_2-purinoceptor population is not homogeneous. This has led to the the proposed subdivision of the P_2-purinoceptor into P_{2x} and P_{2y} subtypes which can be distinguished by the rank order of potency of ATP and its structural analogues. A rank order potency of α,β-methyleneATP (α,β-MeATP) > ATP = 2-methylthioATP (2-Me.S.ATP) is characteristic of the P_{2x} purinoceptor subtype, whilst a rank order potency of 2-Me.S.ATP > ATP > α,β-MeATP is characteristic of the P_{2y} subtype (Burnstock & Kennedy, 1985).

In the vasculature, effects mediated through the P_1-purinoceptor and both P_2-purinoceptor subtypes have been demonstrated in a variety of preparations from several species (Alexander & Eyre, 1985; Burnstock & Kennedy, 1986; Houston *et al.*, 1987). However, in man, although the cardiovascular effects of adenosine are relatively well known (Sollevi, 1986; McCormack *et al.*, 1989), little is known about the effects of ATP or its analogues. Furthermore, even in animal tissues, most of the *in vitro* work on purinoceptors has been done on relatively large arteries that are unlikely to contribute significantly to peripheral vascular resistance (Mulvany, 1987). We have therefore determined the actions of the endogenous purines, adenosine and ATP, in human isolated resistance arteries and have begun to characterize the receptor subtypes involved using ATP analogues. A preliminary account of this work has previously been presented to the British Pharmacological Society (Martin *et al.*, 1988).

Methods

Arteries were obtained from omentum and subcutaneous fat, removed at surgery from 113 patients (aged 17–89 years, median 56 years) and collected into modified Krebs buffer of composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, glucose 11.1, NaH_2PO_4 1.0, $NaHCO_3$ 25, $CaCl_2$ 2.5 and Na_2EDTA 0.03. Artery segments (internal diameter 98–708 μm, median 234 μm) were dissected free of surrounding tissue and mounted on two 40 μm wires in a myograph for the measurement of isometric tension (Mulvany & Halpern, 1977). The myograph chamber contained 10 ml of Krebs buffer maintained at 37°C and aerated with 95% O_2:5% CO_2. The arteries were allowed to equilibrate for at least 1 h and then set to a normalised resting internal circumference equal to 0.9 × L100, where L100 is the internal circumference producing a wall tension equivalent to that produced by a distending wall pressure of 100 mmHg, calculated from the Laplace relationship as described by Mulvany & Halpern (1977). Under these conditions, the arteries generated contractile responses to potassium-induced depolarization that were near to maximal (unpublished data).

Following a further 30 min equilibration, the arteries were exposed to a potassium depolarizing solution (KDS) composed of Krebs buffer in which the NaCl and KCl concentrations were reversed. Arteries that did not generate a tension equivalent to 90 mmHg (calculated by Laplace's relationship) were discarded. After washout and recovery, the arteries were exposed to noradrenaline (10 μM), repeat KDS and noradrenaline (1 μM) with washout and recovery between each. When stable tone had been induced by noradrenaline (1 μM) the functional integrity of the endothelium was assessed by the addition of acetylcholine (1 μM) (Thom *et al.*, 1987). The resultant relaxation was taken as indicative of the release of endothelium derived relaxing factor (EDRF) and the presence of a functional endothelium. In initial experiments, the arteries were preconstricted with noradrenaline (1 μM) and when stable

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tone had been induced, ATP (1 nM–1 mM) or adenosine (1 nM–1 mM) were added cumulatively. In subsequent experiments, the effects of shorter purine concentration-ranges were investigated with and without preconstriction. Concentration-response curves to ATP (1 nM–1 μM), 2-Me.S.ATP (100 pM–1 μM), α,β-MeATP (100 pM–10 nM), and adenosine (10 nM–10 μM) were generated following preconstriction with noradrenaline (1 μM) by cumulative addition of the purine. Concentration-response curves to ATP (1 μM–1 mM), 2-Me.S.ATP (10 nM–1 mM), α,β-MeATP (10 nM–1 mM) and adenosine (1 μM–1 mM) were generated without preconstriction by both cumulative addition of the purine and by the preparation of composite concentration-response curves. For the preparation of composite concentration-response curves, each artery segment was exposed to a single concentration of purine and the resultant responses of several segments exposed to different purine concentrations were grouped together. In this way, each artery segment was exposed to an exogenously applied purine once only and the problem of tachyphylaxis was avoided.

In some experiments, propranolol (4 μM), indomethacin (10 μM), phentolamine (10 μM) or 8-phenyltheophylline (10 μM) was added to the myograph chamber, 30 min before the first purine dose. Concentration-response data from individual artery segments were fitted to a logistic function by a computer programme (Barlow, 1983) and values for EC_{50}, namely the concentration of purine producing 50% of the maximum response to that purine, and maximum responses were derived. Results obtained following preconstriction are expressed as percentage reduction in preconstricted tone and those obtained without preconstriction as a percentage of the KDS response. EC_{50} and maximum responses are expressed as mean ± s.e.mean.

Drugs

Acetylcholine chloride (Sigma), adenosine (Sigma), adenosine 5'-triphosphate disodium salt (Sigma), indomethacin (Sigma), α,β-methyleneadenosine 5'-triphosphate lithium salt (Sigma), 2-methylthioadenosine 5'-triphosphate sodium salt (Research Biochemicals Inc.), noradrenaline bitartrate (Sigma), phentolamine mesylate (Ciba Geigy), 8-phenyltheophylline (Sigma) and (±)-propranolol hydrochloride (Sigma) were used. Indomethacin and 8-phenyltheophylline stock solutions were made up in 80% v/v methanol containing 0.2 mM NaOH and dilutions made in distilled water. All other drugs were dissolved and diluted in Krebs buffer.

Results

Following preconstriction with noradrenaline, the addition of ATP typically produced concentration-dependent relaxation at low concentrations. When the concentration of ATP reached about 1 μM, the effect of ATP reversed and transient concentration-dependent contraction occurred. In contrast, adenosine produced concentration-dependent relaxation which was not reversed at high concentrations (Figure 1).

After preconstriction, both subcutaneous and omental arteries relaxed similarly in response to low concentrations of ATP (1 nM–1 μM) with a maximum response of 83% of the preconstricted tone and EC_{50} of 52 nM (Figure 2, inset). This response did not show tachyphylaxis. 2-Me.S.ATP (100 pM–1 μM) also produced relaxations in preconstricted arteries and was more potent than ATP, having an EC_{50} of 12 nM (Figure 2). However, α,β-MeATP (100 pM–10 nM) failed to relax these arteries reproducibly. Arteries that relaxed well to ATP (100 nM) were not necessarily those that relaxed well to acetylcholine (1 μM) (Figure 3). This lack of relationship was evident in both omental and subcutaneous arteries. When added after noradrenaline, adenosine (10 nM–10 μM) also produced a concentration-dependent relaxation with a maximum response of 87% of the preconstricted tone and EC_{50} of 6.2 μM. Adenosine relaxation showed a similar lack of relationship to acetylcholine relaxation as did ATP. Neither the ATP nor the adenosine relaxant response were significantly altered by indomethacin (10 μM) or propranolol (4 μM) whilst 8-phenyltheophylline (10 μM) competitively antagonized the adenosine response, but not the ATP response (Figure 4).

Both omental and subcutaneous arteries contracted similarly in response to high concentrations of ATP (1 μM–1 mM) (Figure 5, inset). This contraction required the presence of extracellular calcium, was poorly sustained and showed marked tachyphylaxis. Exposure of the arteries to ATP (1 mM) greatly reduced subsequent contractile responses to ATP and prior exposure to α,β-MeATP (3 μM) abolished contractile responses to subsequent exposure to ATP. Taking the transient maximum tension developed in response to ATP as a

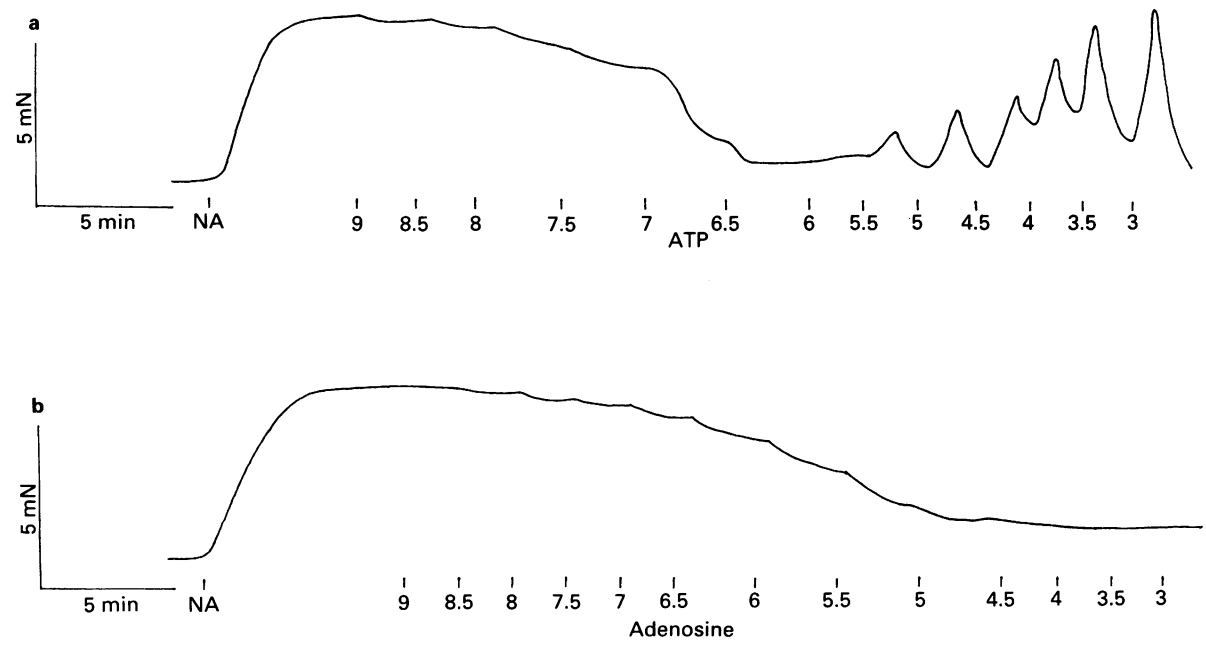

Figure 1 Representative traces showing the effects of ATP and adenosine on human isolated resistance arteries. (a) Omental artery with an internal diameter of 229 μm. (b) Omental artery with an internal diameter of 255 μm. Noradrenaline (NA; 1 μM), ATP and adenosine were added at the points shown. Concentrations are shown as −log molar concentrations.

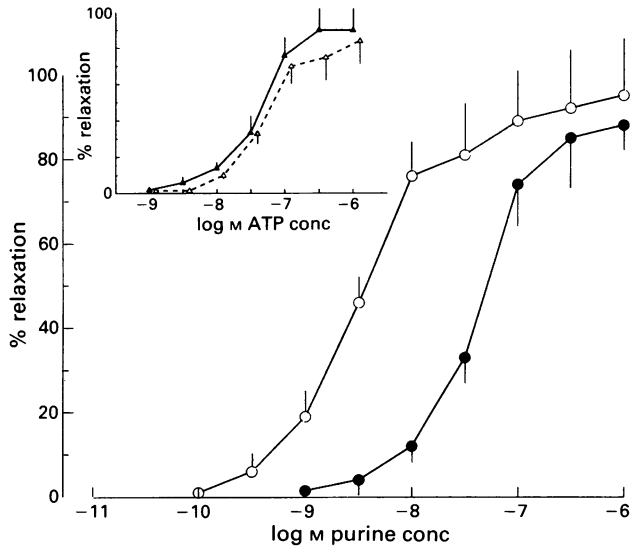

Figure 2 Concentration-response curves (CRC) for ATP (●) ($n = 20$) and 2-methylthioATP (○) ($n = 8$) in pooled human resistance arteries. Inset: CRC for ATP in omental arteries (▲) ($n = 17$) and subcutaneous arteries (△, broken line) ($n = 12$). (Points represent the mean % relaxation of NA-induced tone with s.e.mean shown by vertical lines).

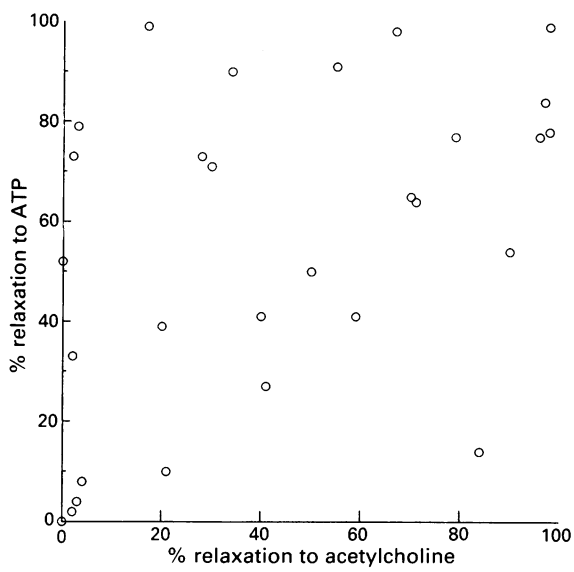

Figure 3 Percentage relaxation of pooled human resistance arteries to ATP (100 nM) against % relaxation of the same arteries to acetylcholine (1 μM) ($n = 29$). Points represent the % relaxation of NA-induced tone. Determinations for acetylcholine were followed immediately by washout, re-constriction and determination of ATP responses.

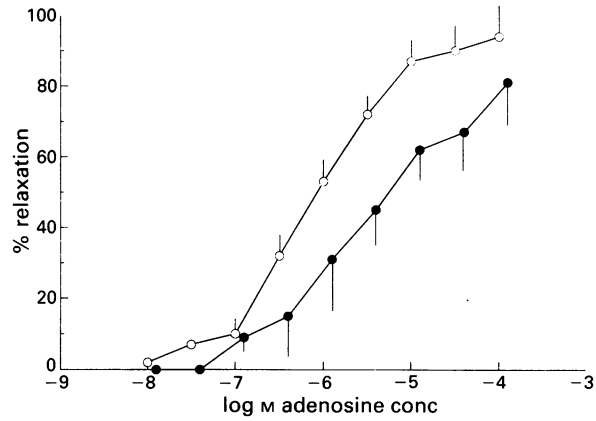

Figure 4 Concentration-response curves for adenosine in the absence (○) ($n = 10$) and presence of 10 μM 8-phenyltheophylline (●) ($n = 6$) in pooled human resistance arteries. Points represent the mean % relaxation of NA-induced tone with s.e.mean shown by vertical lines. ANOVA, $P < 0.05$.

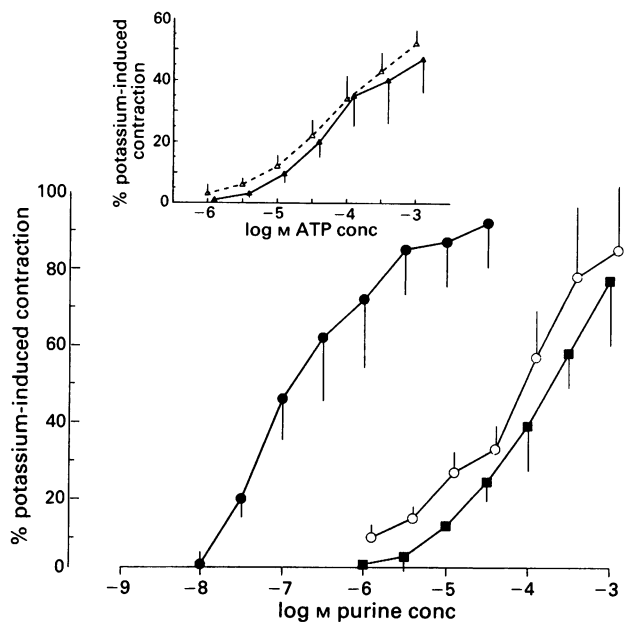

Figure 5 Concentration-response curves (CRC) to high concentrations of ATP (■) ($n = 21$, 3 each dose), α,β-methyleneATP (●) ($n = 24$, 3–5 each dose) and 2-methylthioATP (○) ($n = 21$, 3 each dose) in pooled human resistance arteries determined by a composite method. Points are percentages of the KDS-induced contraction determined in the same artery and are shown as means with s.e.mean shown by vertical lines. Inset: CRC to high concentrations of ATP in omental arteries (△, broken line) ($n = 14$) and subcutaneous arteries (▲) ($n = 11$). Points represent % of the KDS-induced contraction determined in the same artery and are shown as means ± s.e.mean.

measure of the maximum response, this was 55% of the KDS response. The maximum response was increased to 82% when the composite method was used but the EC_{50} was unaltered, being 72 μM and 74 μM by the cumulative and composite methods respectively (Figure 6).

α,β-MeATP (10 nM–1 mM) also produced contractions and was more potent (EC_{50} 0.12 μM) than ATP, whilst 2-Me.S.ATP (10 nM–1 mM) was approximately equipotent (EC_{50} 60 μM) with ATP in this respect (Figure 5). The contractile responses to ATP were unaffected by indomethacin (10 μM), 8-phenyltheophylline (10 μM) or phentolamine (10 μM). When added alone, adenosine (1 μM–1 mM) did not produce contractions.

Discussion

The greater potency of 2-Me.S.ATP in comparison with α,β-MeATP regarding relaxation of noradrenaline preconstricted human resistance arteries, suggests an action at the P_{2y}-purinoceptor. Unlike the response to ATP, the relaxation response to adenosine was antagonized by the P_1-purinoceptor antagonist, 8-phenyltheophylline (Griffith *et al.*, 1981), suggesting an action of adenosine at the P_1-purinoreceptor. Low concentrations of ATP (less than 1 μM) produced relaxant responses but higher concentrations of adenosine were required to produce a comparable effect.

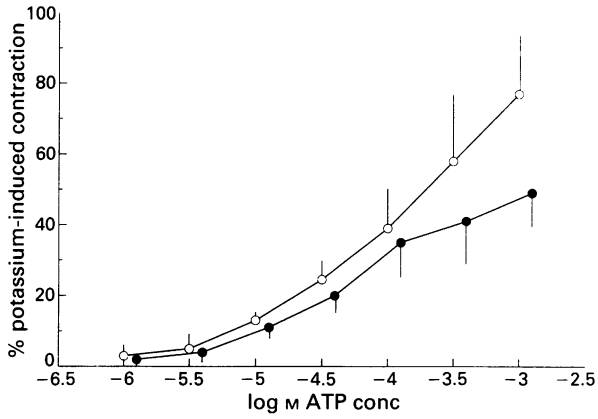

Figure 6 Concentration-response curves to high concentrations of ATP in pooled human resistance arteries determined by a cumulative method (●) ($n = 25$) and a composite method (○) ($n = 21$, 3 each dose). Points represent % of the KDS-induced contraction determined in the same artery, and are shown as means with s.e.mean shown by vertical lines.

This greater potency of ATP, together with the insensitivity to methylxanthine blockade, suggests that in these arteries *in vitro*, relaxant responses to ATP do not occur solely following the hydrolysis of ATP to adenosine. This might have been expected due to the nucleotidase enzymes present in vascular endothelial cells (Pearson & Gordon, 1985). This alone does not necessarily exclude the possibility that the effects of ATP are partially mediated via the P_1-purinoceptor as in the guinea-pig atrium (Moody *et al.*, 1984) and rat coronary vasculature (Hopwood & Burnstock, 1987). Nevertheless, the potent effect of the slowly degradable analogue, 2-Me.S.ATP, provides strong evidence for a substantial P_{2y}-purinoceptor-mediated component to the relaxant response and the lack of effect of 8-phenyltheophylline suggests that any P_1-purinoceptor-mediated component must be small.

In a variety of arteries, including the rat mesenteric bed (Ralevic & Burnstock, 1988), dog coronary artery (Houston *et al.*, 1987), pig aorta (Martin *et al.*, 1985) and rat femoral artery (Kennedy *et al.*, 1985), P_2-purinoceptors mediating relaxation to ATP and its analogues, with orders of agonist potency similar to that determined in this study, have been located on the vascular endothelium and are presumed to produce relaxation via the release of endothelium-derived relaxing factor (EDRF) (Furchgott, 1983). Cautious extrapolation of these animal data may suggest that the P_{2y}-purinoceptor-mediated responses of the human arteries used in this study are also mediated via the release of EDRF. However, there was no evidence that arteries with a functional endothelium relaxed any better to ATP than those lacking a functional endothelium. ATP may be inducing relaxation in an endothelium-independent manner (Mathieson & Burnstock, 1985; Burnstock, 1987). No deliberate attempt was made to remove the endothelium, but some damage was inevitable during the dissection and mounting of the arteries. Previous attempts in our hands to disrupt the endothelium deliberately by passing a third 40 μm wire through the lumen of mounted arteries have given variable results. Reversible loss has been shown to occur in mouse cerebral microvessels exposed to endothelial damage by a laser (Rosenblum, 1988). White & Angus (1987) have also expressed difficulty in reliably removing the endothelium from small calibre arteries, but Osol *et al.* (1989) have described a successful method.

Adenosine also produced relaxation, following preconstriction with noradrenaline, by an essentially endothelium-independent mechanism, as determined by the same criteria used for the ATP-induced relaxations. This observation is consistent with the proposed smooth muscle location of the post-junctional P_1-purinoceptor determined in a variety of vascular preparations (Mathieson & Burnstock, 1985; Su, 1985a; McCormack *et al.*, 1989). When added to arteries at basal tone, ATP had no effect at low concentrations, but produced contraction at high concentrations (greater than 1 μM). These were characteristically poorly sustained and, when cumulative concentration-response curves were constructed, necessitated the addition of incremental doses of agonist at arbitrary 2 min intervals. Once a full concentration-response curve to ATP had been prepared and the myograph chamber washed-out, further addition of ATP either failed to elicit a response or produced only a greatly diminished response. This tachyphylaxis was even more pronounced following exposure to α,β-MeATP and, together with the order of agonist potencies and insensitivity to 8-phenyltheophylline, suggests an action at the P_{2x}-purinoceptor (Burnstock & Kennedy, 1985).

The tachyphylaxis of the contractile response to ATP may invalidate the cumulative concentration-response curves. Composite concentration-response curves were therefore prepared in order to avoid this problem. The maximum response to ATP obtained by the composite method was increased compared with that obtained by the cumulative method. This suggests that tachyphylaxis to the ATP contractile response can occur within 12 min (6 × 2 min dose intervals). It is consequently of note that the EC_{50} values obtained by the two methods were similar. In contrast to ATP, adenosine consistently failed to produce contraction. This is apparent in the majority of arteries described (Burnstock, 1978), except some renal arteries (Williams, 1987). Ramagopal *et al.* (1988), using human epicardial coronary arteries, reported only relaxant responses to adenosine. On the other hand adenosine has been shown to contract strips of the guinea-pig pulmonary artery, and to enhance the contractile response of this tissue to transmural electrical stimulation and applied noradrenaline probably by a postjunctional mechanism (Wiklund *et al.*, 1987).

Purines are stored in endothelial cells, platelets and sympathetic nerve endings and can be released in response to physiological stimuli as well as ischaemia and injury (Pearson & Gordon, 1985; Born & Krater, 1985; Jennings & Steenbergen, 1985; Gordon, 1986). Despite rapid degradation of ATP to adenosine and re-uptake of adenosine into the cells, plasma purine levels, particularly levels of ATP, are likely to exceed those required to initiate P_{2y}-purinoceptor-mediated vasodilatation (Su, 1985b). ATP is released, with noradrenaline, from perivascular sympathetic nerves in a variety of tissues (Kugelgen & Starke, 1985; Kennedy *et al.*, 1986; Muramatsu & Kigoshi, 1987; Machaly *et al.*, 1988) and local concentrations may reach levels sufficient to initiate P_{2x}-purinoceptor-mediated effects. The rate of adenosine outflow from canine subcutaneous adipose tissue increases following nerve stimulation (Fredholm & Sollevi, 1981) and, in addition, adenosine and the P_1-purinoceptor antagonist, theophylline, affect responses to nerve stimulation in this tissue (Sollevi & Fredholm, 1983).

This study has identified vasodilator P_{2y}- and P_1-purinoceptors and vasoconstrictor P_{2x}-purinoceptors in human isolated arteries small enough to contribute significantly to peripheral vascular resistance (Mulvany, 1987). These receptors have similarly been demonstrated in a variety of animal studies (Burnstock & Kennedy, 1986; Williams, 1987; Hourani *et al.*, 1988). These data suggest that purines may play a significant role in human vasoregulation. Other studies of the effects of purines on resistance calibre arteries have almost entirely been restricted to those using perfused animal tissue preparations (Taylor *et al.*, 1989). As in the present study, Ralevic & Burnstock (1988) were unable to produce a vasodilator response to α,β-MeATP in the perfused rat mesentery and, due to the weak vasodilator action of adenosine in this preparation, concluded that the breakdown of ATP to adenosine does not contribute significantly to the vasodilator action of ATP in this preparation. In a study using isolated myograph mounted arteries, White & Angus (1987) demonstrated relaxant effects of ATP and adenosine in

small as well as large dog coronary arteries. Liu *et al.* (1989) have shown preliminary evidence for the presence of P_{2y}- and P_{2x}-purinoceptors in human small pulmonary arteries. Hardebo *et al.* (1987) have demonstrated the presence of both P_1 and P_2-purinoceptors in human pial arteries of about 300–600 μm in diameter, using arteries from 11 patients. These authors observed a P_1-purinoceptor mediated relaxant response to adenosine with an EC_{50} of 1.8 μM and maximum response of 73%, values similar to those obtained in the present study. They also identified a P_{2y}-like relaxant response, but no contractile response, to ATP. These data complement the present study in providing evidence for the widespread distribution of several purinoceptor subtypes in the human resistance vasculature.

In conclusion, the results of this study demonstrate the presence of both P_{2x}-purinoceptors mediating contraction and P_{2y}-purinoceptors mediating relaxation of human resistance arteries. The greater potency of α,β-MeATP compared to ATP and 2-Me.S.ATP for the contraction response suggests an action at the P_{2x}-purinoceptor and the greater potency of 2-Me.S.ATP compared to ATP and α,β-MeATP for the relaxant response suggests an action at the P_{2y}-purinoceptor. Relaxation produced by adenosine, but not ATP, was antagonized by 8-phenyltheophylline suggestive of a role for P_1-purinoceptors in these arteries. There is also evidence that the relaxant responses to ATP and adenosine are, at least in part, independent of endothelium in human resistance arteries.

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References

ALEXANDER, I. & EYRE, P. (1985). P_1-purinoceptors mediate relaxation of the bovine bronchial artery. *Eur. J. Pharmacol.*, **107**, 359–362.

BARLOW, R.B. (1983). *Biodata Handling with Microcomputers.* Cambridge: Elsevier.

BORN, G.V.R. & KRATZER, M.A.A. (1985). Source and concentration of extracellular adenosine triphosphate during haemostasis in rats, rabbits and man. *J. Physiol.*, **354**, 419–429.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptor. In *Cell Membrane Receptors for Drugs and Hormones: a Multidisciplinary Approach.* ed. Straub, R.W. & Bollis, L. pp. 107–118. New York: Raven Press.

BURNSTOCK, G. (1987). Local control of blood pressure by purines. *Blood Vessels*, **24**, 156–160.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

BURNSTOCK, G. & KENNEDY, C. (1986). A dual function of adenosine 5'-triphosphate in the regulation of vascular tone. *Circ. Res.*, **58**, 319–330.

FREDHOLM, B.B. & SOLLEVI, A. (1981). The release of adenosine and inosine from canine subcutaneous tissue by nerve stimulation and noradrenaline. *J. Physiol.*, **313**, 351–367.

FURCHGOTT, R.F. (1983). Role of endothelium in responses of vascular smooth muscle. *Circ. Res.*, **53**, 557–573.

GORDON, J.L. (1986). Review article. Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

GRIFFITH, S.G., MEGHJI, P. & BURNSTOCK, G. (1981). 8-Phenyltheophylline: a potent P_1-purinoceptor antagonist. *Eur. J. Pharmacol.*, **75**, 61–64.

HARDEBO, J.E., KÅHRSTRÖM, J. & OWMAN, C. (1987). P_1- and P_2-purine receptors in brain circulation. *Eur. J. Pharmacol.*, **144**, 343–352.

HOPWOOD, A.M. & BURNSTOCK, G. (1987). ATP mediates coronary vasoconstriction via P_{2x}-purinoceptors and coronary vasodilatation via P_{2y}-purinoceptors in the isolated perfused rat heart. *Eur. J. Pharmacol.*, **136**, 49–54.

HOURANI, S.M.O., WELFORD, L.A., LOIZOU, G.D. & CUSACK, N.J. (1988). Adenosine 5'-(2-fluorodiphosphate) is a selective agonist at P_2-purinoceptors mediating relaxation of smooth muscle. *Eur. J. Pharmacol.*, **147**, 131–136.

HOUSTON, D.A., BURNSTOCK, G. & VANHOUTTE, P.M. (1987). Different P_2-purinergic receptor subtypes of endothelium and smooth muscle in canine blood vessels. *J. Pharmacol. Exp. Ther.*, **241**, 501–506.

JENNINGS, R.B. & STEENBERGEN, C. (1985). Nucleotide metabolism and cellular damage in myocardial ischaemia. *Annu. Rev. Physiol.*, **47**, 727–749.

KENNEDY, C., DELBRO, D. & BURNSTOCK, G. (1985). P_2-purinoceptors mediate both vasodilation (via the endothelium) and vasoconstriction of the isolated rat femoral artery. *Eur. J. Pharmacol.*, **107**, 161–168.

KENNEDY, C., SAVILLE, V.L. & BURNSTOCK, G. (1986). The contributions of noradrenaline and ATP to the responses of the rabbit central ear artery to sympathetic nerve stimulation depend on the parameters of stimulation. *Eur. J. Pharmacol.*, **122**, 291–300.

KUGELGEN, I.V. & STARKE, K. (1985). Noradrenaline and adenosine triphosphate as co-transmitters of neurogenic vasoconstriction in rabbit mesenteric artery. *J. Physiol.*, **367**, 435–455.

LIU, S.F., McCORMACK, D.G., EVANS, T.W. & BARNES, P.J. (1989). Characterisation of P_2-purinoceptors in human small pulmonary artery. *Br. J. Pharmacol.*, **97**, 498P.

MACHALY, M., DALZIEL, H.H. & SNEDDEN, P. (1988). Evidence for ATP as a co-transmitter in dog mesenteric artery. *Eur. J. Pharmacol.*, **147**, 83–91.

MARTIN, G., THOM, S. & SEVER, P. (1988). Purine mediated relaxation and contraction of human resistance arteries. *Br. J. Pharmacol.*, **95**, 863P.

MARTIN, W., CUSACK, N.J., CARLETON, S.J. & GORDON, J.L. (1985). Specificity of the P_2-purinoceptor that mediates endothelium-dependent relaxation of the pig aorta. *Eur. J. Pharmacol.*, **108**, 295–299.

MATHIESON, J.J. & BURNSTOCK, G. (1985). Purine-mediated relaxation and constriction of isolated rabbit mesenteric artery are not endothelium-dependent. *Eur. J. Pharmacol.*, **118**, 221–229.

McCORMACK, D.G., CLARKE, B. & BARNES, P.J. (1989). Characterisation of adenosine receptors in human pulmonary arteries. *Am. J. Physiol.*, **256**, H41–H46.

MOODY, C.J., MEGHJI, P. & BURNSTOCK, G. (1984). Stimulation of P_1-purinoceptors by ATP depends partly on its conversion to AMP and adenosine and partly on direct action. *Eur. J. Pharmacol.*, **97**, 47–54.

MULVANY, M.J. (1987). Editorial review: The Fourth Sir George Pickering Memorial Lecture. The structure of the resistance vasculature in essential hypertension. *J. Hypertens.*, **5**, 129–136.

MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive rats. *Circ. Res.*, **41**, 19–26.

MURAMATSU, I. & KIGOSHI, S. (1987). Purinergic and non-purinergic innervation of the cerebral arteries of the dog. *Br. J. Pharmacol.*, **92**, 901–908.

OSOL, G., CIPOLLA, M. & KNUTSON, S. (1989). A new method for mechanically denuding the endothelium of small (50–150 μm) arteries with a human hair. *Blood Vessels*, **26**, 320–324.

PEARSON, J.D. & GORDON, J.L. (1985). Nucleotide metabolism by the endothelium. *Annu. Rev. Physiol.*, **47**, 617–627.

RALEVIC, V. & BURNSTOCK, G. (1988). Actions mediated by P_2-purinoceptor subtypes in the isolated perfused mesenteric bed of the rat. *Br. J. Pharmacol.*, **95**, 637–645.

RAMAGOPAL, M.V., CHITWOOD, R.W. & MUSTAFA, S.J. (1988). Evidence for an A_2 adenosine receptor in human coronary arteries. *Eur. J. Pharmacol.*, **151**, 483–486.

ROSENBLUM, W.I. (1988). Loss of endothelium-dependent relaxation in mouse cerebral microvessels may be rapidly reversible. *Microvasc. Res.*, **35**, 132–138.

SU, C. (1985a). Purines in hypertension. In *Purines: Pharmacology and Physiological Roles.* ed. Stone, T.W. pp. 143–151. London: Macmillan.

SU, C. (1985b). Extracellular functions of nucleotides in heart and blood vessels. *Annu. Rev. Physiol.*, **47**, 665–676.

SOLLEVI, A. (1986). Cardiovascular effects of adenosine in man; possible clinical implications. *Prog. Neurobiol.*, **27**, 319–349.

SOLLEVI, A. & FREDHOLM, B.B. (1983). Influence of adenosine on the vascular response to sympathetic nerve stimulation in canine subcutaneous adipose tissue. *Acta Physiol. Scand.*, **119**, 15–24.

TAYLOR, E.M., PARSONS, M.E., WRIGHT, P., PIPKIN, M.A. & HOWSON, W. (1989). The effects of adenosine triphosphate and related purines on arterial resistance vessels *in vitro* and *in vivo*. *Eur. J. Pharmacol.*, **161**, 121–133.

THOM, S., HUGHES, A., MARTIN, G. & SEVER, P. (1987). Endothelium-dependent relaxation in human arteries and veins. *Clin. Sci.*, **73**, 547–552.

WHITE, T.D. & ANGUS, J.A. (1987). Relaxant effects of ATP and adenosine on canine large and small coronary arteries *in vitro*. *Eur. J. Pharmacol.*, **143**, 119–126.

WIKLUND, N.P., CEDEQVIST, B., MATSUDA, H. & GUSTAFSSON, L.E. (1987). Adenosine can excite pulmonary artery. *Acta Physiol. Scand.*, **131**, 477–478.

WILLIAMS, M. (1987). Purine receptors in mammalian tissues: pharmacology and functional significance. *Annu. Rev. Pharmacol. Toxicol.*, **27**, 315–345.

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Block of cardiac sodium channels by amiodarone studied by using $\dot{V}_{max}$ of action potential in single ventricular myocytes

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1 Acute effects of amiodarone on cardiac sodium channels were investigated in ventricular myocytes isolated from guinea-pig hearts, and compared with those of lignocaine.

2 Transmembrane potential was recorded and controlled by whole-cell current-clamp and voltage-clamp respectively through suction pipette electrodes. The maximum upstroke velocity ($\dot{V}_{max}$) of the action potential was used as a qualitative index of sodium channel availability.

3 In myocytes treated with amiodarone (1 μM) or lignocaine (40 μM), $\dot{V}_{max}$ of reference action potential elicited at 0.03 Hz was decreased by 6 ~ 11%, indicating minimal tonic block of sodium channels.

4 Application of a single conditioning depolarization to those myocytes resulted in a significant decrease in $\dot{V}_{max}$ of a subsequent test action potential. The $\dot{V}_{max}$ reduction was enhanced in a single exponential function as the clamp pulse duration was prolonged. Time constants at 0 mV clamp were 25 ms for amiodarone and 122 ms for lignocaine.

5 $\dot{V}_{max}$ recovery of test action potential following a 1000 ms 0 mV clamp was approximated by a dual exponential function. Time constants for the late slow component (τ_R) at the resting potential level were 418 ms for amiodarone and 178 ms for lignocaine. τ_R values were shortened in a voltage-dependent manner by hyperpolarization during the coupling interval.

6 These findings suggested that amiodarone, like lignocaine, blocks the sodium channel primarily when it is in the inactivated state. Both onset and offset kinetics of the block are very rapid. Such sodium channel blocking characteristics may contribute to its potent antiarrhythmic activity.

Introduction

Amiodarone is one of the most effective drugs currently available for the treatment of both supraventricular and ventricular tachyarrhythmias (Mason, 1987; Singh, 1989). This drug has long been classified as a Class-III antiarrhythmic drug, because it prolongs action potential duration and refractory period of cardiac muscle (Singh & Vaughan Williams, 1970; Vaughan Williams, 1984). However, recent studies have shown that amiodarone also possesses an inhibitory action against the fast sodium channels (Class-I action). This was initially suggested by clinical observations that chronically administered amiodarone increases the His-ventricular interval in the His-bundle electrograms (Heger *et al.*, 1981). Mason *et al.* (1983, 1984) demonstrated that amiodarone depressed the maximum upstroke velocity ($\dot{V}_{max}$) of action potential in guinea-pig papillary muscles in a frequency- and voltage-dependent manner. Similar $\dot{V}_{max}$ inhibition at high stimulation frequencies was reported by Varro *et al.* (1985), Yabek *et al.* (1985, 1986) and Pallandi & Campbell (1987) in Purkinje fibres as well as in papillary muscles isolated from dog and guinea-pig hearts. More direct evidence of sodium channel block by amiodarone was presented by Follmer *et al.* (1987) in voltage-clamp experiments on single canine Purkinje cells and cat ventricular myocytes. This effect was also shown by single channel recording in cultured cardiocytes of neonatal rats (Kohlhardt & Fichtner, 1988), and in radioligand assay of sodium channel receptors for Class-I antiarrhythmic drugs using [^{3}H]-batrachotoxin A 20 α-benzoate (Sheldon *et al.*, 1989). However, much remains to be elucidated as to the mode of action of amiodarone on cardiac sodium channels in relation to its antiarrhythmic activity.

In the present study, we investigated the effects of amiodarone on $\dot{V}_{max}$ of action potentials in single ventricular myocytes isolated from guinea-pig hearts, and compared them with those of lignocaine. In order to clarify the binding and unbinding characteristics of these drugs for the different states of the sodium channel, the conditioning membrane potential was controlled through the whole-cell voltage clamp technique. This allowed us to test the drug action more precisely than earlier studies using multicellular tissue preparations, and under much more physiological conditions than those employed for direct measurement of sodium inward current (Follmer *et al.*, 1987; Kohlhardt & Fichtner, 1988).

Methods

Single ventricular myocytes were enzymatically isolated from guinea-pig hearts by a procedure described in our previous papers (Honjo *et al.*, 1989; Kodama *et al.*, 1990). A few drops of cell suspension in KB medium (Isenberg & Klöckner, 1982) was placed in a recording chamber attached to an inverted microscope. The chamber was then perfused with Tyrode solution having the following composition (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, HEPES 5.0 and glucose 5.0. pH was adjusted to 7.4 by adding NaOH. The temperature of the perfusate was maintained at 34°C. Following a step increase in calcium concentration of the medium to 1.8 mM (Tyrode solution), 20 ~ 30% of myocytes deteriorated into round-shaped cells due to irreversible contracture, whereas the remaining cells were tolerant to calcium; their intact rod-shape was maintained without spontaneous beating. The experiments were carried out in the latter myocytes.

The single-pipette whole-cell clamp method was employed to control and record membrane potential with a single cell/single channel amplifier (Nihon Kohden, CEZ-2200). The pipettes were heat polished, and filled with internal solution to produce a resistance ranging from 2 to 3 megohm. The pipette solution consisted of (mM): KCl 140.0, NaH_2PO_4 10.0, EGTA 1.0, MgATP 5.0 and HEPES 10.0. pH was adjusted to 7.2 by adding KOH. Cell capacitance and series resistance were compensated by analogue circuits. Command potentials were generated by a multichannel stimulator (Nihon Kohden,

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SEN-7103). When the action potential was elicited, the voltage-clamp mode was switched to the current-clamp mode by use of an electronic relay regulated by the stimulator, and a square depolarizing suprathreshold current (2 ~ 4 ms) was passed through the electrode. The action potential was differentiated electronically in order to measure the maximum upstroke velocity ($\dot{V}_{max}$).

Our control experiments have revealed that $\dot{V}_{max}$ of single ventricular myocytes is unaffected by stimulus intensity provided the latency from the end of stimulus to $\dot{V}_{max}$ was less than 2 ms. Further prolongation of the latency, however, resulted in a slight but significant decrease in $\dot{V}_{max}$. In all the experiments described below, the latency was kept constant (0.5 ~ 1.5 ms) by adjusting the intensity of stimulus current. The membrane potential at which $\dot{V}_{max}$ occurred was also kept constant (±3 mV) by such adjustment.

Single clamp pulse protocols to assess the relative contribution of activation-dependent and inactivation-dependent block were similar to those employed in our previous studies (Kodama *et al.*, 1987; 1990). Following a rest period of 30 s allowing full recovery of $\dot{V}_{max}$ at the resting potential level (−82 mV), the membrane potential was clamped up to 0 mV for 10 to 1000 ms. At the end of conditioning clamp pulse, membrane potentional was clamped back to the resting level for 100 ms, which is long enough for a drug-free channel to reactivate fully (Carmeliet & Vereecke, 1979; Ebihara & Johnson, 1980), but short enough so that only partial dissociation of the drug from the blocked channel occurs (Grant *et al.*, 1984). The voltage-clamp was then released, and a stimulus was applied to elicit a test action potential. $\dot{V}_{max}$ of the test action potential was measured as an index of sodium channel availability.

Recovery from block (unblocking) was studied by the addition of a stimulus to elicit test action potential from the resting potential level (−82 mV) after a 1000 ms conditioning clamp pulse to 0 mV. The coupling interval between the clamp pulse and the test stimulus, during which the myocyte was clamped to a potential ranging from −82 to −120 mV, was varied from 10 ms to 2 s.

Amiodarone HCl was kindly donated by Taisho Pharmaceutical Co. Ltd. (Tokyo, Japan). Lignocaine HCl was purchased from Sigma Chemical Co. Since amiodarone is almost insoluble in physiological saline, it was dissolved into Tyrode solution containing ethanol (0.005 ~ 0.05 mg 100 ml^{-1}) and bovine serum albumin (0.1 ~ 1%) as described by Polster & Broekhuysen (1976). At the concentrations employed in the present study (1 ~ 10 μM), there was no visible precipitation of amiodarone in the perfusate.

Values obtained in experiments were presented as means or means ± s.e. unless otherwise stated. Data were analyzed by the *t* test, analysis of variance, Dunnett's test and regression analysis. Differences were considered significant at $P < 0.05$.

Results

The results were obtained from a total of 31 ventricular myocytes. Baseline action potentials elicited in these cells at a long interstimulus interval (30 s) had a resting potential (RP) of −82.1 ± 0.2 mV and $\dot{V}_{max}$ of 232 ± 6 V s^{-1}. These values were kept constant in untreated myocytes for longer than 60 min. There were no significant differences in the two parameters between the subgroups of cells exposed to amiodarone and lignocaine. We did not analyze the change of action potential duration, because it shortened gradually in untreated myocytes after 30 ~ 40 min of potential recording. This may probably reflect 'run-down' of calcium channels.

Amiodarone at 1 μM caused a slight but significant decrease in $\dot{V}_{max}$ of such reference action potentials without affecting RP. This $\dot{V}_{max}$ reduction (tonic block) was enhanced as the drug exposure was prolonged, and reached an almost steady level (10.7%) within 20 min (Table 1). All the results with amiodarone given below were, therefore, recorded after 20 to 30 min of the drug application. Amiodarone at 3 μM or 10 μM caused a larger tonic block of $\dot{V}_{max}$, but the change was not complete even after 30 min of drug exposure, giving rise to difficulty in obtaining constant reference values. This inhibited further experiments with such high concentrations of the drug.

Lignocaine at 40 μM caused a comparable tonic decrease in $\dot{V}_{max}$ without affecting RP (Table 1). The lignocaine action reached a steady state within 5 min of the drug exposure.

Influence of single conditioning clamp pulse

In untreated myocytes, a single conditioning depolarizing clamp pulse up to 0 mV caused only a slight decrease in $\dot{V}_{max}$ of the test action potential, which was elicited 100 ms after the termination of the clamp pulse, when the clamp pulse duration was longer than 200 ms. For instance, $\dot{V}_{max}$ following a 0 mV clamp for 1000 ms was decreased by 6.1 ± 0.3% ($n = 11$) from the reference level. This small $\dot{V}_{max}$ reduction probably refelcts 'slow inactivation' of cardiac sodium channels (Saikawa & Carmeliet, 1982; Clarkson *et al.*, 1984).

After treatment with 1 μM amiodarone for 20 min, similar clamp pulses caused much greater $\dot{V}_{max}$ reduction, which was dependent on duration and voltage of the plateau clamp (Figure 1). The $\dot{V}_{max}$ decay with prolongation of the conditioning clamp to 0 mV fitted well to a single exponential function with a time constant of 25 ± 2 ms ($n = 5$). Such exponential $\dot{V}_{max}$ decay was induced by a conditioning clamp to a level more positive than −70 mV, and its extent reached a maximal plateau level at around −10 mV.

Figure 2 shows an experiment where the effects of conditioning clamp voltage were examined more extensively. A test action potential was elicited 100 ms after the conditioning clamp to a voltage ranging from −120 to +30 mV. In the presence of amiodarone (1 μM), an appreciable decrease in $\dot{V}_{max}$ was induced at −70 ~ −80 mV, and it was enhanced at a more positive potential range. The $\dot{V}_{max}$ inhibition reached the maximal plateau level at −10 ~ −20 mV. The data points normalized by the maximal $\dot{V}_{max}$ value fitted the Boltzmann equation:

$$y = (1 + \exp(V_m - V_h)/k)^{-1} + C \quad (1)$$

where V_m is the conditioning clamp potential, V_h is conditioning clamp potential showing a half maximal $\dot{V}_{max}$ inhibition, k is the slope factor and C is constant. Average values of V_h and k obtained from five myocytes treated with amiodarone (1 μM) were −65 ± 2 mV and 8.5 ± 0.8 mV, respectively.

Qualitatively similar results were obtained with lignocaine. Thus, in the presence of 40 μM lignocaine, a single conditioning plateau clamp to a voltage more positive than −70 mV

Table 1 Activation and inactivation-dependent block

		Tonic block (%)	*ACB* (%)	*ICB* (%)	*ICB/ACB*
Amiodarone	1 μM	10.7 ± 1.7	6.2 ± 2.2	46.2 ± 4.6	7.7 ± 1.2
Lignocaine	40 μM	6.2 ± 2.4	5.5 ± 2.3	33.3 ± 4.3	6.1 ± 0.8

Values are means ± s.e. of five experiments for both drugs. ACB: activated channel block defined as percentage decrease in $\dot{V}_{max}$ from the reference value (tonic block subtracted) to 0 time intercept of the exponential fit. ICB: inactivated channel block defined as percentage decrease in $\dot{V}_{max}$ from the 0 time intercept to the value following a 500 ms clamp pulse.

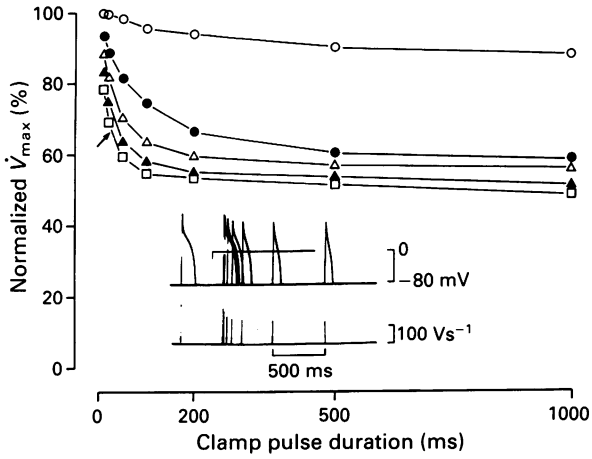

Figure 1 Influence of conditioning depolarization on $\dot{V}_{max}$ inhibition by amiodarone. Inset shows superimposed records of action potentials (upper trace) and their differentiated upstroke spikes (lower trace). Action potentials were elicited without clamp pulse as a reference level (left end) or 100 ms after a conditioning clamp to 0 mV for 10 to 1000 ms. Each conditioning depolarization was preceded by a 30 s rest period. The records were obtained 20 min after application of amiodarone (1 μM). The baseline $\dot{V}_{max}$ of the cell was 226 Vs^{-1}. In the graph, $\dot{V}_{max}$ of test action potential normalized to the reference level (referenced at the tonic block subtracted value) was plotted against the conditioning clamp pulse duration. Voltage level of the conditioning clamp was −70 mV (○), −50 mV (●), −30 mV (△), −10 mV (▲), and 0 mV (□). An arrow indicates time constant (29 ms) for the exponential decay of $\dot{V}_{max}$ at 0 mV conditioning depolarization.

caused a progressive decrease in $\dot{V}_{max}$ of subsequent test action potential (Figure 3). The $\dot{V}_{max}$ decline associated with prolongation of the clamp pulse duration fitted well to a single exponential function. Average time constant of the $\dot{V}_{max}$ decay at 0 mV was calculated to be 122 ± 8 ms (n = 5).

We also tested the influence of the conditioning clamp voltage on the $\dot{V}_{max}$ inhibition with lignocaine by using the

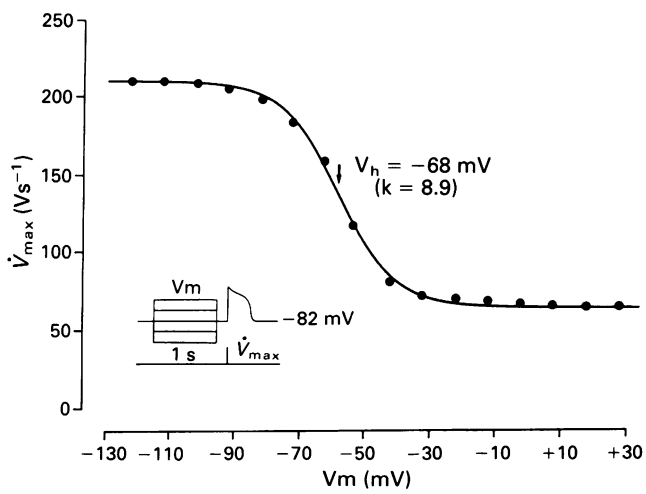

Figure 2 Influence of conditioning clamp voltage on $\dot{V}_{max}$ in a myocyte treated with amiodarone. Following a 30 s rest period, the myocyte was clamped at various voltages (−120 to +30 mV) for 1000 ms. A test action potential was elicited 100 ms after termination of the conditioning clamp (inset). Ordinate scale indicates $\dot{V}_{max}$ of the test action potential. Abscissa scale shows voltage level of the conditioning clamp. Smooth curve is a least squares fit of data points normalized by the maximal $\dot{V}_{max}$ value to the equation:

$$y = (1 + \exp(V_m - V_h)/k)^{-1} + C$$

where V_m is the conditioning clamp voltage, V_h is the value of V_m causing a half maximal inhibition of $\dot{V}_{max}$, k is the slope factor and C is constant. Data were obtained 20 min after application of amiodarone (1 μM).

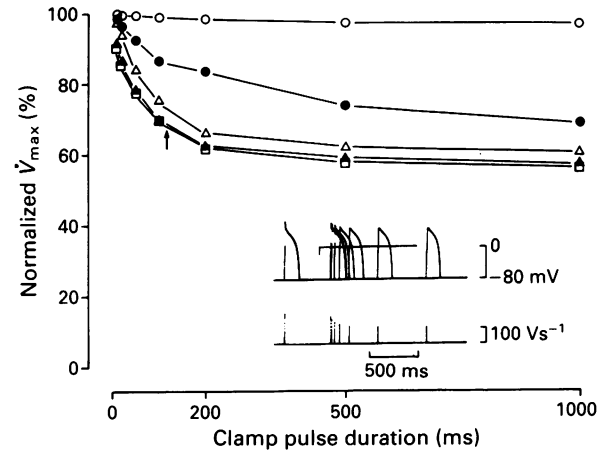

Figure 3 Influence of conditioning depolarization on $\dot{V}_{max}$ inhibition by lignocaine. Inset shows superimposed records of action potentials (upper trace) and their differentiated upstroke spikes (lower trace) 10 min after application of lignocaine (40 μM). The baseline $\dot{V}_{max}$ of the cell was 223 Vs^{-1}. Protocol of the conditioning clamp was the same as in Figure 1. In the graph, $\dot{V}_{max}$ of test action potential normalized to the reference level was plotted against the conditioning clamp pulse duration. Voltage level of the conditioning clamp was −70 mV (○), −50 mV (●), −30 mV (△), −10 mV (▲), and 0 mV (□). An arrow indicates time constant (128 ms) for the exponential decay of $\dot{V}_{max}$ at 0 mV conditioning depolarization.

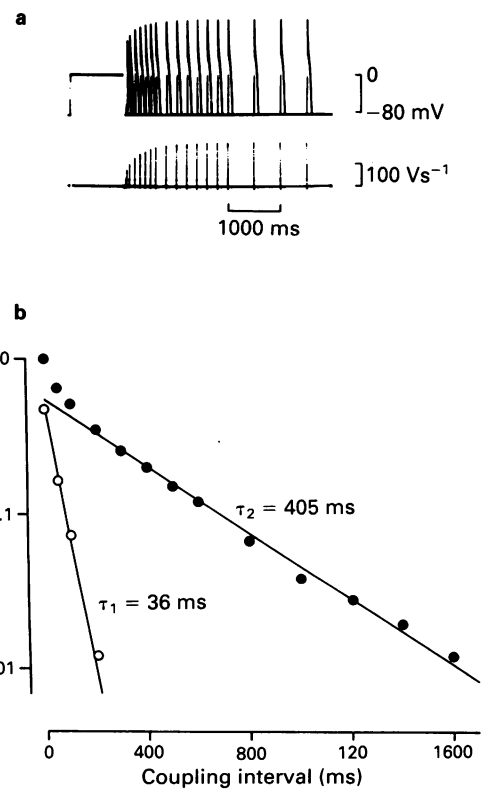

Figure 4 Recovery of $\dot{V}_{max}$ in the presence of amiodarone. (a) Superimposed records of action potentials (upper trace) elicited after a conditioning clamp to 0 mV for 1000 ms, and their differentiated upstroke spikes (lower trace) 20 min after application of amiodarone (1 μM). The baseline $\dot{V}_{max}$ of the cell was 214 Vs^{-1}. The coupling interval between the clamp pulse and the test action potential, during which the cell was clamped to −82 mV, was varied, and each clamp pulse was preceded by a 30 s rest period. (b) Recovery process of $\dot{V}_{max}$ of test action potential. Ordinate scale, fractional $\dot{V}_{max}$ reduction of test action potential compared to the $\dot{V}_{max}$ value of action potential without conditioning clamp pulse (reference level). Abscissa scale, coupling interval between the clamp pulse and the estimated action potential. The time course was approximated by a dual exponential function with τ_1 of 36 ms and τ_2 of 405 ms.

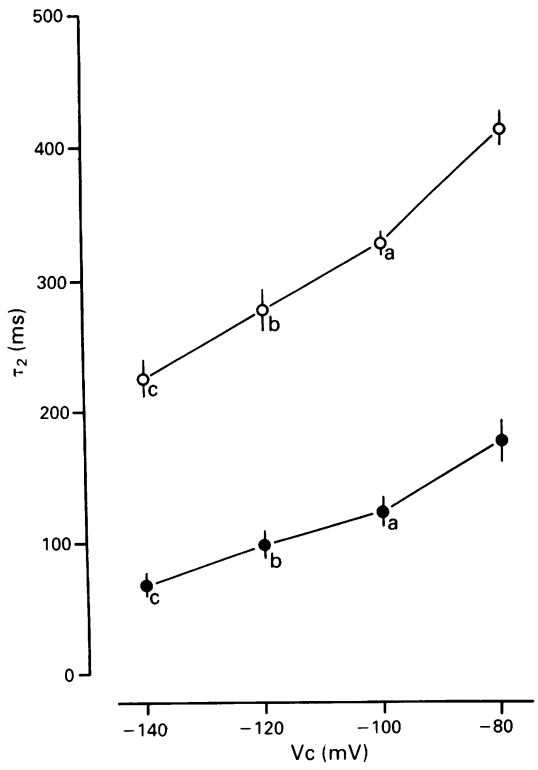

Figure 5 Voltage-dependence of $\dot{V}_{max}$ recovery in myocytes treated with amiodarone (1 μM) or lignocaine (40 μM). Ordinate scale, time constant for the late slow component of $\dot{V}_{max}$ recovery (τ_2). Abscissa scale, membrane potential (V_c) during the coupling interval between the conditioning clamp (0 mV, 1 s) and the test action potential. Values are means ± s.e. of five experiments with amiodarone (○) and with lignocaine (●). Symbols indicate significant difference at $P < 0.05$ from the values at −82 mV (a), −100 mV (b) and −120 mV (c).

same protocol as employed in Figure 2. In myocytes treated with lignocaine (40 μM), $\dot{V}_{max}$ of the test action potential was decreased in a voltage-dependent manner, as in the experiments with amiodarone. The data points relating the conditioning voltage and the $\dot{V}_{max}$ reduction fitted equation (1). Average values of V_h and k were −61 ± 4 mV and 7.3 ± 0.9 mV, respectively ($n = 5$).

Recovery of $\dot{V}_{max}$

The recovery process of $\dot{V}_{max}$ following a long (1 s) conditioning clamp to 0 mV was examined by introducing a test action potential with various coupling intervals, during which the membrane was clamped back to −82 to −140 mV.

A representative experiment with amiodarone is shown in Figure 4. Under drug-free control conditions, the $\dot{V}_{max}$ of the test action potential recovered almost completely (~95%) within 100 ms. The average time constant was in a range from 22 to 41 ms depending on the membrane potential during the coupling interval; being shorter at the more negative potential. In the presence of amiodarone (1 μM), the recovery of $\dot{V}_{max}$ was approximated by a dual exponential function. The early fast component may reflect recovery from inactivation of the drug-free sodium channels, since its time constants (τ_{R1}, 26 to 46 ms) corresponded with the values under control condition. The late slow component reflecting unblocking process of amiodarone had time constants (τ_{R2}) ranging from 227 to 404 ms. The τ_{R2} values showed an appreciable voltage-dependence; being shorter at the more negative potential (Figure 5).

Such dual exponential recovery of $\dot{V}_{max}$ was also observed in experiments with lignocaine at 40 μM (not shown in figures). τ_{R1} values ranged from 24 to 50 ms, whereas τ_{R2} values ranged from 86 to 172 ms. As with amiodarone, τ_{R2} was shorter at the more negative potential (Figure 5).

Discussion

In the present study, we used $\dot{V}_{max}$ as an indirect index of sodium channel availability to infer block of channels with drugs. The probable convex-shaped nonlinear relationship between $\dot{V}_{max}$ and the peak sodium inward current (I_{Na}) in cardiac cells (Bean *et al.*, 1982; Cohen *et al.*, 1984; Sheets *et al.*, 1988) might introduce variable errors in estimating the precise amount of sodium channel block. Nevertheless, such a limitation does not invalidate $\dot{V}_{max}$ as a qualitative index, since voltage-clamp techniques currently available for I_{Na} measurement require more artificial experimental conditions (low temperature and low extracellular sodium concentration) than those for $\dot{V}_{max}$ measurement.

Both amiodarone and lignocaine caused only a slight decrease in $\dot{V}_{max}$ of reference action potentials at the concentrations tested (Table 1). Similar small 'tonic' or 'resting' block in combination with a large 'use-dependent' block of $\dot{V}_{max}$ by amiodarone at 5 to 50 μM was reported by earlier investigators in multicellular cardiac tissue preparations (Yabek *et al.*, 1985; Pallandi & Campbell, 1987). This seems to indicate that amiodarone, like lignocaine, has a very low affinity for the sodium channel receptors in the resting state compared with those in the activated and/or inactivated states. In contrast, Follmer *et al.* (1987) observed in their single cell experiments that amiodarone (0.073 ~ 7.3 μM) produced marked resting as well as use-dependent block of I_{Na}, suggesting a relatively high affinity of the drug for the resting state of sodium channels. Such discrepancies could be the result of different experimental conditions and/or different parameters used to assess the channel availability. I_{Na} measurement by Follmer *et al.* (1987) was carried out at 19 ~ 20°C. It has been shown in nerve fibres that the blocking effects of local anaesthetic agents are enhanced by lowering the temperature (Rosenberg & Heavner, 1980; Sanchez *et al.*, 1987). Amiodarone was also reported to have temperature-dependent effects on the fluidity of the phospholipid membrane (Chatelain *et al.*, 1986).

In myocytes treated with amiodarone (1 μM), application of a single conditioning depolarization caused a significant decrease in $\dot{V}_{max}$ of subsequent test action potential. The $\dot{V}_{max}$ reduction from the reference level was minimal at the shortest (10 ms) clamp pulse, while it was enhanced in a single exponential function as the clamp pulse duration was prolonged. The more positive conditioning depolarization was associated with the greater $\dot{V}_{max}$ inhibition within the range from −70 to −10 mV. These findings may strongly suggest that amiodarone, like lignocaine, blocks the sodium channel primarily when it is in the inactivated state.

We evaluated the approximate extent of activated channel block (ACB) and that of inactivated channel block (ICB) by extrapolating the single exponential curve of $\dot{V}_{max}$ decay with prolongation of 0 mV conditioning depolarization (Figures 1, 3). ACB was defined as a percentage decrease in $\dot{V}_{max}$ from the reference level to 0-time intercept of the exponential fit. ICB was the percentage decrease in $\dot{V}_{max}$ from the 0-time intercept to the value following a 500 ms clamp (Table 1). The average ratio of ICB to ACB was 7.7 for amiodarone and 6.1 for lignocaine. Thus ICB with amiodarone was, like lignocaine, much larger than ACB.

In the presence of either amiodarone or lignocaine, the $\dot{V}_{max}$ recovery of test action potential following a long (1 s) 0 mV clamp was expressed by dual exponential functions. The early fast component may reflect a repriming process (recovery from inactivation) of drug-free (unblocked) sodium channels, because its time constant (τ_{R1}) was similar to that for $\dot{V}_{max}$ recovery in untreated myocytes. The late slow component may reflect the dissociation of the drug molecules (unbinding) from the sodium channels. Time constants of the latter component (τ_{R2}) showed a similar voltage-dependence for both amiodarone and lignocaine, being shorter at the more nega-

tive potential within the range from −82 to −140 mV. This voltage-dependence can be interpreted by their higher affinity for the inactivated state of sodium channels than the resting one. Under such conditions, hyperpolarization would enhance drug dissociation from channel receptors by increasing the resting fraction of drug-associated channels at the expense of their inactivated fraction (Hondeghem & Katzung, 1984; Grant *et al.*, 1984).

There are some discrepancies among earlier investigators as to the recovery time constant of $\dot{V}_{max}$ from the use-dependent block with amiodarone. Mason *et al.* (1984) and Pallandi & Campbell (1987) reported the values of 1.48 ~ 1.63 s in guinea-pig papillary muscles at the resting membrane potential of −84 ~ −85 mV. These values fall between those reported for the fast kinetic Class-I drugs (200 ~ 500 ms) and intermediate kinetic Class-I drugs (2 ~ 12 s, Campbell, 1983). Varro *et al.* (1985) demonstrated much shorter values in canine Purkinje fibres (289 ms) and in guinea-pig papillary muscles (282 ms) at the resting membrane potential of −95 mV. Our data for amiodarone (τ_{R2} 418 ms at −82 mV; 322 ms at −100 mV) are close to the latter report. The values are somewhat longer than lignocaine (τ_{R2} 178 ms at −82 mV; 128 ms at −100 mV), but still comparable to other fast kinetic Class-I drugs (Kodama *et al.*, 1990).

Based on all the results described above, it seems reasonable to conclude that the effects of amiodarone on cardiac sodium channels are similar to lignocaine in terms of their blocking and unblocking kinetics (Kodama *et al.*, 1990). The concentration of amiodarone (1 μM) used in the present study is certainly within the range of therapeutic plasma levels (approximately 0.1 ~ 7.6 μM; Harris *et al.*, 1983; Raeder *et al.*, 1985). Accordingly, such lignocaine-like Class-I actions of amiodarone may play a very important role for the development of its potent antiarrhythmic activity especially when combined with the well-known Class-III action.

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References

BEAN, B.P., COHEN, C.J. & TSIEN, R.W. (1982). Block of cardiac sodium channels by tetrodotoxin and lidocaine: sodium current and V_{max} experiments. In *Normal and Abnormal Conduction in the Heart*. ed. Paes de Carvalho, A., Hoffman, B.F. & Lieberman, M. pp. 189–209. Mount Kisko: Futura.

CAMPBELL, T.J. (1983). Kinetics of onset of rate-dependent effects of Class-I antiarrhythmic drugs are important in determining their effects of refractoriness in guinea-pig ventricle, and provide a theoretical basis for their subclassification. *Cardiovasc. Res.*, **17**, 344–352.

CARMELIET, E. & VEREECKE, J. (1979). Electrogenesis of the action potential and automaticity. In *Handbook of Physiology. The Cardiovascular System, I*. ed. Berne, R.M. & Geiger, S.R. pp. 269–334. Bethesda, Maryland: American Physiological Society.

CHATELAIN, P., FERREIRA, J., LARUEL, R. & RUYSSCHAERT, M. (1986). Amiodarone induced modifications of the phospholipid physical state. A fluorescence polarization study. *Biochem. Pharmacol.*, **35**, 3007–3013.

CLARKSON, C.W., MATSUBARA, T. & HONDEGHEM, L.M. (1984). Slow inactivation of $\dot{V}_{max}$ in guinea pig ventricular myocardium. *Am. J. Physiol.*, **247**, H645–H654.

COHEN, C.J., BEAN, B.P. & TSIEN, R.W. (1984). Maximal upstroke velocity as an index of available sodium conductance. Comparison of maximal upstroke velocity and voltage clamp measurements of sodium current in rabbit Purkinje fibers. *Circ. Res.*, **54**, 636–651.

EBIHARA, L. & JOHNSON, E.A. (1980). Fast sodium current in cardiac muscle. A quantitative description. *Biophys. J.*, **32**, 779–790.

FOLLMER, C.H., AOMINE, M., YEH, J.Z. & SINGER, D.H. (1987). Amiodarone-induced block of sodium current in isolated cardiac cells. *J. Pharmacol. Exp. Ther.*, **243**, 187–194.

GRANT, A.O., STARMER, C.F. & STRAUSS, H.C. (1984). Antiarrhythmic drug action. Blockade of the inward sodium current. *Circ. Res.*, **55**, 427–439.

HARRIS, L., McKENNA, W.J., ROWLAND, E., HOLT, D.W., STOREY, G.C.A. & KRIKLER, D.M. (1983). Side effects of long-term amiodarone therapy. *Circulation*, **67**, 45–51.

HEGER, J.J., PRYSTOWSKY, E.N., JACKMAN, W.M., NACCARELLI, G.V., WARFEL, K.A., RINKENBERGER, R.L. & ZIPES, D.P. (1981). Amiodarone. Clinical efficacy and electrophysiology during long-term therapy for recurrent ventricular tachycardia or ventricular fibrillation. *N. Engl. J. Med.*, **305**, 539–545.

HONDEGHEM, L.M. & KATZUNG, B.G. (1984). Antiarrhythmic agents: The modulated receptor mechanism of action of sodium and calcium channel-blocking drugs. *Annu. Rev. Pharmacol. Toxicol.*, **24**, 387–423.

HONJO, H., TOYAMA, J., KODAMA, I., SATO, T., WATANABE, T. & YAMADA, K. (1989). Fluctuations of membrane potential in isolated single ventricular myocytes of guinea-pig upon resumption of oxidative phosphorylation. *J. Mol. Cell. Cardiol.*, **21**, 241–252.

ISENBERG, G. & KLÖCKNER, U. (1982). Calcium tolerant ventricular myocytes prepared by preincubation in a 'KB medium'. *Pflügers Arch.*, **395**, 6–18.

KODAMA, I., HONJO, H., KAMIYA, K. & TOYAMA, J. (1990). Two types of sodium channel block by Class-I antiarrhythmic drugs studied by using $\dot{V}_{max}$ of action potential in single ventricular myocytes. *J. Mol. Cell. Cardiol.*, **22**, 1–12.

KODAMA, I., TOYAMA, J., TAKANAKA, C. & YAMADA, K. (1987). Block of activated and inactivated sodium channels by Class-I antiarrhythmic drugs studied by using the maximum upstroke velocity ($\dot{V}_{max}$) of action potential in guinea-pig cardiac muscles. *J. Mol. Cell. Cardiol.*, **19**, 367–377.

KOHLHARDT, M. & FICHTNER, H. (1988). Block of single cardiac Na^+ channels by antiarrhythmic drugs: The effect of amiodarone, propafenone and diprafenone. *J. Membr. Biol.*, **102**, 105–119.

MASON, J.W. (1987). Amiodarone. *N. Engl. J. Med.*, **316**, 455–466.

MASON, J.W., HONDEGHEM, L.M. & KATZUNG, B.G. (1983). Amiodarone blocks inactivated cardiac sodium channels. *Pflügers Arch.*, **396**, 79–81.

MASON, J.W., HONDEGHEM, L.M. & KATZUNG, B.G. (1984). Block of inactivated sodium channels and of depolarization-induced automaticity in guinea pig papillary muscle by amiodarone. *Circ. Res.*, **55**, 277–285.

PALLANDI, R.T. & CAMPBELL, T.J. (1987). Resting and rate-dependent depression of $\dot{V}_{max}$ of guinea-pig ventricular action potentials by amiodarone and desethylamiodarone. *Br. J. Pharmacol.*, **92**, 97–103.

POLSTER, P. & BROEKHUYSEN, J. (1976). The adrenergic antagonism of amiodarone. *Biochem. Pharmacol.*, **25**, 131–134.

RAEDER, E.A., PODRID, P.J. & LOWN, B. (1985). Side effects and complications of amiodarone therapy. *Am. Heart J.*, **109**, 975–983.

ROSENBERG, P.J. & HEAVNER, J.E. (1980). Temperature-dependent nerve-blocking action of lidocaine and halothane. *Acta Anaesth. Scand.*, **24**, 314–320.

SAIKAWA, T. & CARMELIET, E. (1982). Slow recovery of the maximum rate of rise ($\dot{V}_{max}$) of the action potential in sheep cardiac Purkinje fibers. *Pflügers Arch.*, **394**, 90–93.

SANCHEZ, V., ARTHUR, G.R. & STRICHARTZ, G.R. (1987). Fundamental properties of local anesthetics. I. The dependence of lidocaine's ionization and octanol: buffer partitioning on solvent and temperature. *Anesth. Analg.*, **66**, 159–165.

SHEETS, M.F., HANCK, D.A. & FOZZARD, H.A. (1988). Nonlinear relation between $\dot{V}_{max}$ and I_{Na} in canine cardiac Purkinje cells. *Circ. Res.*, **63**, 386–398.

SHELDON, R.S., HILL, R.J., CANNON, N.J. & DUFF, H.J. (1989). Amiodarone: biomedical evidence for binding to a receptor for Class I drugs associated with the rat cardiac sodium channel. *Circ. Res.*, **65**, 477–482.

SINGH, B.N. (1989). Amiodarone: electropharmacologic properties. In *Antiarrhythmic Drugs*. ed. Vaughan Williams, E.M. pp. 335–364. Berlin, Heidelberg, New York, London, Paris, Tokyo: Springer-Verlag.

SINGH, B.N. & VAUGHAN WILLIAMS, E.M. (1970). The effect of amiodarone, a new anti-anginal drug, on cardiac muscle. *Br. J. Pharmacol.*, **39**, 657–667.

VARRO, A., NAKAYA, Y., ELHARRAR, V. & SURAWICZ, B. (1985). Use-dependent effects of amiodarone on $\dot{V}_{max}$ in cardiac Purkinje and ventricular muscle fibers. *Eur. J. Pharmacol.*, **112**, 419–422.

VAUGHAN WILLIAMS, E.M. (1984). A classification of antiarrhythmic

actions reassessed after a decade of new drugs. *J. Clin. Pharmacol.*, **24,** 129–147.

YABEK, S.M., KATO, R. & SINGH, B.N. (1986). Effects of amiodarone and its metabolite, desethylamiodarone, on the electrophysiologic properties of isolated cardiac muscle. *J. Cardiovasc. Pharmacol.*, **8,** 197–207.

YABEK, S.M., KATO, R. & SINGH, B.N. (1985). Acute effects of amiodarone on the electrophysiologic properties of isolated neonatal and adult cardiac fibers. *J. Am. Coll. Cardiol.*, **5,** 1109–1115.

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The use of 4-diphenylacetoxy-N-(2-chloroethyl)-piperidine (4-DAMP mustard) for estimating the apparent affinities of some agonists acting at muscarinic receptors in guinea-pig ileum

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1 4-Diphenylacetoxy-*N*-(2-chloroethyl)-piperidine (4-DAMP mustard), which is known to block muscarinic M_3 receptors in preference to muscarinic M_2 receptors, was used to estimate the apparent affinity constants of some agonists acting at muscarinic receptors in guinea-pig ileum. Estimates for carbachol and *n*-pentyl-trimethyl ammonium iodide were similar to published values obtained in similar conditions: those for *n*-hexyl-trimethyl ammonium iodide were slightly lower.

2 The results for the agonists, *n*-pentyl- and *n*-hexyl-trimethyl ammonium iodides and for the partial agonist, *n*-heptyl-trimethyl ammonium iodide were not as regular as was suggested by Stephenson, though there is an overall increase in apparent affinity with chain length.

3 Estimates of apparent affinity may be affected by hexamethonium, usually present in experiments on ileum. Its absence had little effect on the results with carbachol but reduced the estimates obtained with *n*-pentyl trimethyl ammonium, which has strong nicotinic effects compared with its muscarinic effects. On ileum treated with tetrodotoxin the values for *n*-pentyl trimethyl ammonium were similar to those obtained in the presence of hexamethonium (0.28 mM): slightly higher estimates of affinity were obtained in the presence of indomethacin (2.8 μM). The nicotinic effects of *n*-pentyl ammonium may involve the release of prostaglandins.

4 The estimates of apparent affinity did not depend on the method used to calculate them as the 'null' method and the 'operational' method give similar answers. Estimates of the transducer-ratio for the partial agonist, *n*-heptyl-trimethyl ammonium iodide, were numerically the same as those of its efficacy.

5 This work illustrates the use of 4-DAMP mustard as a tool for measuring the apparent affinity of agonists acting at muscarinic M_3 receptors.

Introduction

4-Diphenylacetoxy-*N*-(2-chloroethyl)-piperidine (4-DAMP mustard) hydrochloride reacts with muscarinic receptors and is selective for those in guinea-pig ileum (Barlow *et al.*, 1990). At 30°C the effects are irreversible and at 37°C recovery is extremely slow, so it seemed a suitable compound for trying to measure the affinity of some agonists on guinea-pig isolated ileum and the results were compared with published values using less selective antagonists, such as phenoxybenzamine. The agonists tested were carbachol and *n*-pentyl-, and *n*-hexyl-trimethyl ammonium iodides. Some experiments were done at 30°C as well as at 37°C because tests on atria for the assessment of selectivity are usually done at the lower temperature and the selectivity of 4-DAMP mustard is of particular interest.

n-Pentyl- and *n*-hexyl-trimethyl ammonium iodides were chosen because they were part of the series of alkyltrimethyl ammonium iodides studied by Stephenson (1956). The longer compounds, *n*-heptyl- to *n*-decyl-, were partial agonists and antagonists and it seemed that log affinity increased linearly with chain length. Stephenson (1956) estimated the affinities of the agonists by extrapolation but values were later obtained from kinetic experiments with *n*-hexyl-trimethyl ammonium (Rang, 1966) and by using an irreversible blocking agent with *n*-pentyl-trimethyl ammonium (Abramson *et al.*, 1969). The new compound was used to check these values and fresh experiments were also done with the partial agonist, *n*-heptyl-trimethyl ammonium iodide.

In the experiments of Stephenson (1956) and Rang (1966) the physiological salt solution contained hexamethonium to block the nicotinic effects of the agonists. Hexamethonium, however, has detectable effects at muscarinic receptors (Barlow *et al.*, 1972; Eglen *et al.*, 1989) and an attempt was made to see if it could be omitted.

The results might also be affected by the method used to calculate them. All methods involve obtaining control dose-response curves and dose-response curves in the presence of the blocking agent (or with the partial agonist). With the 'null' method (Furchgott, 1966; Mackay, 1966) these are used to calculate the concentrations, A (for the control) and A′ (after block), which produce the same response. These can then be fitted to a rectangular hyperbola (Parker & Waud, 1971). With the 'operational' model (Black & Leff, 1983) it is possible to write an equation relating response to dose for the two curves (see below). From a comparison of estimates of the affinity of 5-hydroxytryptamine for receptors on rabbit aorta calculated by the 'operational model' and the 'null method', Black *et al.* (1985) concluded that 'the model provided an estimate of K_A which was not significantly different from the estimate obtained using Furchgott's null method'. But although the mean estimates of log K were very similar (6.95 and 6.81), the value obtained by the null method was lower in each of the 9 pairs of estimates reported, which suggested a systematic difference (1 in 2^9 gives $P < 0.002$). It therefore seemed worth comparing the results obtained by the two methods with fresh data.

The aims were to try to obtain answers to the following questions:

(1) Are the results with this blocking agent similar to those by other workers (e.g. Furchgott & Bursztyn, 1967)?

(2) Does log affinity for the agonists in the alkyl-trimethyl ammonium series increase linearly with chain length, as assumed by Stephenson?

(3) Why are the results with some compounds affected by the presence or absence of hexamethonium?

(4) Does the method of calculation (null method or operational model) affect the estimate?

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Methods

All preparations were obtained from male Dunkin-Hartley guinea-pigs with weights ranging from 500–700 g.

Guinea-pig isolated ileum

The guinea-pig ileum was prepared as described by Edinburgh Staff (1974), but each piece of ileum was cut longitudinally to produce two separate preparations (longitudinal sheets). These were set up in Krebs solution, aerated with a mixture of 95% O_2 and 5% CO_2; the responses were recorded isotonically and a load of about 0.5 g was used. In most experiments the Krebs solution contained hexamethonium, 0.28 mM but experiments were also done with hexamethonium, 0.14 mM, with hexamethonium omitted, in the presence of indomethacin, 2.8 μM, and in the presence of tetrodotoxin (0.1 μM: after initial exposure to 1.0 μM for 30 min).

The agonists were allowed to act for 30 s and added once every 90 s by relays controlled from a PET microcomputer. Experiments were done at 29.8 ± 0.3°C and 37.0 ± 0.1°C.

In early work the control responses of the ileum were obtained with at least 4 concentrations of agonist which covered the dose-response curve. In further work, 5 concentrations were used with a fixed dose of agonist interpolated between experimental doses, to reduce the influence of the preceding dose on the response (Barlow *et al.*, 1963). The responses to the interpolated doses were not used in fitting the dose-response curve.

When the tissue appeared to be producing reproducible contractions it was exposed to the mustard (usually 10 nM for at least 1 h) and as the block developed the concentrations of agonist were increased so as to obtain the dose-response curve in the presence of the antagonist. When this started to flatten, the mustard was replaced by ordinary Krebs solution. Recovery was very slow indeed, allowing time to obtain stable responses to a range of concentrations of the agonist. An example is shown in Figure 1.

The control responses (Y) were fitted by least-squares to the concentration of agonist, A, using the logistic expression:

$$Y = M\frac{A^P}{A^P + A_{50}{}^P}$$

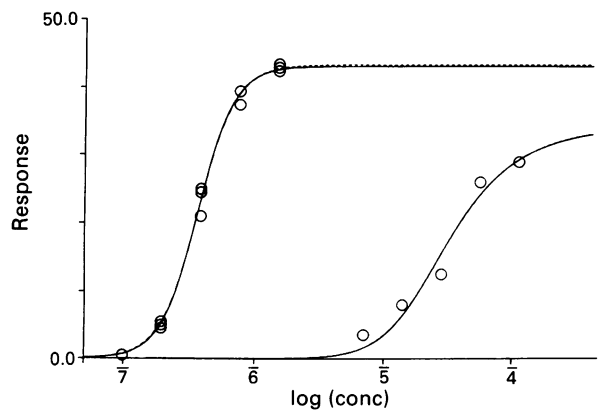

Figure 1 Graphs of response, in arbitrary units, and log dose for carbachol before and after exposure to 4-diphenylacetoxy-*N*-(2-chloroethyl)-piperidine (4-DAMP mustard). The Krebs solution contained hexamethonium (0.28 mM) and responses (not included in the graph) were obtained with 4 μM carbachol (before block) and 30 μM carbachol (after block) between each dose. The temperature was 37°C. The full lines represent a least-squares fit of the controls to the logistic equation (with M = 43.2, A_{50} = 3.81, P = 3.09) and the fit of the responses after block to the operational model having the same values of M and P as the controls and with K_d = 262, τ = 1.55 (method B1). The dotted lines are the least-squares fit to the operational model in which all five parameters are calculated from the data for both curves (method B2) and have the values M = 43.5, P = 3.06, K_d = 264, $\tau 1$ = 70.1, $\tau 2$ = 1.55. Note that the lines are almost identical.

where M is the maximum response, A_{50} is the concentration producing a half-maximum response, and the exponent P determines the steepness of the curve (if P = 1 the expression is a rectangular hyperbola). The fitting procedures have been described by Parker & Waud (1971) and Barlow (1983).

With the null method the control dose-response curve was used to calculate matching concentrations, A and A', producing the same response before and after treatment with the mustard, respectively. If K_a is the agonist affinity constant and z is the proportion of receptors blocked irreversibily,

$$\frac{AK_a}{1 + AK_a} = (1 - z)\frac{A'K_a}{1 + A'K_a}$$

and if K_d is the agonist dissociation constant ($1/K_a$) this can be written:

$$A = \frac{(1 - z)}{z}\frac{K_d A'}{K_d/z + A'}$$

so the graph of A against A' should be a hyperbola with a maximum, $M_h = K_d(1 - z)/z$ and the value of A' corresponding to half the maximum, $A'_{50} = K_d/z$. An example is shown in Figure 2. If the agonist has high efficacy and it is necessary to block more than 90% of the receptors ($z > 0.9$) before the dose-response curve flattens, A'_{50} is approximately the same as K_d: the exact value is obtained by subtracting M_h.

The affinity can also be measured from the same data using the 'operational' model of Black & Leff (1983). With this the response

$$Y = \frac{M\tau^P A^P}{(K_d + A)^P + \tau^P A^P}$$

where M is the maximum response obtainable from the tissue, τ is the 'transducer-ratio', K_d is the agonist dissociation constant (as above) and P is an exponent (if P = 1 the relation is a hyperbola). With the irreversible blocking agent estimates of τ and K_d can be obtained by fitting values of Y (response) by least-squares to the agonist concentration (A), assuming that M and P are the same as with the controls. This method

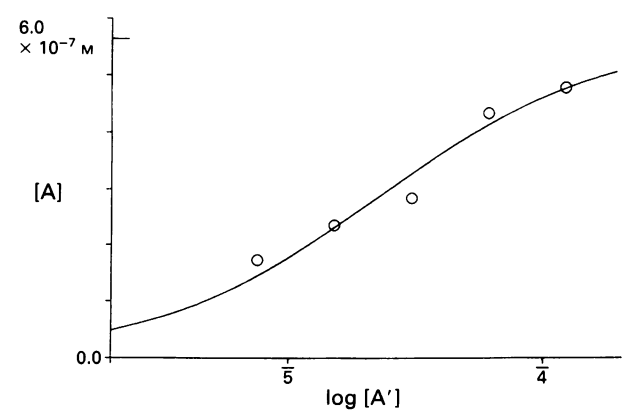

Figure 2 Calculation of affinity by the null method for the dose-response curves in Figure 1. The responses in the absence of the antagonist were used to calculate the concentration of agonist, A ($\times 10^{-7}$ M), which would produce the same response as a concentration A' of agonist after block: A' is plotted on a logarithmic scale. The line shows the least-squares fit of values of A to values of A' according to the hyperbola $A = M\ A'/(A'_{50} + A')$. The maximum response achieved after block matched the response to 5.64 × 10^{-7} M carbachol on the control curve and this concentration is marked on the Y-axis: A'_{50} is 216 × 10^{-7} M so the dissociation constant of the agonist, K_d is $A'_{50} - M$ = 21 μM.

(referred to as 'B1') can also be used for data obtained with a partial agonist. Alternatively, the values for both dose-response curves (before and after block) obtained with a full agonist may be used to calculate the common values of M, K_d and P as well as the individual value of τ for each curve. This procedure (referred to as 'B2') was followed by Black *et al.* (1985) and in the later part of this work the experimental data have been analysed in both ways, as well as by the null method. As can be seen in Figure 1 there is usually little difference between the fit obtained with 'B1' and 'B2'. Copies of the programme used (for Amiga or IBM machines) will be supplied on request.

Compounds

The preparation of 4-diphenylacetoxy-*N*-(2-chloroethyl)-piperidine (4-DAMP mustard) hydrochloride and evidence for its irreversible blockade of muscarinic receptors in guinea-pig ileum has been described by Barlow *et al.* (1990). Acetylcholine iodide, carbachol chloride, hexamethonium bromide, tetrodotoxin and indomethacin were obtained from Sigma. The sample of *n*-pentyl-trimethyl ammonium iodide had m.p. 226–228°C and had been used in earlier work (Edinburgh Staff, 1974).

n-Hexyl- and *n*-heptyl-trimethyl ammonium iodides were prepared from iodo-*n*-hexane and iodo-*n*-heptane (obtained from Aldrich) and trimethylamine in ethanol. The products were recrystallized from mixtures of butanone and ethanol and had m.p. 162.8–163.2°C and 139.5–141°C, respectively, compared with 159–160°C and 138°C for the material used by Stephenson (1956).

Results

The results of the first group of experiments are shown in Table 1. There was a difference of an order of magnitude between the results obtained with *n*-pentyl-trimethyl ammonium in the presence and in the absence of hexamethonium. The value in the presence of hexamethonium (0.28 mM) at 37°C (log K = 3.63) is comparable with that obtained by Abramson *et al.* (1969: log K = 3.73) and slightly less than the value calculated by extrapolating from the results with higher homologues (Stephenson 1956: log K = 3.93). The results for *n*-hexyl-trimethyl ammonium (log K = 3.56) are appreciably lower than the value (log K = 4.28) extrapolated from Stehenson's data, and very much lower than the value (log K = 4.63) calculated by Rang (1966). As *n*-heptyl-trimethyl ammonium is a partial agonist it was not necessary to use 4-DAMP mustard to measure its affinity. The value (log K = 4.63) is very close to the value (log K = 4.61) obtained by Stephenson (1956) and slightly less than the value (log K = 4.80) obtained by Rang (1966). The experiments with this compound were also used to calculate the affinity using the operational model ('B1') and the results are almost identical with those obtained with the 'null' method (Table 1): the values of mean log K ($\pm$ s.e.) were 4.65 $\pm$ 0.10 (3) at 30°C and 4.63 $\pm$ 0.07 (4) at 37°C.

Table 1 Log affinity constant (or $-\log K_d$), calculated by the null method, $\pm$ s.e. (*n* results): experiments were carried out in the presence of hexamethonium bromide (0.28 mM) except where indicated

30°C	*37°C*
Pentyl-trimethyl ammonium iodide (no hexamethonium)	
2.65 $\pm$ 0.05 (3)	2.51 $\pm$ 0.05 (4)
Pentyl-trimethyl ammonium iodide	
4.00 $\pm$ 0.19 (4)	3.63 $\pm$ 0.06 (2)
Hexyl-trimethyl ammonium iodide	
4.26 $\pm$ 0.02 (3)	3.56 $\pm$ 0.18 (4)
Heptyl-trimethyl ammonium iodide	
4.67 $\pm$ 0.10 (3)	4.63 $\pm$ 0.07 (4)

Table 2 summarizes the results obtained in the second part of the work (done many months later) which was concerned with checking the effects of hexamethonium and seeing if the method of data analysis affected the result by making a direct comparison of the estimates obtained with the same data analysed in three ways.

The apparent affinity of carbachol was not greatly affected by hexamethonium. The value at 37°C (log K = 4.45) is slightly lower than that obtained by Furchgott & Bursztyn (1967: log K = 4.78) and considerably lower than that obtained by Grana *et al.* (1987: log K = 5.32). Hexamethonium had marked effects, however, on the results obtained with *n*-pentyl trimethyl ammonium. If these involved the known nicotine-like properties of this compound leading to the release of a substance which contracted smooth muscle, this might cause the tissue to contract even when all the muscarinic receptors had been blocked. The log dose-response curve would not flatten when it should and the affinity would appear too low (the efficacy would be over-estimated).

To test this idea the preparation was treated with tetrodotoxin and the estimates of affinity were found to be similar to those obtained in the presence of hexamethonium (0.28 mM). In the presence of indomethacin (2.8 μM) the estimates were slightly higher. A two-factor analysis of variance of the results in Table 2 in which the same data were analysed by three different methods failed to show any difference between them (F = 0.33 compared with 4.26 for significance at the 5% level: $P > 0.1$ using a non-parametric test). There was, however, a highly significant difference (F = 46.4: $P < 0.001$ using a nonparametric test) between the results obtained in different experimental conditions: the estimates in the presence of indomethacin were different from those obtained in the presence of hexamethonium or tetrodotoxin.

From Table 1 it can be seen that the apparent affinities of *n*-pentyl- and *n*-hexyl-trimethylammonium were higher at 30°C whereas that of *n*-heptyl-trimethyl ammonium was little different as was the affinity of carbachol, for which the estimates were 4.55 $\pm$ 0.34 (3) at 30°C, and 4.48 $\pm$ 0.11 (4) at 37°C.

Table 2 Effects of hexamethonium and method of calculation on estimates of log affinity constant (or $-\log K_d$) $\pm$ s.e. (*n* results) for *n*-pentyl-trimethyl ammonium and carbachol

	N	*B1*	*B2*
Pentyl-trimethyl ammonium iodide			
+ Hex (0.14 mM)	3.66 $\pm$ 0.05 (8)		
+ Hex (0.28 mM)	4.07 $\pm$ 0.09	4.09 $\pm$ 0.07	3.99 $\pm$ 0.14 (4)
+ indomethacin (2.8 μM)	4.63 $\pm$ 0.06	4.59 $\pm$ 0.07	4.61 $\pm$ 0.06 (8)
+ tetrodotoxin (0.1 μM*)	4.20 $\pm$ 0.06	4.12 $\pm$ 0.05	4.14 $\pm$ 0.07 (5)
Carbachol (no hexamethonium)	4.40 $\pm$ 0.09	4.48 $\pm$ 0.07 (5)	
+ Hex (0.28 mM)	4.48 $\pm$ 0.11	4.42 $\pm$ 0.09 (4)	

N indicates results calculated by the null method, B1 indicates results calculated with the operational model using M and P taken from the controls, B2 indicates results calculated by allowing these to vary. The asterisk indicates initial exposure to 1 μM tetrodotoxin for 30 min. Hex = hexamethonium.

Discussion

The term 'apparent affinity' has been used to describe what is being measured in these experiments with agonists acting on tissues which have been treated with an irreversible blocking agent, because the binding mechanism for agonists is not clear. The uncertainty about what estimates of the affinity of an agonist actually measure was noted by Colquhoun (1973) and as more has become known about the stages between agonist-receptor interaction and the tissue response and more detailed schemes have been proposed (e.g. Mackay, 1990), it has appeared increasingly unlikely that the apparent affinity measured in this type of experiment (with an irreversible blocking agent) involves only a single equilibrium process. The work with 4-DAMP mustard does not help to explain what is going on but the finding that, with suitable precautions, it gives results similar to those obtained with other irreversible blocking agents is important because it justifies using the compound to identify muscarinic M_3 receptors. It may be particularly valuable in binding experiments with membrane fragments or purified receptor preparations.

The results obtained with the alkyl-trimethyl ammonium salts are illustrated in Figure 3, which includes the values for the antagonists and partial agonists obtained by Stephenson (1956) and shows his extrapolation to agonists. There is an overall trend to increase affinity with chain length, though the results are not as regular as suggested by the extrapolation, with values for *n*-hexyl trimethyl ammonium being less than expected.

If there are regular increases in log affinity there must be regular increases in the free energy of binding which must be the result of regular changes in the enthalpy and entropy of binding. The results obtained in this work suggest that the effects of temperature on affinity are different for *n*-heptyl-trimethyl ammonium from those for *n*-pentyl- and *n*-hexyl-trimethyl ammonium, so the changes in the enthalpy of binding are not regular and regular increases in log affinity are unlikely. The binding of these compounds to acetylcholinesterase certainly shows discontinuities (Belleau *et al.*, 1965).

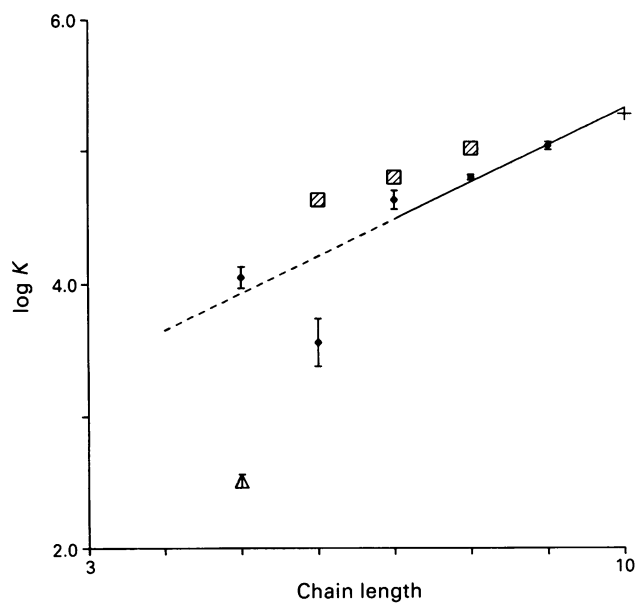

Figure 3 The affinity of *n*-alkyl-trimethyl ammonium iodides. The affinity constant is plotted on a logarithmic scale against chain length. The results in this work are shown together with those of Stephenson (1956) for the *n*-octyl-, nonyl- and decyl- compounds, which appear to lie on a straight line. The hatched squares indicate results obtained by Rang (1966) in the presence of hexamethonium (0.55 mM).

Note (i) the effect of hexamethonium on the values for *n*-pentyl-trimethyl ammonium: the triangle shows results obtained in the absence of hexamethonium; (ii) the result for *n*-pentyl-trimethyl ammonium in the presence of hexamethonium (0.28 mM: Table 2) is close to the value obtained by extrapolating the line drawn by Stephenson (1956). Pooling all the results for *n*-pentyl-trimethyl ammonium in the presence of hexamethonium (0.28 mM) or tetrodotoxin gives an almost identical mean and standard error but it is doubtful whether the relation between chain length and affinity is really as regular as the line implies.

The results provide some explanation for the effects of hexamethonium on the measurement of the apparent affinity of an agonist. Hexamethonium has weak atropine-like activity (Barlow *et al.*, 1972: Eglen *et al.*, 1989) but in experiments with 13 antagonists on guinea-pig ileum and carbachol as agonist there was, on average, no effect of hexamethonium (0.28 mM) on the estimate of affinity. With the concentration (0.55 mM) used by Rang (1966) the expected dose-ratio at muscarinic receptors is about 2, so estimates of affinity should be slightly over-estimated. At nicotinic receptors in guinea-pig ileum, 0.28 mM hexamethonium should produce a dose-ratio of around 74 (Barlow & Franks, 1971: Barlow *et al.*, 1974).

The importance of the hexamethonium should depend on the nicotinic activity of the agonist, relative to its muscarinic activity. Carbachol affects guinea-pig ileum in concentrations around 10^{-7} M and affects the frog rectus preparation in concentrations around 8×10^{-6} M (Edinburgh Staff, 1974), whereas with *n*-pentyl-trimethyl ammonium the respective concentrations are 10^{-6} M and 2×10^{-5} M. A muscarinic antagonist could therefore produce dose-ratios of 80 before the concentrations of carbachol might be big enough to produce nicotinic effects whereas with *n*-pentyl-trimethyl ammonium these should occur with dose-ratios above 20. Even if the concentrations active on the frog rectus are only a rough guide to effects on nicotinic receptors in the ileum, this could explain why the results obtained with *n*-pentyl-trimethyl ammonium (Figure 4) are affected by hexamethonium much more than those obtained with carbachol (Figure 1). The results obtained in the presence of tetrodotoxin confirm that stimulation of the nicotinic receptors leads to the neuronal release of a substance which causes the ileum to contract, so the tissue responds even though the muscarinic receptors are blocked and the experiments with indomethacin suggest that this substance might be prostaglandin.

The estimates of apparent affinity are not greatly affected by the way they are calculated. In Figure 5a and b the estimates are shown plotted against each other (B1 *vs* null method, B2 *vs* B1). There is a close correlation with the slopes not significantly different from 1 ($P < 0.01$ using both parametric and nonparametric tests). We have not found that the 'null'

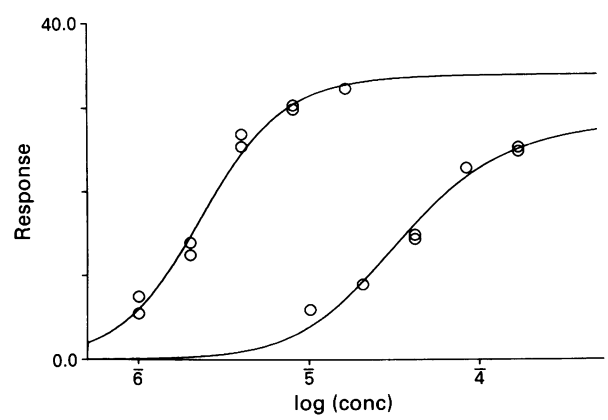

Figure 4 Graphs of response, in arbitrary units, and log dose for *n*-pentyl-trimethyl ammonium before and after exposure to 4-diphenylacetoxy-*N*-(2-chloroethyl)-piperidine (4-DAMP mustard). The Krebs solution contained tetrodotoxin (0.1 μM) and the preparation was treated initially with tetrodotoxin (1.0 μM) for 30 min. The temperature was 37°C. The lines represent a least-squares fit using the operational model (B2). Note that the treatment with 4-DAMP mustard is producing dose-ratios greater than 20.

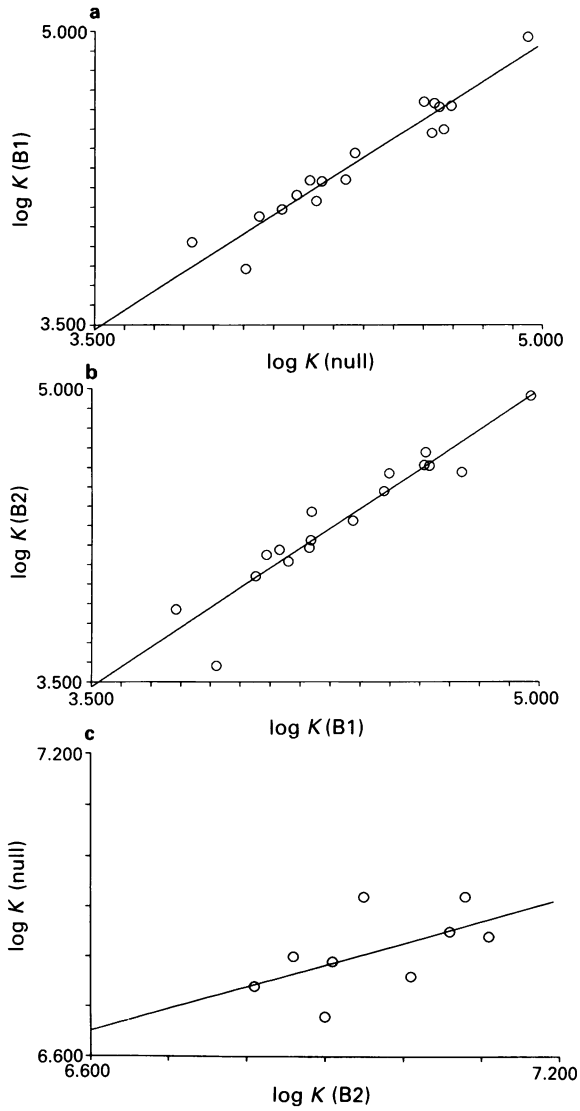

Figure 5 (a) Estimates of log K for n-pentyl-trimethyl ammonium iodide (Table 2) with the value obtained by the method B1 plotted against that obtained by the null method. The slope of the line is 0.98 (s.e. 0.08, 15 d.f.) (b) Estimates of log K with the value obtained by the method B2 plotted against that obtained by method B1. The slope of the line is 1.01 (s.e. 0.09, 15 d.f.). (c) Results (Black *et al.*, 1985) for 5-hydroxytryptamine on rabbit aorta with the value of log K obtained by the null method plotted against that obtained by the operational model (B2). The slope of the line is 0.44 (s.e. 0.24, 7 d.f.).

method gives systematically lower estimates of affinity (Figure 5c) as was observed by Black *et al.* (1985).

A comparison can be made of estimates of the 'transducer-ratio' and of 'efficacy'. With a potent agonist it is very difficult to estimate the 'transducer-ratio' with any precision. Black *et al.* (1985) have shown that the maximum for the agonist ('asymptote parameter') = $M\tau^P/(1 + \tau^P)$, where M is the maximum for the tissue. An agonist with $\tau = 9$ should produce 90% of the tissue maximum if $p = 1$ and over 98% if $p = 2$ (as is more usual in our experiments). With potent agonists the estimates of the transducer ratio (calculated by 'B2') have large standard errors calculated from the within-experiment variance and vary greatly. With n-pentyl trimethyl ammonium iodide the range in the experiments with hexamethonium was from 8–50, in the experiments with tetrodotoxin it was from 19–43 and in the experiments with indomethacin it was from 30–700.

There should be less variation when the value is smaller and with the partial agonist, n-heptyl-trimethyl ammonium iodide the range was 0.7–0.9 at 30°C and 0.4–1.1 at 37°C. The dose-response curves were also used to calculate the efficacy. Stephenson (1956) defined one unit of stimulus as that which produced a half-maximum response, which will be that produced by the concentration A_{50} in the logistic fit of responses to carbachol. In most experiments with the partial agonist the maximum was less than this but greater than that produced by $A_{50}/4$, which should produce a stimulus of 0.25. The concentration, P*, of partial agonist which produces the same response as $A_{50}/4$ (so S = 0.25) was calculated from the hyperbola relating the doses of agonist and partial agonist which produce the same response, $A = M_h P/(P_{50} + P)$, where P_{50} is the concentration which produces half M_h (and is the dissociation constant for the partial agonist or $1/K_P$). So for the partial agonist, $S = e_P P^* K_P/(1 + p^* K_P) = 0.25$ and e_P can be calculated. For the results with n-heptyl-trimethyl ammonium the individual values of efficacy, with the estimate of the transducer-ratio shown in parentheses, were: 0.46 (0.46), 0.40 (0.40), 1.06 (1.07) and 0.77 (0.77) at 37°C and 0.72 (0.72), 0.71 (0.71) and 0.95 (0.94) at 30°C. The transducer-ratio and the efficacy are numerically identical. Perhaps the reason for this and also why the 'null' and 'operational' methods give similar estimates of the apparent affinity is because the mathematical operations involved are similar.

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Note: Samples of 4-DAMP mustard may be obtained from the authors.

References

ABRAMSON, F.B., BARLOW, R.B., MUSTAFA, M.G. & STEPHENSON, R.P. (1969). Relationship between chemical structure and affinity for acetylcholine receptors. *Br. J. Pharmacol.*, **37**, 207–233.

BARLOW, R.B. (1983). *Biodata Handling with Microcomputers.* pp. 124–133. Cambridge: Elsevier Biosoft.

BARLOW, R.B., BOWMAN, F., ISON, R.R. & McQUEEN, D.S. (1974). The specificity of some agonists and antagonists for nicotine-sensitive receptors in ganglia. *Br. J. Pharmacol.*, **51**, 585–597.

BARLOW, R.B. & FRANKS, F.M. (1971). Specificity of some ganglion stimulants. *Br. J. Pharmacol.*, **42**, 137–142.

BARLOW, R.B., FRANKS, F.M. & PEARSON, J.D.M. (1972). A comparison of the affinities of antagonists for acetylcholine receptors in the ileum, bronchial muscle and iris of the guinea-pig. *Br. J. Pharmacol.*, **46**, 300–312.

BARLOW, R.B., SCOTT, K.A. & STEPHENSON, R.P. (1963). An attempt to study the effects of chemical structure on the affinity and efficacy of compounds related to acetylcholine. *Br. J. Pharmacol. Chemother.*, **21**, 509–522.

BARLOW, R.B., SHEPHERD, M.K. & VEALE, M.A. (1990). Some differential effects of 4-diphenylacetoxy-*N*-(2-chloroethyl)-piperidine hydrochloride on guinea-pig atria and ileum. *J. Pharm. Pharmacol.*, **42**, 412–418.

BELLEAU, B., TANI, H. & LIE, F. (1965). A correlation between the biological activity of alkyl trimethylammonium ions and their mode of interaction with acetylcholinesterase. *J. Am. Chem. Soc.*, **87**, 2283–2285.

BLACK, J.M. & LEFF, P. (1983). Operational models of pharmacological agonism. *Proc. R. Soc.* B, **220**, 141–162.

BLACK, J.M., LEFF, P. & SHANKLEY, N.P., with an appendix by Wood, J. (1985). An operational model of pharmacological agonism: the effect of E/[A] curve shape on agonist dissociation constant estimation. *Br. J. Pharmacol.*, **84**, 561–571.

COLQUHOUN, D. (1973). The relation between classical and cooperative models for drug action. In *Drug Receptors* ed. Rang, H.P. pp. 149–182. London: Macmillan.

EDINBURGH STAFF (1974). *Pharmacological Experiments on Isolated Preparations.* 2nd edition. pp. 2, 44, 58–62 & 71. Edinburgh: Churchill Livingstone.

EGLEN, R.M., MICHEL, A.D. & WHITING, R.L. (1989). Characterization of the muscarinic receptor subtype mediating contractions of the

guinea-pig uterus. *Br. J. Pharmacol.*, **96,** 497–499.

FURCHGOTT, R.F. (1966). The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes. *Adv. Drug Res.*, **3,** 21–55.

FURCHGOTT, R.F. & BURSZTYN, P. (1967). Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. *Ann. N.Y. Acad. Sci.*, **144,** 882–899.

GRANA, E., LUCCHELLI, A., ZONTA, F. & BOSELLI, C. (1987). Determination of dissociation constants and relative efficacies of some potent muscarinic agonists at postjunctional muscarinic receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **335,** 8–11.

MACKAY, D. (1966). A new method for the analysis of drug-receptor interactions. *Adv. Drug Res.*, **3,** 1–19.

MACKAY, D. (1990). Agonist potency and apparent affinity, interpretation using classical and steady-state models. *Trends Pharmacol. Sci.*, **11,** 17–22.

PARKER, R.B. & WAUD, D.R. (1971). Pharmacological estimation of drug-receptor dissociation constants. Statistical evaluation. I. Agonists. *J. Pharmacol. Exp. Ther.*, **177,** 1–12.

RANG, H.P. (1966). The kinetics of action of acetylcholine antagonists in smooth muscle. *Proc. R. Soc.*, **B, 164,** 488–510.

STEPHENSON, R.P. (1956). A modification of receptor theory. *Br. J. Pharmacol. Chemother.*, **11,** 373–393.

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Pharmacokinetic-pharmacodynamic modelling of the EEG effects of midazolam in individual rats: influence of rate and route of administration

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1 The purpose of the present investigation was to quantify the concentration-pharmacological effect relationship of midazolam in individual rats by use of effect parameters derived from aperiodic EEG analysis. By varying the rate and route of administration the role of (inter)active metabolites and development of acute tolerance was evaluated.

2 The pharmacokinetics and pharmacodynamics of midazolam were determined after intravenous administration of 10 mg kg^{-1} during 5, 30 and 60 min and oral administration of 15 mg kg^{-1}. Following intravenous administration the pharmacokinetics were most adequately described by a bi-exponential equation. The values (mean ± s.e.mean, $n = 20$) of clearance, volume of distribution at steady-state and terminal half-life were 67 ± 2 ml min^{-1} kg^{-1}, 1.61 ± 0.07 l kg^{-1} and 27 ± 1 min, respectively. Following oral administration midazolam was rapidly absorbed with a systemic availability of 45 ± 9%.

3 The averaged amplitudes in the 11.5–30 Hz (beta) frequency band of the fronto-central lead on the left-hemisphere, as derived by aperiodic EEG analysis, was selected as a measure of the pharmacological effect of midazolam. By pharmacokinetic-pharmacodynamic modelling the individual concentration-EEG effect relationships of midazolam were derived, which were successfully quantified by the sigmoidal E_{max} model. No marked and systematic differences in pharmacodynamic parameters were found between the rates and routes of administration. The averaged pharmacodynamic parameters of midazolam obtained after combining the results of all rates and routes of administration were (mean ± s.e.mean, $n = 27$): Eo = 61 ± 3 μV s^{-1}, E_{max} = 85 ± 3 μV s^{-1}, EC_{50} = 40 ± 3 ng ml^{-1} and N = 0.84 ± 0.04.

4 The results of the present study show that the concentration-EEG effect relationship of midazolam can be characterized in individual animals using the amplitudes in the 11.5–30 (beta) frequency band as a measure of pharmacological response. Acute tolerance did not develop and (inter)active metabolites did not contribute to this effect parameter within the time span of the experiments.

Introduction

The availability of a quantitative measure of the drug response is a first prerequisite to study the kinetics of drug action *in vivo*. Such effect parameters are not readily available, which may explain the limited data on concentration-effect relationships of benzodiazepines, especially with regard to the effect of various (patho)physiological factors (Dingemanse *et al.*, 1988a). Quantitative EEG parameters have proven to be valuable measures of the central nervous system (CNS) effects of benzodiazepines (Fink *et al.*, 1976; Kurowski *et al.*, 1982; Laurian *et al.*, 1984; Saletu *et al.*, 1986) and recently, in man, the relationships between plasma concentrations and EEG effects of midazolam and diazepam have been quantified (Koopmans *et al.*, 1988; Greenblatt *et al.*, 1989; Breimer *et al.*, 1990). EEG parameters conform to most of the criteria of 'ideal' pharmacodynamic measures (Dingemanse *et al.*, 1988a): continuity, sensitivity, objectivity and reproducibility, allowing in principle the characterization of the complete concentration-effect relationship in individual subjects.

In kinetic-dynamic modelling it is important to establish the uniqueness of the derived concentration-effect relationship. Several potentially confounding pharmacokinetic factors, such as distribution to the site of action and formation of (inter)active metabolites, and pharmacodynamic factors, such as development of (acute) tolerance towards the measured effect, have to be considered (Dingemanse *et al.*, 1988a). The influences of these factors are difficult to assess when only one mode of drug administration is studied.

The purpose of the present investigation was to quantify the concentration-EEG effect relationship of midazolam in individual rats by use of effect parameters derived from aperiodic EEG analysis. By varying the rate and route of drug administration the role of (inter)active metabolites and development of acute tolerance was evaluated.

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Methods

Animals

Male SPF rats of Wistar descent (200–250 g) were used throughout the study. The animals were housed individually in plastic cages with a normal 12-h light-dark cycle and fed on laboratory chow (Standard Laboratory Rat, Mouse and Hamster Diets, RMH-TM, Hope Farms, Woerden, The Netherlands) and water *ad libitum*. From the night before the experiment onwards the animals were deprived of food but had free access to water.

Pharmacokinetic-pharmacodynamic experiments

To measure the EEG signals, EEG electrodes were implanted into the skull of the animals one week before the kinetic-dynamic experiment as described previously (Mandema & Danhof, 1990). Briefly, the electrodes were placed at the locations 11 mm anterior and ±2.5 mm lateral (F_l and F_r), 3 mm anterior and ±3.5 mm lateral (C_l and C_r) and 3 mm posterior and ±2.5 mm lateral (O_l and O_r) to lambda. A reference electrode was placed on lambda. Stainless steel screws were used as electrodes and connected to a miniature connector. The whole assembly was insulated and fixed to the skull with dental acrylic cement. Indwelling cannulae were implanted in the right jugular vein and right femoral artery under light ether anaesthesia one day before the experiment. During the experiment the animals were kept in a restraining cage.

Rats (27) were randomly allocated to four groups that received 10 mg kg^{-1} midazolam intravenously during 5, 30 or 60 min or 15 mg kg^{-1} of the drug orally. Midazolam was dissolved in 0.9% saline with the aid of an equimolar quantity of hydrochloric acid. A total volume of 0.75 ml was administered intravenously and 0.5 ml orally. To determine the pharmacokinetics of midazolam arterial blood samples of 100 μl, or 200 μl near the end of the experiment, were collected at fixed time intervals after drug administration over a period of 240 to 360 min, depending on the rate and route of administration. Heparinized blood samples were centrifuged and plasma was separated and stored at −35°C until the time of analysis. Bipolar EEG leads were continuously measured during the time course of the experiment using a System 50000 EEG recorder (Van Gogh BV, Amsterdam, The Netherlands). The low-pass filter was set at 75 Hz, the time constant at 0.3 s. The EEG recordings were started 10 to 15 min before drug administration. Two EEG leads (F_l–C_l and C_l–O_l) were subjected to on-line aperiodic analysis for quantification, using the Lifescan EEG monitor (Neurometrics Inc., San Diego, U.S.A.). Aperiodic analysis was recently introduced as a new technique to quantify brain electrical activity (Gregory & Pettus, 1986). The aperiodic analysis algorithm calculates the amplitude and period of each EEG signal on a wave by wave basis. The analysed EEG data are then sampled into an IBM-AT computer and stored on magnetic diskette as the frequency in Hz, amplitude in μV, time of occurrence and artifact status of each wave. From these data the average amplitudes (μV s^{-1}) in the following frequency bands: 0.5–2.5 Hz (delta), 2.5–7.5 Hz (theta), 7.5–11.5 Hz (alpha) and 11.5–30 Hz (beta) were calculated and studied as descriptors of the drug effect. A total of 50 data points per animal were used as effect measures in the modelling procedure. The data points were calculated by averaging 1 min or more of consecutive EEG data. EEG effect measures were taken more often while the concentration of midazolam was changing rapidly, i.e. during the infusion and shortly thereafter.

Drug analysis

A sensitive assay was developed to measure midazolam concentrations in small plasma samples of 50 or 100 μl by high performance liquid chromatography (h.p.l.c.) with u.v. detection. A solution of 25 ng diazepam (internal standard) in 20 μl methanol was added to a 50 or 100 μl plasma sample which had been diluted with 0.5 ml 0.1 N NaOH solution. The mixture was extracted with 5 ml dichloromethane-pentane (1:1) for 30 s on a vortex mixer. After centrifugation the organic layer was separated and evaporated to dryness under reduced pressure. The residue was reconstituted with 150 μl of the mobile phase and 75 μl was injected into the chromatographic system. The chromatographic system consisted of a M-45 solvent pump (Waters, Milford, MA, USA), a Promis automatic sample injector (Kratos Analytical Instruments, Ramsey, USA), a Z-module containing a Radial-Pak C-18 column (Waters) and a spectroflow 757 UV detector (Kratos) set at 222 nm. The mobile phase consisted of a mixture of 0.01 M phosphate buffer (pH = 5.0) and acetonitrile (Rathburn) in a ratio 50/50 with a flow rate of 1.0 ml min^{-1}. Retention times were 4.4 and 5.0 min for diazepam and midazolam respectively. Data processing was performed using a Shimadzu C-R3A integrator. Coefficients of variation for identical samples were less than 5% (n = 5) with respect to intra- and inter-assay precision in the range 20–10000 ng ml^{-1}. The detection limit was approximately 10 ng ml^{-1} with a 100 μl sample.

Data analysis

The pharmacokinetics and pharmacodynamics of midazolam were quantified for the individual rats. The plasma concentration-time profiles of midazolam after intravenous infusion (eq. 1) and oral administration (eq. 2) were described by a poly-exponential equation:

$$C(t) = \sum_{i=1}^{n} A_i(e^{-\alpha_i(t-T)_+} - e^{-\alpha_i t}) \quad (1)$$

where $(t - T)_+$ is defined by:

$$(t - T)_+ \equiv \begin{cases} t - T \text{ for } t > T \\ 0 \text{ otherwise} \end{cases}$$

and

$$C(t) = \sum_{i=1}^{n} A_i(e^{-\alpha_i t}) \quad (2)$$

where C(t) is the plasma concentration of midazolam at time t, T is the infusion duration and A_i and α_i are respectively the coefficients and exponents of the equation. Different exponential models were investigated and the most suitable model was chosen according to standard criteria. Basic pharmacokinetic parameters as area under the curve (AUC), total clearance (Cl), volume of distribution at steady-state (Vdss) and terminal half-life ($t_{1/2}$, α_n) were calculated from the coefficients and exponents of the fitted functions according to standard procedures (Gibaldi & Perrier, 1982). The systemic availability (F) after oral administration was calculated from the averaged AUCs after oral and three rates of intravenous administration.

The sigmoidal E_{max} model was selected to describe the relationship between midazolam concentrations and EEG effect:

$$E(C) = Eo + \frac{E_{max} C^N}{EC_{50}{}^N + C^N} \quad (3)$$

where E is the observed EEG effect at plasma concentration C of midazolam, Eo is the baseline EEG value, E_{max} the maximal effect, EC_{50} the plasma concentration at half maximal effect and N is a constant expressing the sigmoidicity of the concentration-effect relationship. The pharmacokinetic model was used to calculate the concentrations of midazolam at the time points of effect measurement. The increase in average amplitude in the 11.5–30 Hz (beta) frequency band (F_l–C_l lead) was used as measure of the pharmacological effect of midazolam.

The equations were fitted to the data by the nonlinear least squares regression programme Siphar (Siphar pharmacokinetic modelling software package, SIMED S.A., Creteil, France). The pharmacokinetic and pharmacodynamic parameters obtained after oral and three rates of intravenous administration were statistically compared by a parametric one-way analysis of variance (ANOVA) or a nonparametric Kruskal-Wallis test if more appropriate. The probability level was set at 5%.

Results

The characteristic EEG changes observed after administration of midazolam are shown in Figure 1 for the fronto-central lead on the left hemisphere (F_l–C_l). The upper trace shows the baseline EEG pattern (trace 1). As the concentration of midazolam increased, first a profound increase in high frequency (beta) activity was observed (trace 2), followed by an increase in EEG slow wave (delta) activity at higher concentrations of the drug (trace 3). The characteristic EEG changes observed in the raw EEG data are reflected in the changes in the calculated EEG parameters. The averaged time-effect profiles of the amplitudes in the 0.5–2.5 Hz and 11.5–30 Hz frequency range of all rats receiving 10 mg kg^{-1} of midazolam in 5 min are shown in Figure 2. During the infusion of midazolam there was a marked increase in the amplitudes in the 11.5–30 Hz frequency band. After the infusion was terminated, the effect returned gradually to pre-infusion values. The amplitudes in the 0.5–2.5 Hz frequency range were also increased after administration of midazolam, but this effect declined to baseline levels much more rapidly. A small increase was observed

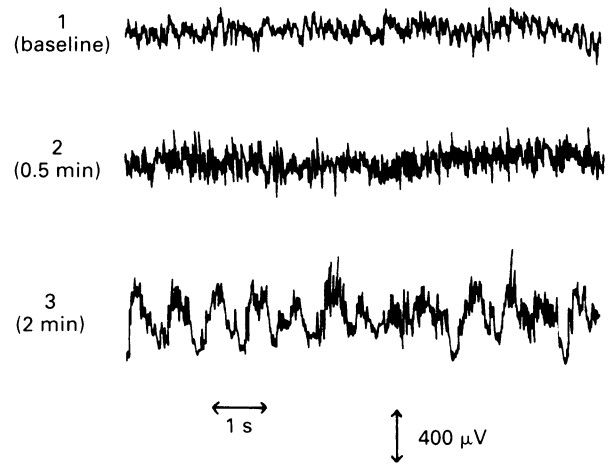

Figure 1 Characteristic EEG changes observed in the fronto-central lead on the left hemisphere (F_l–C_l) during intravenous administration of 10 mg kg^{-1} midazolam during 5 min. Trace 1 represents the baseline EEG pattern. As the concentration of midazolam increases first a substantial increase in high frequency (beta) activity is observed (trace 2; 0.5 min after start of the infusion), followed by an increase in EEG slow wave (delta) activity at higher concentrations of the drug (trace 3; 2 min after start of the infusion).

in the amplitudes in the 2.5–7.5 Hz frequency range and no consistent changes were observed in the amplitudes in the 7.5–11.5 frequency range.

Figure 3 shows the plasma concentration versus time profile of midazolam and the pharmacological effect versus time profile (change in amplitudes in the 11.5–30 Hz frequency band) in two individual rats after intravenous administration of 10 mg kg^{-1} during 5 min (Figure 3a) and 15 mg kg^{-1} orally

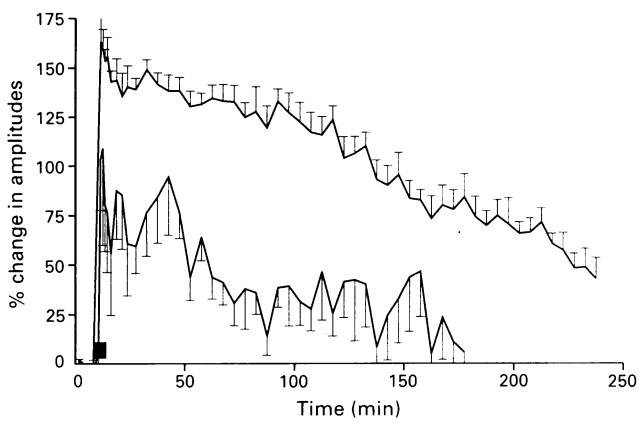

Figure 2 Percentage change over baseline value of the amplitudes in the 0.5–2.5 Hz (lower trace) and 11.5–30 Hz (upper trace) frequency bands. The curves represent the average increase (mean, with s.e.mean shown by vertical bars, $n = 8$) for all rats receiving 10 mg kg^{-1} midazolam intravenously during 5 min. The solid bar represents the infusion duration.

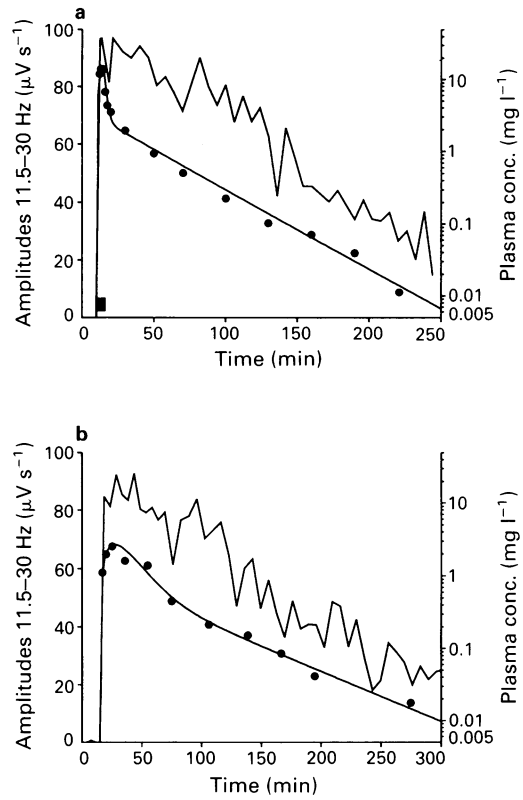

Figure 3 Midazolam plasma concentrations (●) and EEG effect (change in amplitudes in the 11.5–30 Hz frequency band) versus time profiles in two rats which had received 10 mg kg^{-1} intravenously during 5 min (a) and 15 mg kg^{-1} orally (b) respectively. The solid line fitted to the plasma concentrations represents the best fit according to the pharmacokinetic model.

(Figure 3b). After intravenous administration the concentration-time curves were most adequately described by a bi-exponential equation, after oral administration a bi- or tri-exponential equation best fitted the individual rat data. The pharmacokinetic parameters of midazolam following the different rates and routes of administration were summarized in Table 1. The averaged pharmacokinetic parameters calculated from all three rates of intravenous administration were (mean ± s.e.mean, $n = 20$): Cl = 67 ± 2 ml min^{-1} kg^{-1}, Vdss = 1.61 ± 0.07 l kg^{-1} and $t_{1/2}$, α_n = 27 ± 1 min. After oral administration midazolam was rapidly absorbed and peak concentrations were attained between 5 to 15 min. The systemic availability after oral administration was 45 ± 9%. No time delay (hysteresis) between plasma concentrations and EEG effect was observed and the two values were directly correlated to each other by the sigmoidal E_{max} model. Figure 4 shows the derived concentration-EEG effect relationship of the same two rats presented in Figure 3 after the pharmacokinetic-pharmacodynamic modelling procedure. The solid lines in this figure were fitted to the data using the sigmoidal E_{max} model. The pharmacodynamic parameters of

Table 1 Pharmacokinetic parameter estimates of midazolam after different rates and routes of administration

	10 mg kg^{-1} i.v. 5 min	10 mg kg^{-1} i.v. 30 min	10 mg kg^{-1} i.v. 60 min	15 mg kg^{-1} oral
Number of animals	8	6	6	7
Cl (ml min^{-1} kg^{-1})	60 ± 2[a]	71 ± 3	72 ± 5	169 ± 25
Vdss (l kg^{-1})	1.54 ± 0.10	1.42 ± 0.08	1.88 ± 0.10[b]	
$t_{1/2}$, α_n (min)	27 ± 1	24 ± 2	29 ± 2	
F (%)				45 ± 9

Values are means ± s.e.mean.
[a] Significantly different from i.v. 30 and 60 min ($P < 0.05$).
[b] Significantly different from i.v. 5 and 30 min ($P < 0.05$).

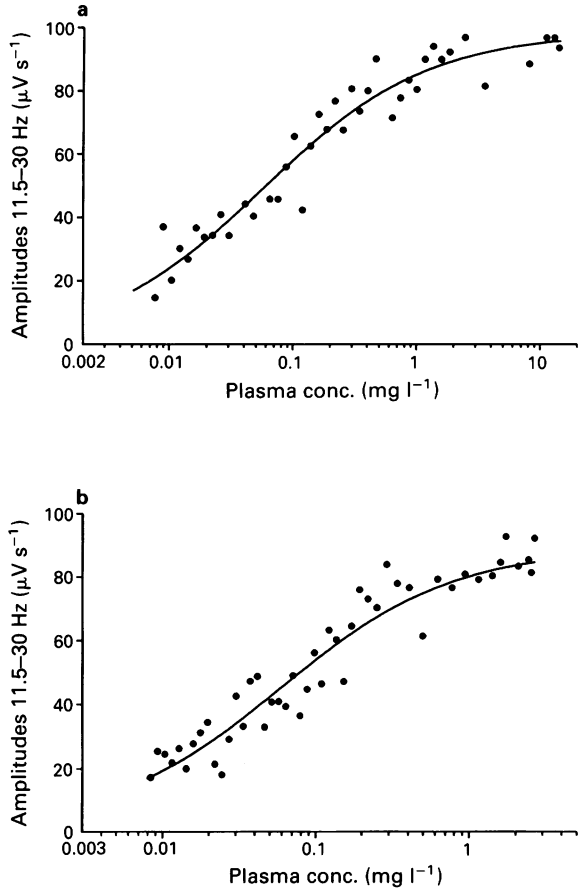

Figure 4 Concentration-EEG effect relationship of midazolam in two rats which had received 10 mg kg^{-1} intravenously during 5 min (a) and 15 mg kg^{-1} orally (b) respectively. The solid line represents the best fit to the actual data points (●) according to the sigmoidal E_{max} model.

midazolam following the different rates and routes of administration are summarized in Table 2. No statistically significant differences in the Eo, EC_{50} and N parameters were found between the different administrations. The E_{max} after intravenous infusion during 60 min and oral administration was found to be statistically significantly different from the values for 5 and 30 min infusion. No statistically significant differences in the pharmacodynamic parameters of midazolam were found between oral and the combined data after all intravenous administrations. The averaged pharmacodynamic parameters of midazolam calculated from all rates and routes of administration were (mean ± s.e.mean, $n = 27$): Eo = 61 ± 3 μV s^{-1}, E_{max} = 85 ± 3 μV s^{-1}, EC_{50} = 40 ± 3 ng ml^{-1} and N = 0.84 ± 0.04.

Discussion

The purpose of the present investigation was to quantify the concentration-EEG effect of relationships of midazolam in individual rats and to examine the influence of rate and route of administration on these relationships. Midazolam was selected as a model compound because of its water solubility, allowing intravenous administration in an aqueous solution, and its lack of a chiral centre.

The characteristic EEG changes of midazolam observed in the rat (Figures 1 and 2) correspond with EEG changes observed in man after benzodiazepine administration (Fink *et al.*, 1976; Kurowski *et al.*, 1982; Saletu *et al.*, 1986; Koopmans *et al.*, 1988). Initially at low benzodiazepine concentrations there is a profound increase in high frequency (beta) activity (11.5–30 Hz). At higher concentrations also, an increase in low frequency (delta) activity is observed (0.5–2.5 Hz). The increase of EEG beta-activity is most commonly used as a measure of the pharmacological effect of benzodiazepines. In the present study the amplitudes in the 11.5–30 Hz frequency range was preferred above the other parameters because it was stable at baseline and a profound effect, up to about 150% increase being observed with little spontaneous fluctuation during the duration of the experiment. Furthermore, in the individual animals the concentration-effect relationship could be described by the sigmoidal E_{max} model, which is preferred on theoretical grounds (Holford & Sheiner, 1982). The relatively low EC_{50} of 40 ng ml^{-1} shows the sensitivity of the EEG parameter for measurement of the CNS effects of benzodiazepines. Following intravenous administration midazolam plasma concentration-time data were most adequately described by a bi-exponential equation. Midazolam is rapidly eliminated from the body with an average elimination half-life of 27 ± 1 min, due to a large plasma clearance of 67 ± 2 ml min^{-1} kg^{-1}. The rapid disappearance of midazolam is another advantage of this drug, as it allows the determination of the complete concentration-effect relationship within a reasonable time span, thus minimizing influences due to diurnal variations.

Modelling concentration-effect relationships of CNS active drugs is often complicated due to an observed time lag between plasma concentrations and effect (hysteresis). In this study no delay between midazolam plasma concentrations and EEG effect was observed (Figure 4). After the relatively rapid intravenous administration of midazolam during 5 min, effects were maximal within 2 min after drug administration (Figure 3), indicating a rapid equilibration between plasma and effect site, which is to be expected considering the highly lipophilic character of the drug (Gerecke, 1983). This is consistent with the rapid equilibration of midazolam concentrations between plasma and cerebrospinal fluid (CSF) observed in the cat (Arendt *et al.*, 1983). Midazolam rapidly entered the CSF, with peak concentrations attained 3 min after dosage. After equilibrium was attained, disappearance of midazolam from the CSF and plasma occurred in parallel. In man a short delay was observed between plasma concentration of midazolam and EEG effects by Breimer *et al.* (1990), with an equilibration half-life ($t_{1/2}$, keo) of only 1.7 min. As no hysteresis was observed in this study, plasma concentrations were directly correlated to the EEG effects and successfully quantified using the sigmoidal E_{max} model (Figure 4).

In kinetic-dynamic modelling it is important to establish the uniqueness of the derived concentration-effect relationship, that is, the independence of this relationship of the pharmacokinetic profile of the drug. Several potentially confounding pharmacokinetic and pharmacodynamic factors

Table 2 Pharmacodynamic parameter estimates of midazolam after different rates and routes of administration

	10 mg kg^{-1} i.v. 5 min	10 mg kg^{-1} i.v. 30 min	10 mg kg^{-1} i.v. 60 min	15 mg kg^{-1} oral
Eo (μV s^{-1})	63 ± 2	75 ± 7	55 ± 5	53 ± 6
E_{max} (μV s^{-1})	92 ± 5	95 ± 5	73 ± 5[a]	78 ± 5[a]
EC_{50} (ng ml^{-1})	38 ± 4	37 ± 8	41 ± 7	44 ± 6
N	0.76 ± 0.08	0.91 ± 0.16	0.83 ± 0.07	0.87 ± 0.05

Values are means ± s.e.mean.
[a] Significantly different from i.v. 5 and 30 min ($P < 0.05$).

have to be considered, including the potential interfering role of (inter)active metabolites, development of acute tolerance and the role of counteracting homeostatic mechanisms (Dingemanse *et al.*, 1988a). These complicating factors are difficult to assess when only one mode of drug administration is studied, and Greenblatt *et al.* (1989) suggested that acute tolerance was likely to have contributed to the termination of the EEG effects of midazolam and diazepam in their study. To elucidate further the importance of these phenomena, the concentration-EEG effect relationship of midazolam was studied after various rates and routes of drug administration.

Acute tolerance is reported to develop towards several actions of benzodiazepines in man (MacLeod *et al.*, 1977; Ellinwood *et al.*, 1983; 1985) and animals (Yoong *et al.*, 1986; File *et al.*, 1989). It has been suggested that the rate of drug administration is an important determinant of the clinical effects of benzodiazepines and development of acute tolerance (Greenblatt *et al.*, 1977; Grundström *et al.*, 1978; Sellers, 1978; Ellinwood *et al.*, 1985). The concentration-EEG effect relationship of midazolam was studied at three rates of infusion. By varying the rate of drug administration the possible development of acute tolerance or role of counteracting homeostatic mechanisms can be elucidated. No marked or systematic differences were found in the pharmacokinetic and pharmacodynamic parameters of midazolam between the three rates of administration (Tables 1 and 2), indicating that acute tolerance towards the EEG effects of midazolam did not occur and that midazolam plasma concentrations and not the rate of drug administration determined the time-effect profile of this drug. Also the absence of 'clockwise' hysteresis in the plasma concentration versus effect profiles of the individual rats suggests that tolerance does not develop rapidly towards the EEG effects of midazolam. The E_{max} was slightly lower after the 60 min administration as compared to the 5 and 30 min (Table 2). This may in part have been caused by the lower baseline effect value in this group. The percentage increase over baseline did not differ between the three rates of administration. With regard to the pharmacokinetics, a significant lower clearance value was observed after the 5 min infusion (Table 1), which may be attributed to more pronounced cardiovascular effects of midazolam associated with higher drug concentrations attained during the rapid drug input (Reves *et al.*, 1985). Midazolam is a highly cleared drug in the rat, thus a reduction in liver blood flow would result in a reduction of the clearance. However, the reduction in clearance is small and not reflected in a change of the terminal half-life.

The possible contribution of metabolites to the pharmacological effect of the parent compound can be examined by studying the concentration-effect relationship under circumstances in which different amounts of the metabolites are formed (Holford & Sheiner, 1982; Dingemanse *et al.*, 1988b). In the present study the systemic availability after oral administration of 15 mg kg^{-1} was only 45% despite the rapid absorption of the drug (Table 2). This indicates the formation of a substantial amount of first-pass metabolites, which was to be expected on the basis of the intravenous data. However, no significant differences in the pharmacodynamic parameters of midazolam were found between oral and intravenous administrations, indicating that in the rat, metabolites of midazolam do not interfere with the effects of midazolam on the EEG. When the different groups were compared the E_{max} was slightly lower after oral administration as compared to 5 and 30 min intravenous administration (Table 2), which may be due to the lower baseline effect value in this group.

Interestingly, in man, metabolites of midazolam were found to contribute to the CNS effects of the drug (Crevoisier *et al.*, 1983). In man, midazolam is rapidly eliminated from the body by biotransformation to two metabolites, 1-hydroxy-midazolam and 4-hydroxy-midazolam, which are both pharmacologically active (Pieri *et al.*, 1981; Ziegler *et al.*, 1983). After oral, but not after intravenous, administration of midazolam, concentrations of 1-hydroxy-midazolam similar to those of the parent compound are observed, due to a substantial first pass metabolism (Heizmann *et al.*, 1983). These high concentrations of 1-hydroxy-midazolam were shown to contribute significantly to the pharmacological effect of the parent drug, which was reflected in a parallel shift to the left of the relationship between plasma concentrations of midazolam and reaction time observed after oral administration in comparison with intravenous dosage. The concentration-effect relationships were superimposable when the plasma concentrations of midazolam plus 1-hydroxy-midazolam were taken into account (Crevoisier *et al.*, 1983). This difference compared with the situation in rats can be explained by a different metabolic pathway of midazolam in the two species. It has been shown that in the rat midazolam is primarily metabolized by aromatic hydroxylation leading to the formation of a phenolic (4'-OH) derivative of midazolam, which is not formed in man (Woo *et al.*, 1981).

The results of the present study show that the concentration-EEG effect relationship of midazolam can be characterized in individual animals using the amplitudes in the 11.5–30 (beta) Hz frequency range as a measure of pharmacological response. It has been shown that acute tolerance did not develop and (inter)active metabolites did not contribute to the EEG effects of midazolam within the time span of the experiments. The EEG effect parameter (amplitudes in the 11.5–30 Hz frequency band) offers in principle the possibility of evaluating the effect of such factors as age, chronic drug use, disease states and drug interactions, on the pharmacokinetics and pharmacodynamics of benzodiazepines. However, the relationship between the benzodiazepine-induced EEG changes and their clinical, anxiolytic, anticonvulsant, muscle relaxant, sedative and hypnotic effects still remains to be established.

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References

AREND T, R.M., GREENBLATT, D.J., DEJONG, R.H., BONIN, J.D., ABERNETHY, D.R., EHRENBERG, B.L., GILES, H.G., SELLERS, E.M. & SHADER, R.I. (1983). *In vitro* correlates of benzodiazepine cerebrospinal fluid uptake, pharmacodynamic action and peripheral distribution. *J. Pharmacol. Exp. Ther.*, **227**, 98–106.

BREIMER, L.T.M., HENNIS, P.J., BURM, A.G.L., DANHOF, M., BOVILL, J.G., SPIERDIJK, JOH. & VLETTER, A.A. (1990). Quantification of the EEG effect of midazolam by aperiodic analysis in volunteers. Pharmacokinetic/pharmacodynamic modelling. *Clin. Pharmacokin.*, **18**, 245–253.

CREVOISIER, C.H., ZIEGLER, W.H., ECKERT, M. & HEIZMANN, P. (1983). Relationship between plasma concentration and effect of midazolam after oral and intravenous administration. *Br. J. Clin. Pharmacol.*, **16**, 51S–61S.

DINGEMANSE, J., DANHOF, M. & BREIMER, D.D. (1988a). Pharmacokinetic pharmacodynamic modelling of CNS drug effects. An overview. *Pharmacol. Ther.*, **38**, 1–52.

DINGEMANSE, J., THOMASSEN, D., MENTINK, B.H. & DANHOF, M. (1988b). Strategy to assess the role of (inter)active metabolites in pharmacodynamic studies *in-vivo*: a model study with heptabarbital. *J. Pharm. Pharmacol.*, **40**, 552–557.

ELLINWOOD, E.H., LINNOILA, M., EASLER, M.E. & MOLTER, D.W. (1983). Profile of acute tolerances to three sedative anxiolytics. *Psychopharmacology*, **79**, 137–141.

ELLINWOOD, E.H., HEATHERLY, D.G., NIKAIDO, A.M., BJORNSSON, T.D. & KILTS, C. (1985). Comparative pharmacokinetics and pharmacodynamics of lorazepam, alprazolam and diazepam. *Psychopharmacology*, **86**, 392–399.

FILE, S.E., WILKS, L.J. & MABBUTT, P.S. (1989). Withdrawal, tolerance and sensitization after a single dose of lorazepam. *Pharmacol. Biochem. Behav.*, **31,** 937–940.

FINK, M., IRWIN, P., WEINFIELD, R.E., SCHWARTZ, M.A. & CONNEY, A.H. (1976). Blood levels and electroencephalographic effects of diazepam and bromazepan. *Clin. Pharmacol. Ther.*, **20,** 184–191.

GERECKE, M. (1983). Chemical structure and properties of midazolam compared with other benzodiazepines. *Br. J. Clin. Pharmacol.*, **16,** 11S–16S.

GIBALDI, M. & PERRIER, D. (1982). Noncompartmental analysis based on statistical moment theory. In *Pharmacokinetics*, 2nd ed., pp. 409–424. New York: Marcel Dekker.

GREENBLATT, D.J., SHADER, R.I., HARMATZ, J.S., FRANKE, K. & KOCH-WESER, J. (1977). Absorption rate, blood concentrations, and early response to oral chlordiazepoxide. *Am. J. Psychiatry*, **134,** 559–562.

GREENBLATT, D.J., EHRENBERG, B.L., GUNDERMAN, J., LOCNISKAR, A., SCAVONE, J.M., HARMATZ, J.S. & SHADER, R.I. (1989). Pharmacokinetic and electroencephalographic study of intravenous diazepam, midazolam and placebo. *Clin. Pharmacol. Ther.*, **45,** 356–365.

GREGORY, T.K. & PETTUS, D.C. (1986). An electroencephalographic processing algorithm specifically intended for analysis of cerebral electrical activity. *J. Clin. Monit.*, **2,** 190–197.

GRUNDSTRÖM, R., HOLMBERG, G. & HANSEN, T. (1978). Degree of sedation obtained with various doses of diazepam and nitrazepam. *Acta Pharmacol. Toxicol.*, **43,** 13–18.

HEIZMANN, P., ECKERT, M. & ZIEGLER, W.H. (1983). Pharmacokinetics and bioavailability of midazolam in man. *Br. J. Clin. Pharmacol.*, **16,** 43S–49S.

HOLFORD, N.H.G. & SHEINER, L.B. (1982). Kinetics of pharmacologic response. *Pharmacol. Ther.*, **16,** 143–166.

KOOPMANS, R., DINGEMANSE, J., DANHOF, M., HORSTEN, G.P.M. & VAN BOXTEL, C.J. (1988). Pharmacokinetic-pharmacodynamic modeling of midazolam effects on the human central nervous system. *Clin. Pharmacol. Ther.*, **44,** 14–22.

KUROWSKI, M., OTT, H. & HERMANN, W.M. (1982). Relationship between EEG dynamics and pharmacokinetics of the benzodiazepine lormetazepam. *Pharmacopsychiatry*, **15,** 77–83.

LAURIAN, S., GAILLARD, J.-M., LE, P.K. & SCHÖPF, J. (1984). Effects of benzodiazepine antagonist on the diazepam-induced electrical brain activity modifications. *Neuropsychobiology*, **11,** 55–58.

MACLEOD, S.M., GILES, H.G., PATZALEK, G., THIESSEN, J.J. & SELLERS, E.M. (1977). Diazepam actions and plasma concentrations following ethanol ingestion. *Eur. J. Clin. Pharmacol.*, **11,** 345–349.

MANDEMA, J.W. & DANHOF, M. (1990). Pharmacokinetic-pharmacodynamic modelling of the central nervous system effects of heptabarbital using aperiodic EEG analysis. *J. Pharmacokin. Biopharmacol.*, **18,** 459–481.

PIERI, L., SCHAFFNER, R., SCHERSCHLICHT, R., POLC, P., SEPINWALL, J., DAVIDSON, A., MÖHLER, H., CUMIN, R., DA PRADA, M., BURKARD, W.P., KELLER, H.H., MÜLLER, R.K.M., GEROLD, M., PIERI, M., COOK, L. & HAEFELY, W. (1981). Pharmacology of midazolam. *Arzneim-forsch./Drug Res.*, **31,** 2180–2201.

REVES, J.G., FRAGEN, R.J., VINIK, H.R. & GREENBLATT, D.J. (1985). Midazolam pharmacology and uses. *Anesthesiology*, **62,** 310–324.

SALETU, B., GRÜNBERGER, J. & SIEGHART, W. (1986). Pharmaco-EEG, behavioural methods and blood levels in the comparison of temazepam and flunitrazepam. *Acta Psychiatr. Scand.*, Suppl. 332, **74,** 67–94.

SELLERS, E.M. (1978). Addictive drugs: disposition, tolerance, and dependence interrelationships. *Drug Metabol. Rev.*, **8,** 5–11.

WOO, G.K., WILLIAMS, T.H., KOLIS, S.J., WARINSKY, D., SASSO, G.J. & SCHWARTZ, M.A. (1981). Biotransformation of [^{14}C]midazolam in the rat *in vitro* and *in vivo*. *Xenobiotica*, **6,** 373–384.

YOONG, Y.L., LEE, H.S., GWEE, M.C.E. & WONG, P.T.-H. (1986). Acute tolerance to diazepam in mice: pharmacokinetic considerations. *Clin. Exp. Pharmacol. Physiol.*, **13,** 153–158.

ZIEGLER, W.H., SCHALCH, E., LEISHMAN, B. & ECKERT, M. (1983). Comparison of the effects of intravenously administered midazolam, triazolam and their hydroxy metabolites. *Br. J. Clin. Pharmacol.*, **16,** 63S–69S.

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The effects of the stereoisomers of propafenone and diprafenone in guinea-pig heart

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1 Optically pure enantiomers of propafenone and diprafenone were prepared from their racemic mixtures and tested for their ability to block β-adrenoceptors and to prolong functional refractory period in the guinea-pig heart. β-Adrenoceptor affinity of the enantiomers was determined by the radioligand binding technique and in functional experiments.

2 Propafenone and diprafenone inhibited specific binding of the β-adrenoceptor antagonist (−)-[^{3}H]-CGP-12177 to guinea-pig myocardial membranes. β-Adrenoceptor affinities of diprafenone enantiomers exceeded those of corresponding propafenone enantiomers by one order of magnitude. Displacement of (−)-[^{3}H]-CGP-12177 by both antiarrhythmics was highly stereoselective, in that the (S)-enantiomers were 40–60 fold, i.e. 1.6–1.8 log units more potent than the (R)-enantiomers.

3 Propafenone and diprafenone antagonized the positive inotropic action of isoprenaline in isolated atria. β-Adrenoceptor antagonist potencies of diprafenone enantiomers were about one order of magnitude higher than those of corresponding propafenone enantiomers. For both drugs the (S)-enantiomer was found to be considerably more potent (14–40 fold) than the (R)-enantiomer.

4 Propafenone and diprafenone prolonged functional refractory period of isolated auricles with equal potency and no difference in the antiarrhythmic activity of purified enantiomers was found.

5 It is concluded that the enantiomers of propafenone and diprafenone exert comparable antiarrhythmic activity, whereas only (S)-enantiomers block cardiac β-adrenoceptors with high affinity, which explains the β-adrenoceptor antagonist effects of the racemic drugs.

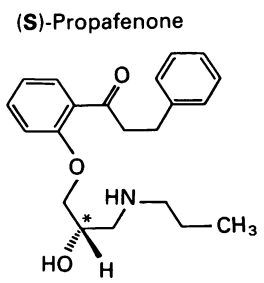
Introduction

Propafenone and diprafenone interact with two different target sites in the heart. Both drugs have been classified as type 1 antiarrhythmics according to the Vaughan Williams (1975) classification, since their antiarrhythmic effects are predominantly due to inhibition of transmembrane sodium current (Kohlhardt & Seifert, 1983; Kohlhardt, 1984; Dukes & Vaughan Williams, 1984). Both drugs display the structural features of β-adrenoceptor antagonists (1-alkylamino-3-aryloxy-2-propanol, see Figure 1), and in line with these structural characteristics, adrenoceptor antagonist effects of propafenone have been demonstrated in various studies (Paietta *et al.*, 1977; Kukovetz *et al.*, 1977; Ledda *et al.*, 1981; Müller-Peltzer *et al.*, 1983; McLeod *et al.*, 1984). A more recent clinical study with diprafenone indicates that this new antiarrhythmic drug is also a potent antagonist at β-adrenoceptors (Wagner *et al.*, 1987).

Binding to β-adrenoceptors is characterized by a high degree of stereoselectivity (Kaumann & Birnbaumer 1974; Wächter *et al.*, 1980; Lemoine & Kaumann, 1983; Morris & Kaumann, 1984). There is also evidence for stereoselective binding of type 1 antiarrhythmics and local anaesthetics to sodium channels (Akermann *et al.*, 1969; Yeh, 1980; Sheldon *et al.*, 1988; Hill *et al.*, 1988). In most previous studies, concerned with stereoselectivity of drug actions, optical rotation was used to describe the stereoisomers. The laevorotatory isomer was generally found to be the more potent β-blocker, whereas no clear cut separation was obtained for type 1 antiarrhythmic drugs (Hill *et al.*, 1988). The description of an optically active compound exclusively by its optical rotation, however, might not be sufficient, since optical rotation may vary and even change its direction when the medium (solvent, pH, etc.) in which the drugs are dissolved is changed, whereas the configuration which determines the interaction with membrane receptors (Ariens, 1984; Ariens *et al.*, 1988) remains unchanged. Therefore, the absolute configuration of the optically active centre has to be defined precisely, by use of the (R)-(S)-nomenclature (Cahn *et al.*, 1956). So far, in all cases studied and examined, β-adrenoceptor agonists and antagonists were more potent in the (S)-configuration (see Figure 1). For instance, recent *in vitro* and *in vivo* experiments with propranolol demonstrated that the laevorotatory stereoisomer with (S)-configuration is a more potent β-blocker (2.25 log units) than the corresponding dextrorotatory isomer, (Stoschitzky *et al.*, 1989).

Since propafenone and diprafenone interact with both cardiac β-adrenoceptors and sodium channels, a divergent pharmacological profile of the enantiomers might be anticipated, based on differences in the stereoselectivity of the two target sites. The present study was designed to compare propafenone and diprafenone enantiomers with respect to β-adrenoceptor affinity and antiarrhythmic activity. In order to perform this study, we had first to resolve the racemic drugs into the optically pure enantiomers, characterize them physico-chemically and assign their absolute configurations.

(S)-Propafenone



(S)-Diprafenone

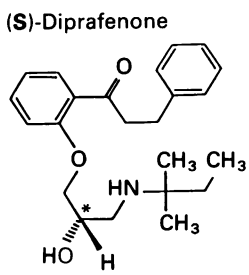


Figure 1 Structural formula of (S)-propafenone and (S)-diprafenone. The asymmetric carbon atom is indicated by an asterisk.

Methods

Guinea-pigs of either sex (250–750 g body weight) were stunned with a blow to the head and exsanguinated. The

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hearts were rapidly removed and rinsed with cold Tyrode solution (mmol l^{-1}: NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and glucose 10). Atria were dissected, and the remaining myocardium was cleared from large vessels and connective tissues and placed in cold Tris-HCl buffer (mmol l^{-1}: Tris 20, sucrose 250, $MgCl_2$ 5, pH 7.5 = buffer A).

Binding experiments

Guinea-pig myocardium (left and right ventricles) was minced with scissors and homogenized for 40 s in a Micro-Dismembrator (Braun, Melsungen, F.R.G.). The homogenate was passed through two sieves (pore sizes 1 and 0.2 mm^2) and centrifuged at 500 *g* for 10 min. The resulting pellet was washed (buffer A) and the combined supernatants were centrifuged at 37,000 *g* for 30 min. The final pellet was resuspended in Tris-HCl buffer (mmol l^{-1}, Tris 20, NaCl 154, $MgCl_2$ 2, pH 7.5 = buffer B) to give a final protein concentration of 6–8 mg ml^{-1}, determined by the method of Bradford (1976). Tissue suspensions were stored in liquid nitrogen.

A volume of 50 μl of the tissue suspension (150–250 μg protein/assay) was incubated in 200 μl buffer B, 50 μl (−)-[^{3}H]-CGP-12177 ([^{3}H]-CGP) and 100 μl H_2O or appropriate drug solution for 30 min at 30°C. Binding was terminated by the addition of 3 ml icecold Tris-HCl buffer (mmol l^{-1}: Tris 20; NaCl 154, pH 7.5 = buffer C) and the membrane-bound radioactivity was separated by filtration through Whatman GF/C glass fibre filters. Filters were rinsed 5 times with 3 ml buffer C, placed in a 5 ml toluene based scintillation cocktail containing 33% Triton-X 100 and counted in a Packard Tricarb 4530 scintillation spectrometer with an efficiency of 47%. Specific binding was calculated as the difference between counts bound in the presence and absence of 50 μmol l^{-1} (±)-isoprenaline hydrochloride, respectively. To characterize the binding properties of [^{3}H]-CGP in this preparation, we performed saturation binding experiments in which the [^{3}H]-CGP concentration was varied from 0.05–6 nmol l^{-1}. In competition binding experiments, the [^{3}H]-CGP concentration was adjusted to 0.4 nmol l^{-1}, which resulted in 600–1000 d.p.m. of total binding. Specific binding in these experiments was 70–85% of total binding. Total binding accounted for less than 10% of the radioactivity in the medium and no correction for free radioligand concentration was made. Saturation experiments were performed in duplicate, competition experiments in triplicate.

Saturation and competition curves were analyzed by a non-linear least squares computer curve fitting programme (Wiemer *et al.*, 1982). Mean K_D and K_I values (geometric means) as well as 95% confidence limits were calculated from 3–4 individual experiments. B_{max} values were calculated as arithmetic means with standard error (s.e.mean). Hill coefficients of competition binding curves were calculated for binding between 90 and 10%.

Experiments in isolated atria

Left atria were suspended in organ baths containing 5 ml Tyrode solution and gassed with 95% O_2, 5% CO_2 at a constant temperature of 32°C and stimulated at a resting tension of 10 mN at a rate of 2 Hz (stimulus-duration 3 ms; twice threshold voltage) by means of a HSE stimulator 215/I (Hugo Sachs Elektronik, Freiburg, F.R.G.). Contractile force was recorded isometrically, with a Gould transducer connected to a Watanabe multi channel recorder. Muscles were allowed to stabilize for 45 min.

Concentration-response curves of the positive inotropic action of isoprenaline were established cumulatively in the absence and presence of propafenone or diprafenone (1, 5, 10, 30 μmol l^{-1}), respectively. Muscles were pretreated with pure enantiomers for at least 45 min. The extracellular Ca^{2+}-concentration was increased to 5.8 mmol l^{-1} subsequent to the addition of drugs in order to obtain an about maximum increase in contractility, which was set at 100% for calculation of percentage changes in contractile force. With this experimental protocol, two dose-response curves could be established with each preparation (control experiments yielded identical ED_{50} values) and the potency of isoprenaline was highly reproducible (95% confidence limits for control ED_{50}: 1.3–4.6 × 10^{-8} mol l^{-1}, $n = 7$). K_B-values were calculated from mean ED_{50} values (4–6 individual experiments) according to the method of Arunlakshana & Schild (1959). For graphical comparison of blocking potencies, the inhibitory effects at a certain isoprenaline concentration were plotted as a function of the antagonist concentration.

The effects of propafenone and diprafenone on the functional refractory period were determined by the paired pulses method according to Govier (1965) by use of a refractory period calculator VR 215/20 (Hugo-Sachs Elektronik, Freiburg, F.R.G.).

Drugs

(−)-[^{3}H]-CGP-12177 (specific activity: 49 Ci $mmol^{-1}$) was purchased from Amersham International (GB), (+)-isoprenaline hydrochloride was from Sigma Chemical Co. (Munich, F.R.G.).

Preparation of optically pure enantiomers of propafenone and diprafenone

Propafenone was resolved by cristallization according to Blaschke & Walther (1987). Yield: 47% (**R**)-propafenone base; optical purity (o.p.) 98.4%: optical rotation ($[\alpha]_{365}^{22}$) = −22.0° (10 mg ml^{-1} in EtOH); m.p. 68°C and 39% (**S**)-propafenone base; o.p. 98.8%; $[\alpha]_{365}^{22}$ = +19.7° (10 mg ml^{-1} in EtOH); m.p. 67°C.

The corresponding hydrochloride salts were made by acidification with HCl-gas. (**R**)-propafenone · HCl: $[\alpha]_{546}^{22}$ = +8.2° (9.7 mg ml^{-1} in MeOH), m.p. 165–168°C. (**S**)-propafenone · HCl: $[\alpha]_{546}^{21}$ = −8.5° (10 mg ml^{-1} in MeOH); m.p. 166–168°C.

Diprafenone was resolved successfully, by a modification of the general method described previously (Lindner, 1985). In brief: 4.8 g (13 mmol) (**R,S**)-diprafenone were dissolved in dry dichlormethane followed by the addition of 3.2 g (19.5 mmol) acetic acid and 5.1 g (15 mmol) (**R,R**)-O,O'-dibenzoyl tartaric acid anhydride (**R,R**)-DBTAAN). After 3 h refluxing, all (**R,S**)-diprafenone was derivatized to the corresponding diastereomeric tartaric acid monoesters. The organic solution was extracted with 3 g $NaHCO_3$ in 30 ml H_2O and the organic layer was evaporated. The residue was stirred with acetonitrile to yield 7.7 g (84%) of a crystalline mixture of diastereoisomers. This product was separated into the optically pure diastereoisomers according to the following procedure. To 5 g product in 50 ml glacial acetic acid 100 ml of H_2O/2-propanol (1:1) was added in small portions. After some minutes of stirring, crystallization occurred and (**R**)-diprafenone-(**R,R**)-O,O'-dibenzoyl-tartaric acid monoester (fraction I) could be isolated. Evaporation of the mother liquor and addition of a great excess of water yielded the optically enriched (**S**)-diprafenone diastereomeric derivative (fraction II). Further purification of fraction I was possible by repeating the crystallization procedure with glacial acetic acid–H_2O/2-propanol (1:1). Repeating the derivatization procedure of optically enriched (**S**)-diprafenone with (**S,S**)-DBTAAN yielded optically pure (**S**)-diprafenone-(**S,S**)-O,O'-dibenzoyl tartaric acid monoester. The diastereomeric derivatives were cleaved to the optically pure parent compound with methanolic KOH, and isolated in the usual way resulting in oily products. These were transferrred into the corresponding hydrochloride salts.

Yield: 64% (**R**)-diprafenone · HCl; o.p. 99.5%; $[\alpha]_{546}^{21}$ = +8.0° (10 mg ml^{-1} in MeOH); m.p. 88°C and 34% (**S**)-

diprafenone · HCl; o.p. 99%; $[\alpha]_{546}^{22} = -7.9°$ (10 mg ml^{-1} in MeOH); m.p. 89°C.

(S)-propafenone base and (S)-diprafenone base were both dextrorotatory in ethanol, but laevorotatory as hydrochloride salts in water. This means that in both cases one gets an inversion of the optical rotation dealing with either the free base or the hydrochloride salts of the drugs.

Optical purity of the resolved and isolated enantiomers of propafenone and diprafenone was checked chromatographically with (**R,R**)-O,O'-diacetyl tartaric acid anhydride ((**R,R**)-DATAAN) as chiral derivatizing agent according to a general procedure described by Lindner *et al.* (1984).

The absolute configuration of (**R**)- and (S)-propafenone was determined according to Blaschke & Walther (1987) with CD spectroscopy and cupra A as agent. In all our previous experiments (Lindner *et al.*, 1984; Lindner, 1985) of resolving enantiomers of aryloxy-propanolamine type drugs as diastereomeric tartaric acid monoesters (e.g. with (**R,R**)-DATAAN as chiral reagent) chromatographically by reversed phase h.p.l.c., we noticed that the diastereomeric derivatives of (**R**)-enantiomers eluted always in front of (S)-enantiomer-derivatives. This was also observed for propafenone enantiomers. Due to the structural analogy between propafenone and diprafenone, the enantiomers of both drugs should behave in a similar manner chromatographically, and we assigned the first eluted peak of the diastereomeric pair to be the derivative of (**R**)-diprafenone.

Results

Binding experiments

The high-affinity ligand [^{3}H]-CGP was used to study the binding of propafenone and diprafenone enantiomers to guinea-pig myocardial β-adrenoceptors. Saturation experiments (data not shown), yielded a mean K_D of 0.15 nmol l^{-1} (0.13–0.20 nmol l^{-1}) and a binding capacity of 28 ± 3 fmol mg^{-1} protein (n = 4). Typical competition binding curves of propafenone and diprafenone enantiomers are shown in Figure 2, binding data are given in Table 1.

Both antiarrhythmics displaced [^{3}H]-CGP, and the binding data were best fitted to a one binding site model. Hill coefficients close to unity were calculated (Table 1). The β-adrenoceptor affinities of (S)-enantiomers were considerably higher than of corresponding (**R**)-forms. Affinity ratios (**R**- versus S-enantiomer) of 1.7 log units for propafenone and 1.8 log units for diprafenone were obtained.

Experiments in isolated atria

β-Adrenoceptor blocking effects of propafenone and diprafenone enantiomers Propafenone and diprafenone stereoisomers dose-dependently (1–30 μmol l^{-1}) antagonized the positive inotropic action of isoprenaline. Isoprenaline concentration-response curves were shifted in a parallel fashion to the right (Figure 3a,b). Figure 3 also shows (d) the graphical analysis of

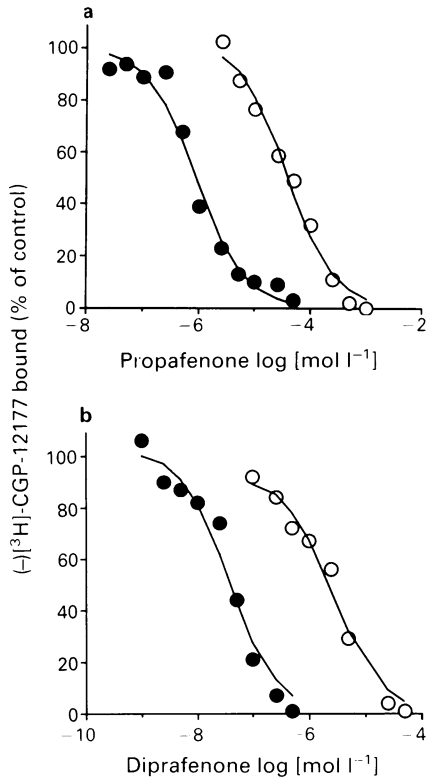

Figure 2 Inhibition of specific (−)-[^{3}H]-CGP-12177 binding to guinea-pig myocardial membranes by the stereoisomers of propafenone (a) and diprafenone (b). Competition curves of (S)-enantiomers (●) and (**R**)-enantiomers (○) are shown. Each point represents the mean of triplicate determinations of a single experiment. Individual competition curves (n = 3–5) were fitted to a one site model and yielded the binding constants shown in Table 1.

diprafenone effects using the method of Arunlakshana & Schild (1959). The β-adrenoceptor antagonist potencies of pure (S)-enantiomers were 1.2 log units (propafenone) and 1.6 log units (diprafenone) higher than that of corresponding (**R**)-enantiomers (Table 1).

Diprafenone enantiomers were potent in antagonizing the positive inotropic action of isoprenaline with higher potency than corresponding propafenone enantiomers (Table 1). Figure 3 (e,f) shows the dose-dependent inhibition of isoprenaline-induced positive inotropism by propafenone and diprafenone (at 10^{-7} and 10^{-6} mol l^{-1} isoprenaline respectively), which clearly demonstrated the higher blocking potency of (S)-enantiomers. Both antiarrhythmics were tested up to concentrations of 30 μmol l^{-1}. Higher concentrations elicited marked cardiodepressant effects (contractility and excitability) which compromised the evaluation of β-adrenoceptor antagonist effects at drug concentrations above 30 μmol l^{-1} in the functional studies.

Table 1 Equilibrium dissociation constants of propafenone and diprafenone enantiomers as calculated from competition binding (K_I) and functional experiments (K_B)

	K_I (mol l^{-1}) (95% confidence limits)	n_H* (range)	K_B (mol l^{-1})	*Schild plot slope*
(**R**)-Propafenone	8.9×10^{-6} (7×10^{-6}–1.1×10^{-5})	1.01 (0.9–1.1)	4.3×10^{-6}	1.1
(S)-Propafenone	1.8×10^{-7} (1.1–2.9×10^{-7})	1.02 (0.9–1.1)	3.1×10^{-7}	1.1
(**R**)-Diprafenone	8.8×10^{-7} (4.4×10^{-7}–1.7×10^{-6})	0.9 (0.8–1.0)	1.6×10^{-6}	0.99
(S)-Diprafenone	1.4×10^{-8} (9×10^{-9}–2.2×10^{-8})	1.06 (1.0–1.1)	3.7×10^{-8}	1.03

* n_H = Hill coefficient of competition binding curves

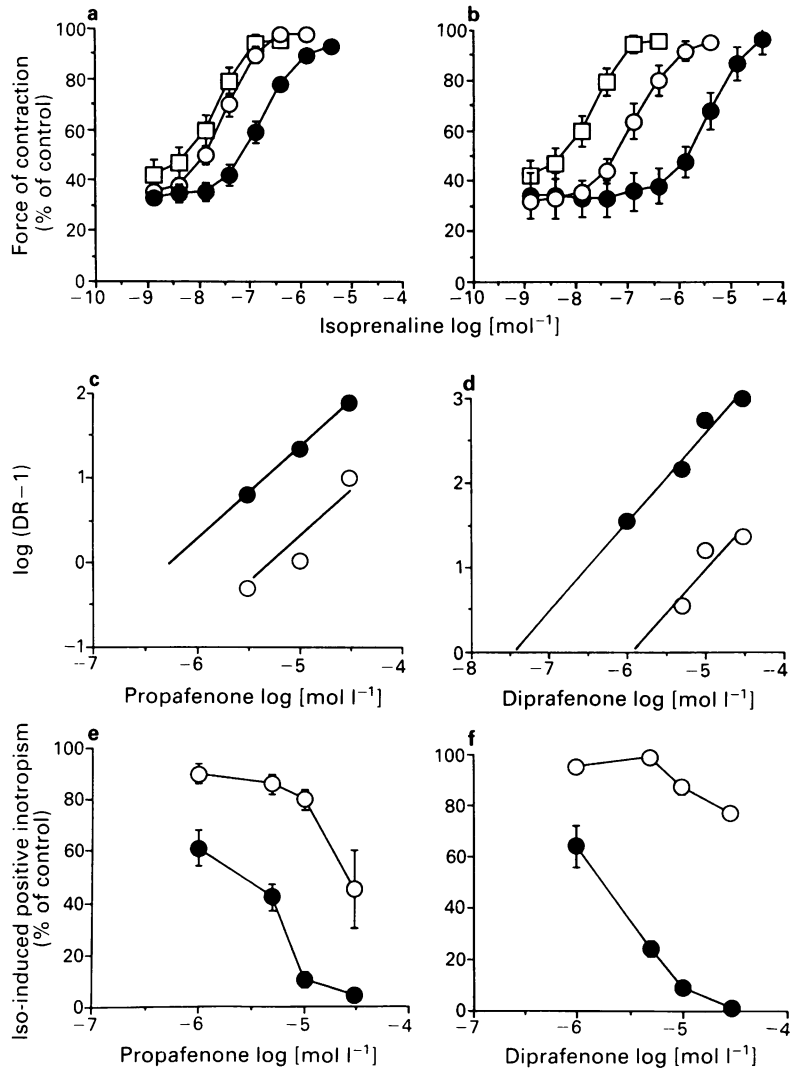

Figure 3 β-Adrenoceptor antagonist effects of propafenone (c,e) and diprafenone enantiomers (d,f) in guinea-pig atria. (a,b): Positive inotropic action of isoprenaline in the absence (□) and presence of 5 μmol l^{-1} (S)- (●) and 5 μmol l^{-1} (R)-enantiomers (○). Each point represents the mean of 4–8 experiments, vertical bars show s.e.mean. (c,d): Analysis of propafenone and diprafenone effects by the method of Arunlakshana & Schild (1959); S- (●) and R- (○) enantiomers. (e,f): Concentration-dependent inhibition of isoprenaline (Iso)-induced positive inotropism by propafenone and diprafenone enantiomers. Effects of propafenone enantiomers at a concentration of 100 nmol l^{-1} isoprenaline, and effects of diprafenone at 1 μmol l^{-1} isoprenaline are shown (mean with s.e.mean shown by vertical bars, n = 4–8): S- (●) and R- (○) enantiomers.

Antiarrhythmic effects of propafenone and diprafenone enantiomers Propafenone and diprafenone dose-dependently prolonged functional refractory period of guinea-pig isolated auricles (Figure 4).

The drugs were approximately equipotent with respect to prolongation of atrial refractory period. (R)- and (S)- enantiomers of each drug exhibited equal antiarrhythmic potency in this model. Prolongation of refractory period by 25% was obtained with propafenone enantiomers at 3.4 × 10^{-6} mol l^{-1} and with diprafenone enantiomers at 2 × 10^{-6} mol l^{-1}.

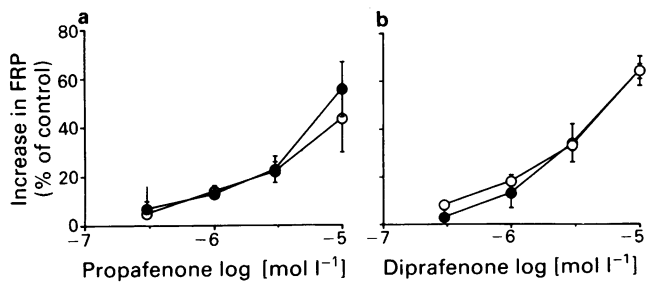

Figure 4 Concentration-response curves of propafenone (a) and diprafenone (b)-induced increase in functional refractory period (FRP) of guinea-pig isolated atria. Effects of (S)-enantiomers (●) and (R)-enantiomers (○) are shown. Each point represents the mean of 4–6 experiments; vertical bars show s.e.mean.

Discussion

The present study demonstrates the highly stereoselective interaction of propafenone and diprafenone with cardiac β-adrenoceptors. Irrespective of dextro- or laevorotatory properties, resolved enantiomers with (S)-configuration, as determined chromatographically and by CD-spectroscopy, exerted considerably higher β-adrenoceptor affinity than their corresponding (R)-enantiomers. A similar pattern of stereoselectivity has recently been reported for the binding of propafenone to human lymphocyte β-adrenoceptors (Kroemer *et al.*, 1989). In the present experiments, enantiomeric affinity ratios of 1.2–1.8 log units were obtained. These values are in keeping with those previously reported for the stereoselectivity of cardiac β-adrenoceptors (Kaumann & Birnbaumer, 1974; Morris & Kaumann, 1984).

In clear contrast to the high stereoselectivity of the β-adrenoceptor antagonist action, purified enantiomers of both

antiarrhythmics prolonged atrial refractory period with equal potency. The antiarrhythmic effects of propafenone and diprafenone are mainly due to inhibition of fast sodium channels (Kohlhardt & Seifert, 1980; 1983; Kohlhardt, 1984). Although propafenone is known also to exert some calcium channel blocking activity, its calcium antagonist action is very weak (Ledda *et al.*, 1981; Dukes & Vaughan Williams, 1984), hence a contribution to the effects on refractory period appears negligible. Modulation of cardiac sodium channel function is thought to be mediated by binding of the drugs to a receptor site directly associated with the ion channel (Catterall, 1987; Sheldon *et al.*, 1987). Stereoselectivity of this putative binding site has been demonstrated in experiments with the local anaesthetic RAC 109 (Yeh, 1980; Postma & Catterall, 1983; Clarkson, 1986) as well as the antiarrhythmic tocainide (Sheldon *et al.*, 1988). A comparative study, however, revealed considerable stereospecific differences in the binding of type 1 antiarrhythmics to cardiac sodium channels, and disopyramide as well as flecainide were found to bind non-stereoselectively (Hill *et al.*, 1988). The latter results were interpreted in terms of two different stereospecific domains within the receptor site and of more complex structural determinants of binding. This might explain the lack of stereoselectivity found for propafenone and diprafenone effects on atrial refractory period in the present study. This is also in agreement with a recent report on the effects of propafenone enantiomers on upstroke velocity (phase 0) in canine Purkinje fibres (Kroemer *et al.*, 1989).

The present results indicate that purified (R)-enantiomers of propafenone and diprafenone have marked antiarrhythmic but negligible β-adrenoceptor antagonist activity. Thus, the use of purified (R)-enantiomers instead of racemic drugs might be of clinical advantage with respect to the antiarrhythmic therapy of patients intolerant to β-blockade. For the treatment of certain types of arrhythmias on the other hand, combination of sodium channel and β-blocking activity is known to be of advantage (Hirsowitz *et al.*, 1986). With the novel drug diprafenone, apparently both actions are perfectly combined. The affinity of (S)-diprafenone for cardiac β-adrenoceptors was found to be 1.4×10^{-8} mol l^{-1} in binding experiments and 3.7×10^{-8} mol l^{-1} in functional studies, which exceeds the β-adrenoceptor affinity of (S)-propafenone by one order of magnitude and is comparable to the β-adrenoceptor affinities reported for atenolol (Abrahamson *et al.*, 1988; Rimele *et al.*, 1988) and metoprolol (Abrahamson *et al.*, 1988).

In summary, we have shown that purified enantiomers of propafenone and diprafenone are equivalent in their antiarrhythmic activity but differ considerably in their β-adrenoceptor antagonist potency. Only enantiomers with (S)-configuration exert appreciable β-blocking effects in the heart. These results favour the idea that the use of purified enantiomers of propafenone and diprafenone could be of therapeutic advantage for antiarrhythmic therapy.

References

ABRAHAMSON, T., EK, B. & NERME, V. (1988). The β_1- and β_2-adrenoceptor affinity of atenolol and metoprolol. *Biochem. Pharmacol.*, **37**, 203–208.

AKERMAN, S.B.A., CAMOUGIS, G. & SANDBERG, R.A. (1969). Stereoisomerism and differential activity in excitation block by local anaesthetics. *Eur. J. Pharmacol.*, **8**, 337–347.

ARIENS, E. (1984). Stereochemistry, a basis of sophisticated nonsens in pharmacokinetics and clinical pharmacology. *Eur. J. Clin. Pharmacol.*, **26**, 663–668.

ARIENS, E., WUIS, E. & VERINGA, E. (1988). Stereoselectivity of bioactive xenobiotics. *Biochem. Pharmacol.*, **37**, 9–18.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BLASCHKE, G. & WALTHER, B. (1987). Razemattrennung von Propafenon und Diprafenon, Konfiguration der Propafenon-Enantiomeren. *Liebigs Ann. Chem.*, 561–563.

BRADFORD, M.M. (1976). a rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.*, **72**, 153–165.

CAHN, R., INGOLD, C. & PRELOG, V. (1956). The specification of asymmetric configuration in organic chemistry. *Experientia*, **12**, 8124.

CATTERALL, W.A. (1987). Common modes of drug action on Na^+ channels: local anesthetics, antiarrhythmics and anticonvulsants. *Trends Pharmacol. Sci.*, **8**, 57–65.

CLARKSON, C.W. (1986). Use-dependent block of sodium channels by RAC 109 in isolated guinea pig ventricular myocytes is stereospecific. *Circulation*, **74** (Suppl. II), 20.

DUKES, I.D. & VAUGHAN WILLIAMS, E.M. (1984). The multiple modes of action of propafenone. *Eur. Heart J.*, **5**, 115–125.

GOVIER, W.C. (1965). The mechanism of the atrial refractory period change produced by ouabain. *J. Pharmacol. Exp. Ther.*, **148**, 100–105.

HILL, R.J., DUFF, H.J. & SHELDON, R.S. (1988). Determinants of stereospecific binding of type 1 antiarrhythmic drugs to cardiac sodium channels. *Mol. Pharmacol.*, **34**, 659–663.

HIRSOWITZ, G., PODRID, P.J., LAMPERT, S., STEIN, J. & LOWN, B. (1986). The role of beta-blocking agents as adjunct therapy to membrane stabilizing drugs in malignant ventricular arrhythmia. *Am. Heart J.*, **111**, 852–860.

KAUMANN, A.J. & BIRNBAUMER, L. (1974). Characteristics of the adrenergic receptor coupled to myocardial adenylyl cyclase. Stereospecifity and determination of apparent affinity constants for beta blockers. *J. Biol. Chem.*, **249**, 7874–7885.

KOHLHARDT, M. (1984). Block of sodium currents by antiarrhythmic agents: Analysis of the electrophysiologic effects of propafenone in heart muscle. *Am. J. Cardiol.*, **54**, 13D–19D.

KOHLHARDT, M. & SEIFERT, C. (1980). Inhibition of V_{max} of the action potential by propafenone and its voltage-, time, and pH-dependence in mammalian ventricular myocardium. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **315**, 55–62.

KOHLHARDT, M. & SEIFERT, C. (1983). Tonic and phasic I_{Na} blockade by antiarrhythmics. Different properties of drug binding to fast sodium channels as judged from V_{max} studies with propafenone and derivatives in mammalian ventricular myocardium. *Pflügers Arch.*, **396**, 199–209.

KROEMER, H.K., FUNCK-BRENTANO, C., SILBERSTEIN, D.J., WOOD, A.J.J., EICHELBAUM, M., WOOSLEY, R.L. & RODEN, D.M. (1989). Stereoselective disposition and pharmacologic activity of propafenone enantiomers. *Circulation*, **79**, 1068–1076.

KUKOVETZ, W.R., PÖCH, G., HOLZMANN, S., WURM, A. & PAIETTA, E. (1977). Wirkung von Propafenon auf Phosphodiesterase, Koronararterien und Herz. In *Fortschritte in der Pharmakotherapie von Herzrhythmusstörungen.* ed. Hochrein, H., Hapke, H.J. & Albrecht, O. pp. 9–20. Stuttgart, New York: Gustav Fischer Verlag.

LEDDA, F., MENTELLI, L., MANZINI, S., AMERINI, S. & MUGELLI, A. (1981). Electrophysiological and antiarrhythmic properties of propafenone in isolated cardiac preparations. *J. Cardiovasc. Pharmacol.*, **3**, 1162–1173.

LEMOINE, H. & KAUMANN, A.J. (1983). A model for the interaction of competitive antagonists with two receptor-subtypes characterized by a Schild-plot with apparent slope unity. Agonist-dependent enantiomeric affinity ratios for bupranolol in tracheae but not in right atria of guinea-pigs. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **322**, 111–120.

LINDNER, W. (1985). *Ger. Offen. DE 3,330,005; CA 103, 178036z.*

LINDNER, W., LEITNER, C. & URAY, G. (1984). Liquid chromatographic separation of enantiomeric alkanolamines via diastereomeric tartaric acid monoesters. *J. Chromatogr.*, **316**, 605–616.

McLEOD, A.A., SZILES, G.L. & SHAND, D.G. (1984). Demonstration of beta-adrenoceptor blockade by propafenone hydrochloride: clinical pharmacologic and adenylate cyclase activation studies. *J. Pharmacol. Exp. Ther.*, **228**, 461–466.

MORRIS, T.H. & KAUMANN, A.J. (1984). Different steric characteristics of β_1- and β_2-adrenoceptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **327**, 176–179.

MÜLLER-PELTZER, M., GREGER, G., NEUGEBAUER, G. & HOLLMAN, M. (1983). Beta blocking and electrophysiological effects of propafenone in volunteers. *Eur. J. Clin. Pharmacol.*, **25**, 831–833.

PAIETTA, E., PÖCH, G. & KUKOVETZ, W.R. (1977). Analyse der

ß-Blockerwirkung von Propafenon (SA 79). In *Fortschritte in der Pharmakotherapie von Herzrhthmusstörungen.* ed. Hochrein, H., Hapke, H.J. & Albrecht, O. pp. 20–28. Stuttgart, New York: Gustav Fischer Verlag.

POSTMAS, S.W. & CATTERALL, W.A. (1983). Inhibition of binding of [^{3}H]batrachotoxin A 20-alpha-benzoate to sodium channels by local anesthetics. *Mol. Pharmacol.*, **25**, 219–227.

RIMELE, T.J., HENRY, D.E., GIESA, F.R., BUCKLEY, S.K., GEIGER, G., HEASLIP, R.J., LEE, D.K. & GRIMES, D. (1988). Comparison of the β-adrenoceptor affinity and selectivity of cetamolol, atenolol, betaxolol, and ICI-118,551. *J. Cardiovasc. Pharmacol.*, **12**, 208–217.

SHELDON, R.S., CANNON, N.J. & DUFF, H.J. (1987). A receptor for type I antiarrhythmic drugs associated with rat cardiac sodium channels. *Circ. Res.*, **61**, 492–497.

SHELDON, R.S., CANNON, N.J. & DUFF, H.J. (1988). Stereospecific interactions of tocainide with the cardiac sodium channel. *Mol. Pharmacol.*, **33**, 327–331.

STOSCHITZKY, K., LINDNER, W., RATH, M., LEITNER, C., URAY, G., ZERNIG, G., MOSHAMMER, T. & KLEIN, W. (1989). Stereoselective haemodynamic effects of (R)- and (S)-propranolol in man. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **339**, 474–478.

VAUGHAN WILLIAMS, E.M. (1975). Classification of antidysrhythmic drugs. *Pharmacol. Ther.*, **B1**, 115–138.

WÄCHTER, W., MÜNCH, U., LEMOINE, H. & KAUMANN, A.J. (1980). Evidence that (+)-bupranolol interacts directly with myocardial β-adrenoceptors: control of optical purity with differential thermal analysis. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **313**, 1–8.

WAGNER, F., SACHS, W., TRENK, D. & JÄHNCHEN, E. (1987). Beta-adrenozeptoren-blockierende Wirkung von Diprafenon im Vergleich zu Propranolol. *Z. Kardiol.*, **76**, (S1), 81.

WIEMER, G., WELLSTEIN, A., PALM, D., VON HATTINGBERG, H.M. & BROCKMEIER, D. (1982). Properties of agonist binding at the β-adrenoceptor of the rat reticulocyte. *Naunyn-Schmidebergs Arch. Pharmacol.*, **321**, 11–19.

YEH, J.Z. (1980). Blockade of sodium channels by stereoisomers of local anaesthetics. In *Molecular Mechanisms of Anesthesia—Progress in Anesthesiologie.* ed. Funk, B.R. vol. 2, pp. 33–44. New York: Raven Press.

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Differential effect of R 56865 on ouabain binding to isolated sarcolemma and intact atrial tissue of guinea-pig

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1 R 56865 (N-[1-[4-(4-fluorophenoxy)-butyl]-4-piperidinyl]-N-methyl-2-benzothiazolamine) is a compound known to antagonize cardiac glycoside intoxication. Therefore, the effect of the compound on ouabain binding to intact cardiac tissue as well as cardiac membrane preparations was investigated.

2 The binding of ouabain to highly purified sarcolemmal membranes was not influenced by R 56865 1×10^{-6} mol l^{-1} (ouabain: $K_D = 1.3 \times 10^{-7}$ mol l^{-1}, $B_{max} = 160$ pmol mg^{-1}; ouabain + R 56865: $K_D = 1.4 \times 10^{-7}$ mol l^{-1}, $B_{max} = 168$ pmol mg^{-1}).

3 In contrast to the results in purified membranes, the binding of ouabain (10^{-8} mol l^{-1} to 5×10^{-7} mol l^{-1}) to intact atria was significantly reduced.

4 Ouabain, 5×10^{-7} mol l^{-1}, led to a transient positive inotropic effect of about 220% followed by a developing negative inotropic effect after 3 h. R 56865, 10^{-7} mol l^{-1}, led to a maximal positive inotropic effect of about 290% also followed by a delayed decline of contractile force. A tenfold higher concentration of R 56865 led to sustained positive inotropic effect of about 250% in the same time interval.

5 The different effects of R 56865 on ouabain binding in subcellular preparations and intact tissue do not support the view that R 56865 interferes directly with the action of ouabain on Na/K-ATPase. An indirect effect, which may be mediated by a lowered intracellular sodium load is discussed.

Introduction

R 56865 (N-[1-[4-(4-fluorophenoxy)-butyl]-4-piperidinyl]-N-methyl-2-benzothiazolamine), a cationic amphiphilic compound shifts the onset of toxic effects of digitoxin in the guinea-pig heart-lung preparation to higher concentrations (Schneider *et al.*, 1988). R 56865 reverses ouabain-induced arrhythmias in dogs (P. Remeysen & R. Xhonneux, personal communication) and diminishes ionic derangement, usually observed under toxic ouabain conditions, such as K^+ loss and Na^+ and Ca^{2+} gain (Peters, 1986). In papillary muscles, R 56865 strongly inhibits cardiac glycoside-induced arrhythmias (Vollmer *et al.*, 1987). In the nanomolar range R 56865 counteracts ouabain-induced shortening of the action potential (AP), decrease of AP amplitude, lowering of the resting membrane potential, and the occurrence of delayed afterdepolarizations, whereas under control conditions it neither influences the resting membrane potential nor the shape of the action potential (Vollmer *et al.*, 1987).

It is generally accepted that the positive inotropic and direct cardiotoxic effects of cardiac glycosides are mediated by inhibition of sarcolemmal Na/K-ATPase (Brody & Akera, 1977; Akera & Brody, 1978). However, it is not clear whether the inhibitory effects of R 56865 on cardiac glycoside intoxication are due to a direct interaction with the digitalis receptor. Therefore, the influence of R 56865 on ouabain binding to the Na/K-ATPase in highly purified guinea-pig sarcolemma and in intact electrically stimulated left atria was investigated. In the latter preparation effects on ouabain binding were compared with changes in contractile force.

A preliminary account of this work was presented as a poster to the Winter Meeting of the British Pharmacological Society (1988).

Methods

Preparation of sarcolemmal membranes

Sarcolemmal membranes were isolated from guinea-pig ventricles according to the method of Bers *et al.* (1986). Na/K-ATPase was enriched 29–40 fold, as estimated from [^{3}H]-ouabain binding in the presence of 0.01% saponin. The concentration of cardiac glycoside was 1×10^{-8} mol l^{-1}. A comparison of ouabain binding in the presence and absence of saponin revealed that more than 70% (72–75%) of the membranes were sealed vesicles.

Binding of ouabain to sarcolemmal membranes

Binding of [^{3}H]-ouabain was measured at 37°C as described by Herzig & Mohr (1984) with some modifications. In particular, saponin was included in the incubation media to permeabilize the large fraction of sealed vesicles. In brief, membranes (10 μg ml^{-1}) were suspended in a medium (final volume 1 ml) consisting of (mmol l^{-1}): NaCl 80, $MgCl_2$ 16, Tris-HCl 50 (final pH 7.3, after the addition of ATP), saponin 0.01% and [^{3}H]-ouabain 0.01–3 μmol l^{-1} in the presence or absence of R 56865 1×10^{-6} mol l^{-1}. Vesicles were preincubated with R 56865 for 20 min before addition of [^{3}H]-ouabain (0.16 μCi ml^{-1}) and the reaction was started with Na_2 ATP (final concentration 2.5 mmol l^{-1}). After an appropriate incubation time, membranes were harvested on glass fibre filters (Schleicher + Schüll, Sorte 6). Filters were washed with 10 ml ice-cold buffer containing (mmol l^{-1}): NaCl 80, $MgCl_2$ 16, Tris-HCl 50 (pH 7.3).

Dried filters were incubated with 1 ml Soluene 350 (Packard Instruments) at 60°C for 1 h. Scintillation cocktail (10 ml) (Hionic Fluor, Packard Instruments) was added and radioactivity was counted in a liquid scintillation counter (Beckman Instruments). Nonspecific binding was measured in the presence of unlabelled ouabain, 1×10^{-4} mol l^{-1}, and was subtracted from the total binding to determine specific binding.

The time course of ouabain binding was measured at a concentration of 1×10^{-7} mol l^{-1} over a period of 75 min. Maximal binding was achieved after 15 min of incubation (data not shown). For the establishment of specific binding curves sarcolemma was incubated with ouabain for 55 min.

Binding of ouabain to left atria

Guinea-pig left atria were suspended in Tyrode solution of the following composition (mmol l^{-1}): NaCl 124, KCl 4, $CaCl_2$ 0.9, $MgCl_2$ 1.1, $NaHCO_3$ 25, NaH_2PO_4 0.4, glucose 11, pH 7.4 which was gassed with carbogen (95% O_2/5% CO_2) and

kept at 32°C. Atria were preloaded with 0.33 g and electrically stimulated at 2 Hz by rectangular pulses of 4 ms duration at twice threshold voltage. After equilibration for 60 min or 30 min plus 30 min in the presence of R 56865, respectively, different concentrations of ouabain ($0.05\,\mu Ci\,ml^{-1}$) were added. At various time intervals atria were removed from the bath, blotted, weighed and processed for liquid scintillation counting.

Values were corrected for extracellular space according to Bentfeld *et al.* (1977).

Force of contraction

Guinea-pig left atria were set up according to a protocol identical to that described above and isometric contractions recorded. Atria were equilibrated for 60 min, or for 30 min plus 30 min with R 56865 before addition of ouabain which was allowed to act for 3 h.

Protein determination

Protein concentrations were determined according to the method of Bradford (1976) with bovine serum albumin used as standard.

Statistics

Data were analysed by Wilcoxon, Mann, Whitney-U-test. Criterion for significance was a P value of less than 0.05. All data are presented as mean ± s.d.

Results

Ouabain binding to sarcolemmal membranes

The time course of [^{3}H]-ouabain binding to purified sarcolemmal membranes was completely unaffected by R 56865, 1×10^{-6} mol l^{-1} (data not shown). Specific ouabain binding to the sarcolemmal vesicles was characterized by K_D-values of $1.31 \pm 0.41 \times 10^{-7}$ mol l^{-1} and $1.42 \pm 0.51 \times 10^{-7}$ M in the absence and presence of R 56865, respectively. Maximal binding amounted to 160.3 ± 12.3 pmol mg^{-1} sarcolemmal protein under control conditions and 167.9 ± 15.2 pmol mg^{-1} sarcolemmal protein in the presence of R 56865 (1×10^{-6} mol l^{-1}) (Figure 1). The difference between the K_D-values and maximal binding was not statistically significant.

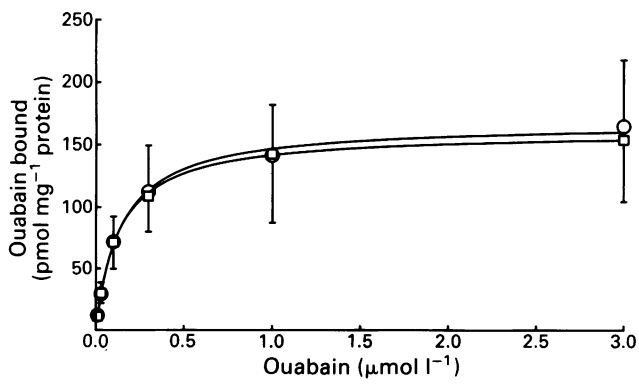

Figure 1 Specific equilibrium binding of [^{3}H]-ouabain to purified cardiac sarcolemmal membranes from guinea-pigs in the absence (□) or presence (○) of R 56865, 1×10^{-6} mol l^{-1}. Membranes were incubated in the reaction medium at 37°C for 55 min with different concentrations of [^{3}H]-ouabain. Specific binding was calculated as the difference between total binding and ouabain binding in the presence of 1×10^{-4} mol l^{-1} unlabelled ouabain. Each point represents the mean of four preparations with s.d. indicated by vertical bars.

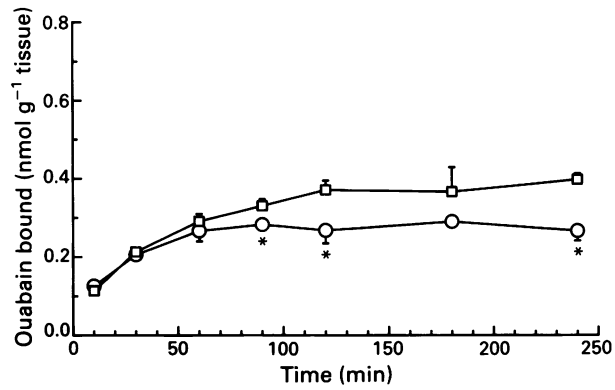

Figure 2 Binding of [^{3}H]-ouabain to paced guinea-pig left atria in the absence (□) or presence (○) of R 56865, 1×10^{-6} mol l^{-1}. Atria were incubated in a Tyrode solution at 32°C containing a low ouabain concentration (2×10^{-7} mol l^{-1}). Values were corrected for the amount of drug present in the extracellular space. Each point represents the mean ± s.d. of four experiments with s.d. shown by vertical bars. Asterisks indicate significant differences ($P < 0.05$) between treated and untreated atria.

Ouabain binding to guinea-pig left atria

At a low ouabain concentration (2×10^{-7} mol l^{-1}; see Figure 2), the time course of binding in the presence of R 56865, 1×10^{-6} mol l^{-1}, was qualitatively similar to that observed in control atria. However, maximal binding was reduced to about 70% of the control value.

At a high concentration of ouabain (5×10^{-7} mol l^{-1}; see Figure 3) major differences in the time course of ouabain binding in R 56865 treated and untreated atria were observed. The control binding curve was triphasic. An initial binding up to about 0.4 nmol g^{-1} wet wt. was followed by a significant decrease to 0.22 nmol g^{-1} wet wt., observed at 45 min. Thereafter, binding increased rapidly to about 0.7 nmol g^{-1} wet wt. This plateau was maintained throughout the period of observation. In contrast, pretreatment with R 56865 resulted in a monophasic type of binding resembling that at low ouabain concentrations. A value of about 0.4 nmol g^{-1} wet wt. was attained after 45 min, remaining constant until the end of the experiment.

Equilibrium binding was achieved in all left atrial experiments after at least 4 h of incubation with ouabain. Therefore, the extent of binding at 4 h of incubation was plotted versus the ouabain concentration. In controls, ouabain binding was augmented with increasing drug concentrations but levelled

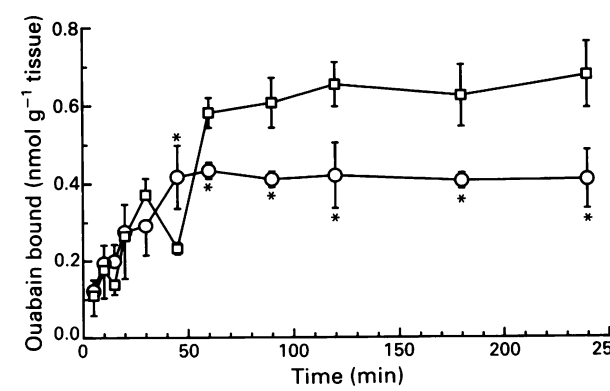

Figure 3 Binding of [^{3}H]-ouabain to paced guinea-pig left atria in the absence (□) or presence (○) of R 56865, 1×10^{-6} mol l^{-1}. Atria were incubated at 32°C for the indicated time in a Tyrode solution containing a high concentration of ouabain (5×10^{-7} mol l^{-1}). Values were corrected for the amount of drug present in the extracellular space. Each point represents the mean of four experiments with s.d. indicated by vertical bars. Asterisks indicate significant differences ($P < 0.05$) between treated and untreated atria.

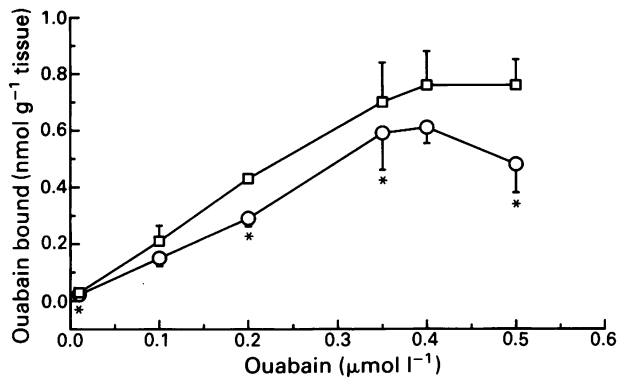

Figure 4 Binding of [^{3}H]-ouabain to paced guinea-pig left atria in the absence (□) or presence (○) of R 56865, 1×10^{-6} mol l^{-1}. Atria were incubated in a Tyrode solution at 32°C for 4 h with different concentrations of [^{3}H]-ouabain. Values were corrected for the amount of drug present in the extracellular space. Each point represents the mean ± s.d. of four to eleven experiments with s.d. shown by vertical bars. Asterisks indicate significant ($P < 0.05$) decrease in [^{3}H]-ouabain-binding.

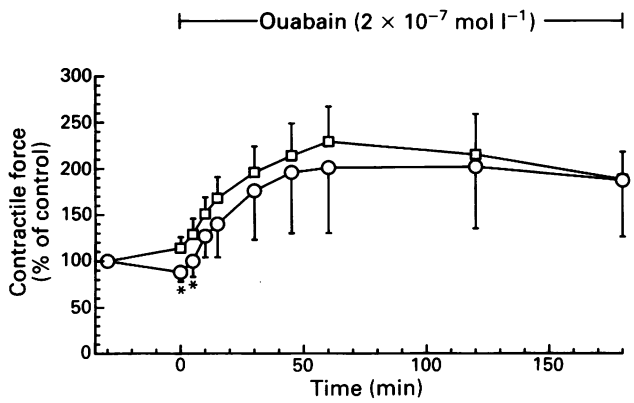

Figure 5 Time course of the inotropic effects of ouabain, 2×10^{-7} mol l^{-1}, in isolated left atria in the absence (□) or presence (○) of R 56865, 1×10^{-7} mol l^{-1}. Atria were suspended in Tyrode solution at 32°C and electrically driven at 2 Hz. Each point represents the mean of six experiments with s.d. shown by vertical bars.

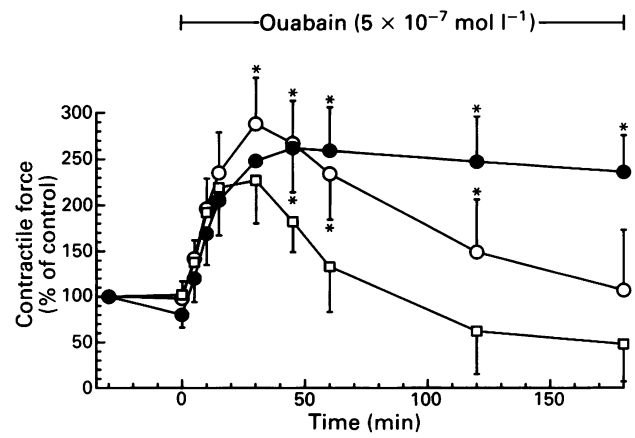

Figure 6 Time course of the effects of ouabain, 5×10^{-7} mol l^{-1}, on the contractile force of isolated left atria in the absence (□) or presence of 1×10^{-7} mol l^{-1} (○) and 1×10^{-6} mol l^{-1} (●) R 56865. Atria were suspended in Tyrode solution at 32°C and paced at a frequency of 2 Hz. Each point represents the mean of six experiments with s.d. shown by vertical bars. Significant differences ($P < 0.05$) of R 56865-treated atria from atria without the compound are indicated by asterisks.

off at high concentrations of the cardiac glycoside (Figure 4). No attempt was made to describe this behaviour by a classical hyperbolic binding isotherm, because ouabain binding to intact tissue opposed to membrane preparations fails to follow this algorithm (Herzig *et al.*, 1985a,b). Pretreatment with R 56865, 1×10^{-6} mol l^{-1}, reduced ouabain binding at all concentrations (1×10^{-8} mol l^{-1} to 5×10^{-7} mol l^{-1}). The reduction ranged between 16 and 37% of the control value. The concentration-dependency of the equilibrium binding of ouabain was not significantly affected by R 56865 (1×10^{-6} mol l^{-1}) (Figure 4).

Force of contraction of guinea-pig left atria

In the presence of R 56865 (1×10^{-7} mol l^{-1}) ouabain, 2×10^{-7} mol l^{-1}, elicited a positive inotropic effect, with peak tensions reaching control values (Figure 5). The slight but significant difference in contractile force observed at 0 and 5 min seems not to be relevant but rather the result of an unusual slight increase in the contractile force of the controls already before ouabain treatment.

The positive inotropic effect of 5×10^{-7} mol l^{-1} ouabain was transient, attaining a maximum of about 220% of control contractile tension after about 30 min.

Thereafter, a progressive loss of tension could be observed over a period of 3 h. Diastolic tension did not increase during the experimental period. Ouabain (5×10^{-7} mol l^{-1}) produced a larger positive inotropic effect and slower rate of decline in contractile force after peak effect in the presence of R 56865, 1×10^{-7} mol l^{-1} (Figure 6). Tenfold higher concentrations of R 56865 totally prevented the decline in the positive inotropic effect of ouabain. Thus in the presence of R 56865, 1×10^{-6} mol l^{-1}, ouabain (5×10^{-7} mol l^{-1}) induced a sustained positive inotropic effect (about 260% of control value) throughout the whole observation period (Figure 6). This effect was not different from the maximal positive inotropic effect of a nontoxic ouabain concentration (Figure 5). At 1×10^{-6} mol l^{-1}, R 56865 alone produced a small decrease in contractile force.

Discussion

The results demonstrate that R 56865, 1×10^{-6} mol l^{-1}, inhibits the development of signs of toxicity like the negative inotropic effect of a high concentration of ouabain. This protection is achieved without compromising the positive inotropic effect of the cardiac glycoside. The equilibrium binding of ouabain to beating atria is reduced significantly by 1×10^{-6} mol l^{-1} R 56865 at subtherapeutic glycoside concentrations as well as at high ouabain concentrations.

The time course of binding shows no qualitative alterations in the presence and absence of R 56865 at low ouabain concentrations. Yet, a difference is obvious at a high concentration of the cardiac glycoside, where it prevents the triphasic binding characteristics which are considered to be a sign of cardiac glycoside toxicity (Busse *et al.*, 1979; Peters, 1986).

In contrast to the results in intact atria, R 56865 does not alter ouabain binding to purified cardiac sarcolemma. This suggests that R 56865 does not interfere with ouabain binding directly to the Na/K-ATPase. Furthermore, R 56865 neither inhibits nor stimulates Na/K-ATPase activity in purified cardiac membranes (F. Tegtmeier, personal communication). There is also no interference of the compound with the ouabain inhibition of erythrocyte Na pumps (Finet *et al.*, 1989). The modification of ouabain binding observed only with beating atria rather suggests an indirect effect of R 56865 on Na/K-ATPase, for which intact cells are a prerequisite.

Under physiological conditions ouabain binds to the so-called Na-induced conformation of the Na/K-ATPase (Lee & Klaus, 1971; Schwartz *et al.*, 1975; Akera & Brody, 1978; Lüllmann & Peters, 1979). Consequently, ouabain binding to intact cells (at low subtherapeutic concentrations, e.g.

10^{-8} mol l^{-1}) is enhanced by raising intracellular [Na^+] (Bentfeld *et al.*, 1977; Temma & Akera, 1982; Kennedy *et al.*, 1983). Alternatively, a compound reducing the 'Na-load' of the cell would be expected to reduce ouabain binding to intact cells. R 56865 may thus reduce ouabain binding to atria by interfering with the regulation of Na-pump activity. Since the intracellular free Na-concentration is the most important factor for the ATPase turnover rate (Eisner *et al.*, 1981; Akera & Brody, 1982), it may be speculated that R 56865 influences the intracellular Na concentration. The reduced equilibrium binding of ouabain to atria over a wide ouabain concentration-range suggests a reduced Na load of the intact cells after pretreatment with R 56865. A comparable situation cannot be achieved in a membrane suspension, where the Na/K-ATPase always faces a constant ionic environment, in particular a constant Na concentration.

The molecular basis on which a reduction of Na-load may be achieved is not clear. An influence of R 56865 on cellular Na-permeability may be considered but needs further experimental investigation.

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References

AKERA, T. & BRODY, T.M. (1978). The role of the Na, K-ATPase in the inotropic action of digitalis. *Pharmacol. Rev.*, **29**, 187–220.

AKERA, T. & BRODY, T.M. (1982). Myocardial membranes: Regulation and function of the sodium pump. *Annu. Rev. Physiol.*, **44**, 375–388.

BENTFELD, M., LÜLLMANN, H., PETERS, T. & PROPPE, D. (1977). Interdependence of ion transport and the action of ouabain in heart muscle. *Br. J. Pharmacol.*, **61**, 19–27.

BERS, D.M., ALLEN, L.-A., H. & KIM, Y. (1986). Calcium binding to cardiac sarcolemmal vesicles: potential role as a modifier of contraction. *Am. J. Physiol.*, **251** (Cell Physiol., 20), C861–C871.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.*, **72**, 248–254.

BRODY, T.M. & AKERA, T. (1977). Relation among Na, K-ATPase activity, sodium pump activity, transmembrane sodium movement, and cardiac contractility. *Fedn. Proc.*, **36**, 2219–2224.

BUSSE, F., LÜLLMANN, H. & PETERS, T. (1979). Concentration dependence of the binding of ouabain to isolated guinea-pig atria. *J. Cardiovasc. Pharmacol.*, **1**, 687–698.

EISNER, D.A., LEDERER, W.J. & VAUGHAN-JONES, R.D. (1981). The dependence of sodium pumping and tension on intracellular sodium activity in voltage-clamped sheep Purkinje fibres. *J. Physiol.*, **317**, 163–187.

FINET, M., BRAVO, G. & GODFRAIND, T. (1989). The protective action of R 56865 against ouabain-induced intoxication in rat heart isolated atria and ventricles. *Eur. J. Pharmacol.*, **164**, 555–563.

HERZIG, S., KREY, U., LÜLLMANN, H. & MOHR, K. (1985a). Is digitalis binding in intact myocardium co-operative? *Trends Pharmacol. Sci.*, **6**, 432–433.

HERZIG, S., LÜLLMANN, H. & MOHR, K. (1985b). On the cooperativity of ouabain-binding to intact myocardium. *J. Mol. Cell. Cardiol.*, **17**, 1095–1104.

HERZIG, S. & MOHR, K. (1984). Action of ouabain on rat heart: comparison with its effect on guinea-pig heart. *Br. J. Pharmacol.*, **82**, 135–142.

KENNEDY, R.H., AKERA, T. & BRODY, T.M. (1983). Suppression of positive inotropic and toxic effects of cardiac glycosides by amiloride. *Eur. J. Pharmacol.*, **89**, 199–209.

LEE, K.S. & KLAUS, W. (1971). The subcellular basis for the mechanism of inotropic action of cardiac glycosides. *Pharmacol. Rev.*, **23**, 193–261.

LÜLLMANN, H. & PETERS, T. (1979). Action of cardiac glycosides on the excitation-contraction coupling in heart muscle. *Prog. Pharmacol.*, **2**, 1–57.

PETERS, T. (1986). Glycoside receptors in the heart. *Prog. Pharmacol.*, **6**, 65–80.

SCHNEIDER, J., BECK, E. & BORGERS, M. (1988). Dose dependent protection by R 56865 against digitalis toxicity in the guinea-pig heart-lung preparation. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **337**, R 260.

SCHWARTZ, A., LINDENMAYER, G.E. & ALLEN, J.C. (1975). The sodium-potassium adenosine triphosphatase: pharmacological, physiological and biochemical aspects. *Pharmacol. Rev.*, **27**, 3–134.

STEMMER, D. & AKERA, T. (1988). Sodium-pump activity and its inhibition by extracellular calcium in cardiac myocytes of guinea-pigs. *Biochim. Biophys. Acta*, **937**, 247–257.

TEMMA, K. & AKERA, T. (1982). Enhancement of cardiac actions of ouabain and its binding to Na^+, K^+-adenosine triphosphatase by increased sodium influx in isolated guinea-pig heart. *J. Pharmacol. Exp. Ther.*, **223**, 490–496.

VOLLMER, B., MEUTER, C. & JANSSEN, P.A.J. (1987). R 56865 prevents electrical and mechanical signs of ouabain intoxication in guinea-pig papillary muscle. *Eur. J. Pharmacol.*, **142**, 137–140.

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Comparison of the effects of several potassium-channel openers on rat bladder and rat portal vein *in vitro*

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1 The ability of several K-channel openers to inhibit KCl-induced contractions of rat bladder detrusor and spontaneous mechanical activity in rat portal vein was examined.

2 Lemakalim, pinacidil, Ro 31-6930, RP 49356, P1060 and S 0121 dose-dependently relaxed rat detrusor, precontracted with 20 mM KCl. With the exception of pinacidil, concentrations of these agents below 30 μM did not inhibit 80 mM KCl-included contractions. Pinacidil (10 μM) produced a small, but significant ($P < 0.05$) relaxation of 80 mM KCl-induced mechanical activity. Minoxidil sulphate and BRL 38226 produced some relaxation of 20 mM but not 80 mM KCl-induced contractions.

3 Glibenclamide (0.3–3 μM) antagonized the relaxant effects of lemakalim, pinacidil, Ro 31-6930, RP 49356, P1060 and S 0121 in a competitive manner (pA_2 values 6.3–6.6). The effects of minoxidil sulphate and BRL 38226 were fully antagonized by 3 μM glibenclamide.

4 Lemakalim, pinacidil, S 0121, BRL 38226 and minoxidil sulphate were each approximately 8 times more potent as inhibitors of the spontaneous contractions of rat portal vein than KCl-induced contractions of the rat detrusor. Minoxidil sulphate was approximately 30 times more potent in the rat portal vein than in the bladder. This may indicate that either minoxidil sulphate is acting at different recognition sites in these two tissues, or that this compound has an additional mechanism of action in the portal vein.

5 With the exception of minoxidil sulphate, all the compounds tested stimulated ^{86}Rb efflux and ^{42}K efflux from preloaded rat detrusor strips. The stimulated ^{86}Rb efflux was qualitatively but not quantitatively similar to the stimulated ^{42}K efflux. Minoxidil sulphate stimulated ^{42}K efflux from rat portal vein but not from rat bladder.

6 It is concluded that all the compounds tested cause relaxation of rat detrusor predominantly by K-channel opening. Selectivity for bladder rather than vascular smooth muscle was not shown by any compound.

Introduction

K-channel opening drugs such as cromakalim and pinacidil are currently being evaluated for the treatment of hypertension (Singer *et al.*, 1989) and asthma (Williams *et al.*, 1990) and they may also prove to be beneficial in the control of other conditions including bladder instability. Typically, the unstable bladder is treated with antimuscarinic drugs (e.g. oxybutinine, terodiline or propantheline) or with tranquilliser-sedative-hypnotic drugs (e.g. imipramine) although these agents are not ideal (Mundy & Stephenson, 1984; Andersson & Mattiasson, 1988). Pinacidil and cromakalim are capable of reducing spontaneous mechanical activity in rat, pig or human detrusor (Foster *et al.*, 1989a,b; Fovaeus *et al.*, 1989; Malmgren *et al.*, 1990) and *in vivo* experiments have demonstrated that cromakalim and pinacidil abolish the contractions associated with bladder outflow obstruction in pig and rat without affecting the ability to void urine (Foster *et al.*, 1989a; Malmgren *et al.*, 1989).

Such data suggest that K-channel openers could be clinically useful in the treatment of the irritable bladder syndrome, a condition which is very prevalent, especially in males with benign prostatic hyperplasia (McGuire, 1984). However, because of their relaxant effect on the vasculature, these agents may also reduce blood pressure in normotensive patients, an effect which has already been demonstrated for cromakalim (Eckl & Greb, 1987; Fox *et al.*, 1987) and for pinacidil (see review by Friedel & Brogden, 1990). To be clinically useful in the irritable bladder syndrome, the K-channel opener should ideally be a selective inhibitor of detrusor muscle contractions in comparison to effects on vascular smooth muscle.

The aim of the present study was to compare the *in vitro* effects of several K-channel openers on bladder and vascular smooth muscle to determine any degree of tissue specificity. Two of the benzopyrans used in this study were the single enantiomeric components of the racemate cromakalim, the relatively inactive BRL 38226 and the biologically active lemakalim (BRL 38227) (Buckingham *et al.*, 1986; Bray *et al.*, 1987; Hof *et al.*, 1988). The two other benzopyrans were S 0121, which differs from BRL 38226 only by the presence of a methyl substituent on the lactam ring, and Ro 31-6930 which is a non-chiral molecule (lacking the hydroxyl group at the 3 position) with a six-membered ring replacing the lactam ring in the 4 position. We also used pinacidil and the related compound P 1060, both K-channel openers of the guanidine group (Edwards & Weston, 1990). In addition, the effects of RP 49356, a substituted thioformamide which opens ATP-sensitive K-channels in isolated cardiac myocytes (Escande *et al.*, 1988), and minoxidil sulphate which opens K-channels in isolated smooth muscle (Meisheri *et al.*, 1988; Leblanc *et al.*, 1989; Newgreen *et al.*, 1990) were studied. Their activity and therapeutic potential have been recently reviewed (Edwards & Weston, 1989; 1990).

Methods

Experiments were performed on bladders or portal veins isolated from male Sprague-Dawley rats (280–350 g) which were killed by stunning and bleeding.

Tissue bath experiments

Bladder detrusor Four longitudinal strips of detrusor were dissected from each bladder and each was mounted for isometric tension recording under a resting tension of 1 g. Tissues were allowed to equilibrate for 1 h in Krebs solution at 37°C, aerated with 95% O_2:5% CO_2. During this period the tissues

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were frequently washed and their resting tension continuously adjusted until it was maintained in the range 0.75–1 g.

To evaluate the spasmolytic effects of the K-channel openers, the detrusor strips were first exposed to 20 mM or 80 mM KCl. Maximal mechanical effects were produced within 15–20 min of exposure to KCl, after which time the tissues were exposed to a single K-channel opener using a cumulative protocol and with concentration increments at 5 min intervals. After a maximum relaxant response to the K-channel opener had been achieved, 1 mM aminophylline was added to produce full relaxation. For each tissue, the mechanical activity in the presence of each concentration of K-channel opener applied was integrated and expressed as a percentage of the response to 20 mM KCl (=100%) with the response to aminophylline defining the mechanical zero point (=0%). Following initial exposure to a K-channel opener, tissues were washed frequently for 15 min and then incubated with glibenclamide for 30 min. During the last 15 min of a glibenclamide incubation, 20 mM KCl was also present. The responses to the K-channel openers were reassessed in the continued presence of glibenclamide. In this manner, each tissue was sequentially exposed to a total of 2 or 3 increasing concentrations of glibenclamide (typically 0.1 μM, 1 μM and 3 μM). Concentration-effect curves for the K-channel openers were also repeated in tissues which were not exposed to glibenclamide to detect any time-dependent changes in tissue sensitivity.

Portal vein Whole portal veins were mounted under 1 g tension for isometric recording. The tissues were allowed to equilibrate in Krebs solution for 1 h before they were exposed at 5 min intervals to increasing concentrations of a single K-channel opener using a cumulative protocol.

Mechanical responses of both the portal vein and bladder were recorded and the activity integrated with respect to time by use of an Apple Macintosh Plus computer in conjunction with a MacLab system (World Precision Instruments) and software (MacLab system chart recorder software programme version 2.5) supplied by Analog Digital Instruments.

Efflux studies

Each rat detrusor was cut into 4 equal pieces as already described and each was allocated to a separate treatment group as follows: (1) vehicle control, (2) K-channel opener, (3) K-channel opener + 1 μM glibenclamide, (4) K-channel opener + 3 μM glibenclamide. Each group contained tissues from 5 different animals. Whole portal veins were similarly grouped (n = 5). Pieces of detrusor or whole portal veins were then impaled on syringe needles and attached to a gassing manifold. The tissues were allowed to equilibrate for about 1 h in normal Krebs at 37°C and then incubated for 3 h in Krebs solution in which the potassium was derived from $^{42}K_2CO_3$ and to which ^{86}Rb had been added to give final activities of 1.57 μCi ml^{-1} ^{42}K and 5 μCi ml^{-1} ^{86}Rb. The final concentration of Rb in the Krebs solution did not exceed 60 μM. Following this loading period, each tissue was passed at 4 min intervals through a series of tubes containing 3 ml normal Krebs together with K-channel opener or glibenclamide as appropriate for the treatment group. The tissues were then blotted and the total (^{42}K and ^{86}Rb) activity of the tissue and of the efflux samples corresponding to each collection period was determined in a γ-counter. After approximately 10 half-lives of ^{42}K (5 days), the samples and tissues were recounted to determine the activity associated with the ^{86}Rb (using a correction factor to compensate for ^{86}Rb decay). The values obtained were subtracted from the total activity counts to yield the counts due to ^{42}K. The fractional loss of ^{42}K and ^{86}Rb from each tissue was standardized for a 1 min period and expressed as a rate coefficient (% per min).

Drugs and solutions

The following substances were used: BRL 38226 ((+)-3**R**,4**S**-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-pyrrolidin-1'-yl)-2*H*-1-benzopyran-3-ol) and lemakalim ((−)-3**S**,4**R**-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2'-oxopyrrolidin-1'-yl)-2*H*-1-benzopyran-3-ol; SmithKline Beecham), pinacidil ((±)-N''-cyano-*N*-4-pyridyl-N'-1,2,2-trimethylpropyl-guanidine mono-hydrate; Leo), P1060 (N'-cyano-*N*-(3-pyridyl)-N''-(*t*-butyl) guanidine; Pfizer), Ro 31-6930 (2-(6-cyano-2,2-dimethyl-2H-1benzopyran-4-yl)pyridine 1-oxide; Roche), RP 49356 ((±)-N-methyl-2(3-pyridinyl)-tetrahydrothiopyran-2-carbothioamide-1-oxide; Rhône-Poulenc), S 0121 ((−)-3**R**,4**S**, 5'**R**-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2'-oxo-5'methyl-pyrrolidin-1'-yl)-2*H*-1-benzopyran-3-ol; Hoechst), minoxidil sulphate (2,4-diamino-6-piperidinyl-pyrimidine-3-oxide sulphate; ICI), glibenclamide and aminophylline (Sigma), $^{42}K_2CO_3$ (University of Manchester Reactor Facilities, Risley). Glibenclamide was made as a 1 mM stock solution in absolute ethanol and aminophylline as a 100 mM stock solution in distilled water. All other compounds were prepared as stock solutions in 70% or 100% ethanol and diluted with twice distilled water. Minoxidil sulphate was dissolved immediately before use.

The Krebs solution had the following composition (mM): Na^+ 142.7, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 127.6, $H_2PO_4^-$ 1.2, HCO_3^- 25, SO_4^{2-} 1.2 and glucose 11.1.

Statistical analysis

The significance of differences between two means was assessed by Student's t test.

Results

Effects of K-channel openers on mechanical activity

In vitro, strips of detrusor muscle showed spontaneous contractions, although the tension generated during each contraction was small. The addition of 20 mM KCl increased the frequency and amplitude of the phasic tension waves. Figure 1a shows that lemakalim produced a concentration-dependent reduction in the amplitude of contractions generated by 20 mM KCl, an inhibitory effect that was antagonized by glibenclamide in a manner suggestive of competitive antagonism. Time-matched concentration-effect curves to lemakalim showed that there was no alteration in the tissue response with time or previous exposure to lemakalim (data not shown). Pinacidil, Ro 31-6930, RP 49356, P1060 and S 0121 each inhibited contractions produced by 20 mM KCl, effects which were antagonized by glibenclamide in a competitive-like manner (Figure 1b–f). Minoxidil sulphate was relatively impotent, and was inhibited by glibenclamide in a non-competitive manner (Figure 1g). The highest concentration of BRL 38226 tested in detrusor, 30 μM, produced only a slight inhibition in the response to 20 mM KCl, an effect that was antagonized by glibenclamide (Figure 1h). Table 1 summarises some of the characteristics of the K-channel openers in relaxing a 20 mM KCl-induced contraction of rat detrusor. The rank order of potency was Ro 31-6930 > P1060 = lemakalim ≥ pinacidil > RP 49356 > S 0121 > minoxidil sulphate > BRL 38226.

The effects of lemakalim, pinacidil, S 0121, BRL 38226 and minoxidil sulphate were also examined in portal veins (Figure 2), providing some comparison of the relative potency of these compounds in the two smooth muscle types. A comparison of the IC_{50} values (Table 1) shows that, with the exception of minoxidil sulphate, all the compounds tested were approximately 8 times more potent in the rat portal vein than in rat detrusor. Schild analysis of the inhibition of the relaxant responses of detrusor to the K-channel openers by glibenclamide yielded similar pA_2 values and, with the exception of pinacidil, slopes greater than 1 (Table 1). The pA_2 value for the antagonism by glibenclamide was derived from dose ratios of the mean responses of 4 tissues in the absence and in the presence of two (S 0121 – portal vein) or three concentrations

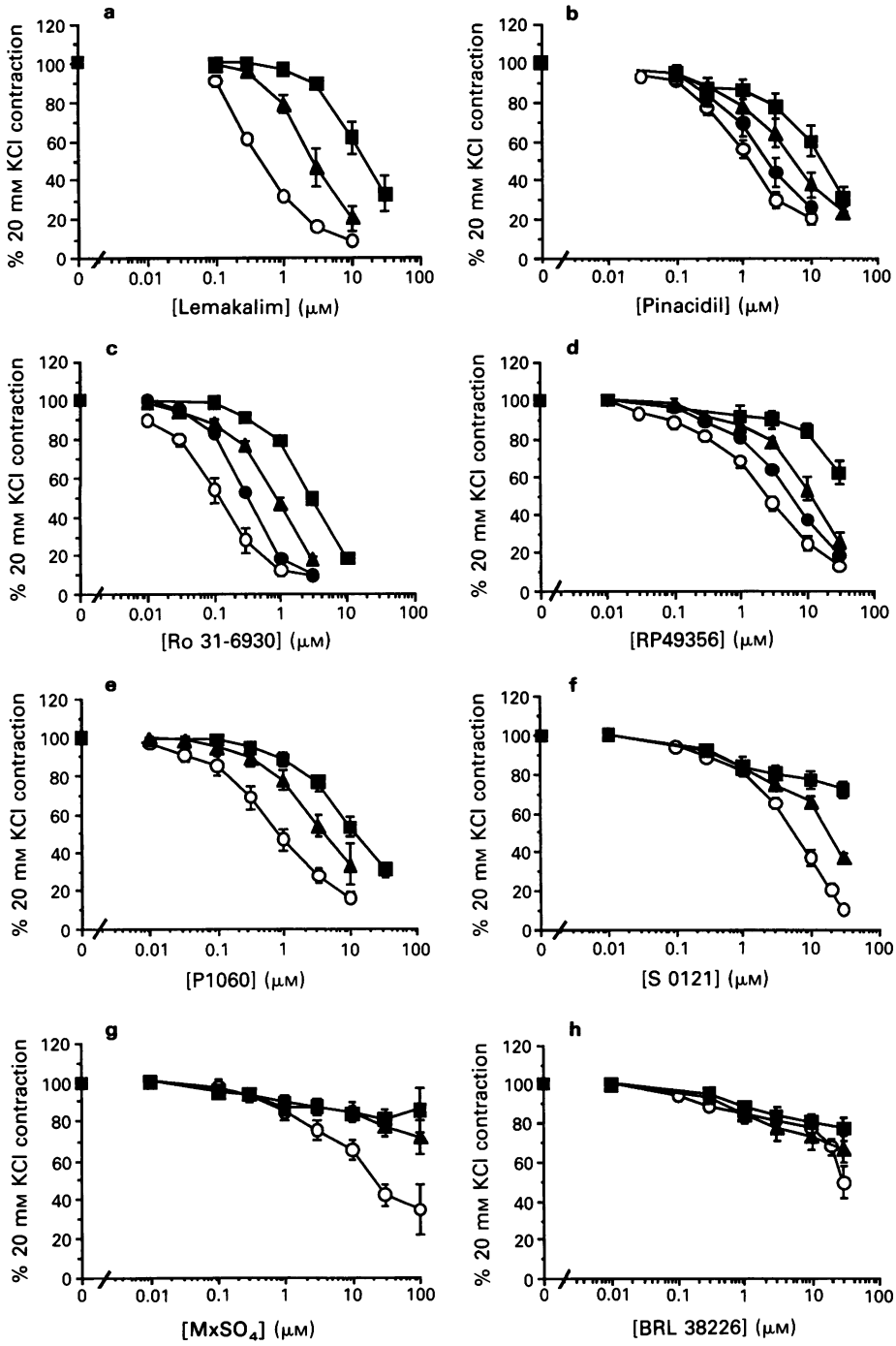

Figure 1 Suppression of 20 mM KCl-induced mechanical activity in rat detrusor by K-channel openers. Symbols represent the drug alone (○) or in the presence of 0.3 μM (●), 1 μM (▲) or 3 μM (■) glibenclamide. Data points represent the mean from at least 4 animals. Vertical bars indicate ± s.e.mean.

of glibenclamide. The pA_2 for glibenclamide against lemakalim in the bladder (6.61 ± 0.11) was similar to that in the portal vein (6.79 ± 0.11). In contrast the pA_2 for glibenclamide against pinacidil in the bladder (6.51 ± 0.11) was lower than that in the portal vein (7.21 ± 0.17: $P < 0.01$). The low potencies of BRL 38226 and S 0121 in the detrusor, and of BRL 38226 in portal vein, prevented determination of pA_2 values. The pA_2 for glibenclamide against S 0121 in the portal vein (7.01 ± 0.28) did not differ from that against pinacidil in this tissue, but was significantly higher than the value against lemakalim.

At concentrations below 30 μM neither RP 49356, lemakalim, P1060, S 0121 nor Ro 31-6930 had an effect on the response to 80 mM KCl (Figure 3). Each of these agents produced a small, but significant ($P < 0.05$; Student's t test), reduction in the response to 80 mM KCl at the highest tested concentration (100 μM). Figure 3 shows that minoxidil sulphate caused no reduction in the magnitude of the contractions induced by 80 mM KCl. Concentrations of pinacidil above 10 μM however, produced a small but significant concentration-dependent decrease in the 80 mM KCl-induced contractions ($P < 0.05$).

Effects of K-channel openers on ^{42}K and ^{86}Rb efflux

Figure 4 shows stimulation of ^{42}K and ^{86}Rb efflux from bladder detrusor strips by lemakalim and antagonism of this effect by glibenclamide. Such a result is typical of K-channel openers in a variety of tissues. With the exception of minoxidil sulphate, all the agents tested produced similar effects on the

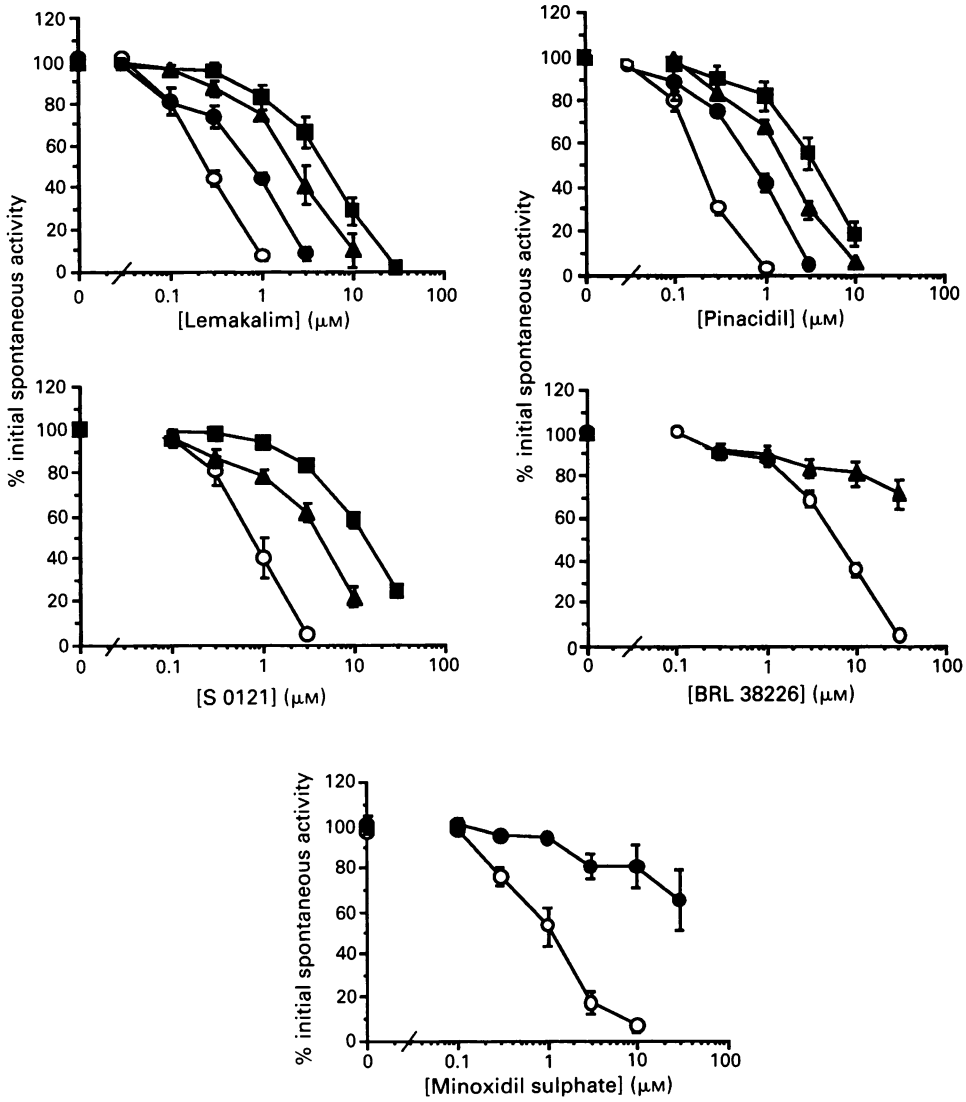

Figure 2 Suppression of spontaneous mechanical activity in rat portal vein by K-channel openers. Symbols represent the drug alone (○) or in the presesnce of 0.3 μM (●), 1 μM (▲) or 3 μM (■) glibenclamide. Data points represent the mean from at least 4 animals. Vertical bars indicate ± s.e.mean.

Table 1 A comparison of the IC_{50} values of some K-channel openers, pA_2 values and slopes for antagonism by glibenclamide in rat bladder strips and rat portal vein

	IC_{50} (μM)	pA_2	Slope
Rat bladder			
Ro 31-6930	0.12 (0.09–0.16)	6.54 ± 0.21	1.69
Lemakalim	0.86 (0.44–1.68)	6.61 ± 0.11	1.55
P1060	0.82 (0.52–1.29)	6.33 ± 0.15	1.36
*Pinacidil	1.59 (1.21–2.09)	6.51 ± 0.11	1.04
*RP 49356	2.69 (1.99–3.62)	6.30 ± 0.17	1.49
S 0121	5.84 (4.95–6.89)	ND	—
$MxSO_4$	23.51 (16.84–32.82)	ND	—
BRL 38226	>20.00*	ND	—
Rat portal vein			
Lemakalim	0.08 (0.07–0.10)	6.79 ± 0.11	1.01
*Pinacidil	0.21 (0.15–0.30)	7.21 ± 0.17	0.75
S 0121	0.75 (0.64–0.89)	7.01 ± 0.28	0.71
$MxSO_4$ (n = 2)	1.02 (0.34–3.08)	ND	—
BRL 38226	5.93 (5.27–6.67)	ND	—

Values are geometric means (95% confidence limits) or arithmetic means ± s.e.mean (n = 4–8). In some instances the low potency of the K-channel opener prevented determination of the pA_2 value for antagonism by glibenclamide (ND). *Racemic mixtures; if the active enantiomeric component had been available, the IC_{50} value would theoretically have been halved.

detrusor strips. Glibenclamide alone was without effect on either ^{42}K or ^{86}Rb basal efflux but produced a concentration-dependent reduction in the efflux stimulated by lemakalim, P1060, Ro 31-6930, RP 49356, S 0121 and pinacidil. Figure 5 provides a comparison of the maximum increases in the ^{42}K and ^{86}Rb efflux rate coefficients (as a percentage of the efflux rate coefficient for each tissue at the time point immediately before addition of the K-channel opener) produced by approximately three times the maximum relaxant concentration of each K-channel opener. The corresponding drug-stimulated increases in the presence of 1 μM and 3 μM glibenclamide are also indicated. As the efflux rate coefficient falls during the efflux collection period (see Figure 5), the efflux rate coefficient for the control tissues in the time period which corresponded to the peak efflux collection period for drug-treated tissues was less than that which corresponded to the last collection period before addition of the drug. This is expressed as a fall in the 'max % increase in efflux rate coefficient' in Figure 5.

Unfortunately, insufficient minoxidil sulphate was available to allow investigation of the effect of three times the maximum relaxant concentration on efflux rates. The concentration used (30 μM) was less than the maximum relaxant concentration (100 μM). Compared with the vehicle control, this concentration of minoxidil sulphate had no effect on either ^{86}Rb efflux (data not shown) or ^{42}K efflux from detrusor strips (Figure 6a), but, in the same experiment, significantly increased ^{42}K

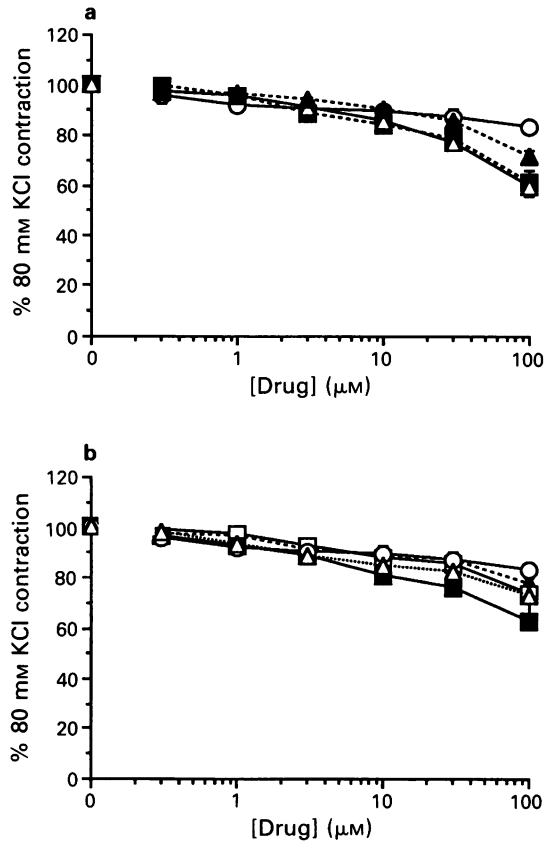

Figure 3 Effects of K-channel openers on mechanical activity of rat detrusor pre-contracted with 80 mM KCl. In (a), control (○); lemakalim (■); pinacidil (△) and S 0121 (▲). In (b), control (○); Ro 31-6930 (■); RP 49356 (□); minoxidil sulphate (▲) and P1060 (△). Data points represent the mean from 4 animals. Vertical bars indicate ±s.e.mean.

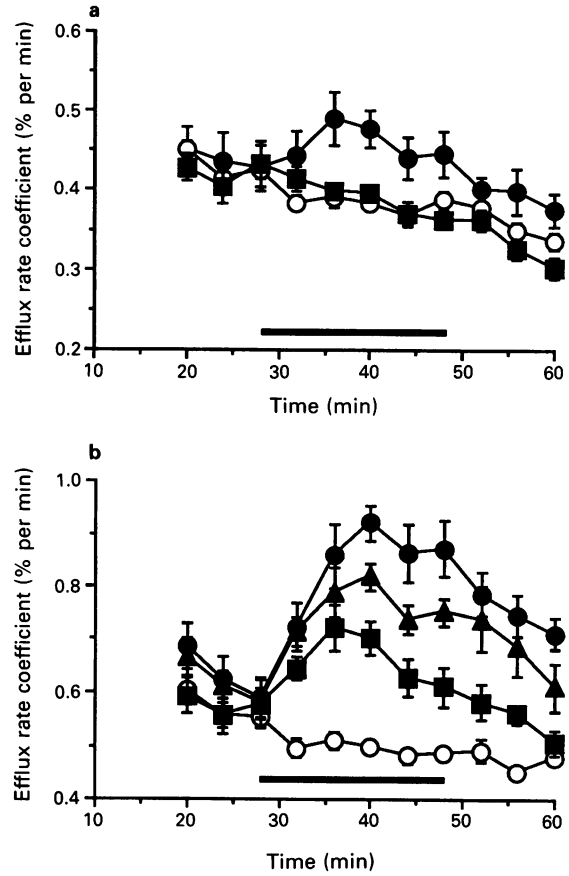

Figure 4 Rat detrusor: effect of 1 μM (▲) or 3 μM (■) glibenclamide on (a) ^{86}Rb or (b) ^{42}K efflux stimulated by 5 μM lemakalim (●); (○) indicates vehicle controls. The horizontal bar indicates the time during which the tissues were exposed to lemakalim or vehicle. Glibenclamide was present for 28 min before and during the exposure to lemakalim. Each point represents the mean from 5 animals. Vertical bars indicate ± s.e.mean.

efflux from rat portal vein, an effect which was inhibited by 1 μM glibenclamide (Figure 6b). The low potency of BRL 38226 also prevented the effects of an equipotent concentration of this K-channel opener from being tested. This concentration of the compound had no effect on ^{86}Rb efflux. Nevertheless, it was possible to show that BRL 38226 (100 μM) increased ^{42}K efflux (Figure 5).

Discussion

With the exception of minoxidil sulphate and high concentrations of pinacidil and lemakalim, the compounds tested in the rat bladder in this study acted in a manner typical of potassium channel openers. They relaxed 20 mM but not 80 mM KCl-induced contractions and stimulated ^{42}K and ^{86}Rb efflux from preloaded tissues (Hamilton *et al.*, 1986; Weir & Weston, 1986). Although the K-channel openers were less potent in the bladder than in other tissues, their relative potencies were, in general, similar. Thus, the compound Ro 31-6930, which in this study was about 5 times more potent than lemakalim (biologically the more active enantiomer of the racemate cromakalim; Bray *et al.*, 1987; Hof *et al.*, 1988) in relaxing rat bladder, is approximately 6 times more potent than cromakalim in relaxing rat portal vein and 8–10 times more potent *in vivo* in reducing blood pressure in rats or cats (Paciorek *et al.*, 1990a,b). Similarly, the potency of RP 49356 in relation to lemakalim in rat bladder was comparable to the relative potencies of RP 49356 and cromakalim in guinea-pig pulmonary artery (Eltze, 1989a) or rat portal vein (Longmore *et al.*, 1990). Pinacidil, which is approximately 3 times less potent than lemakalim in rat aorta (Bray *et al.*, 1987) was also 3 times less potent in the bladder.

In contrast to blood vessels, in which minoxidil sulphate is approximately 3 times less potent than cromakalim (Winquist *et al.*, 1989; Newgreen *et al.*, 1990), this agent was approximately 50 times less potent than lemakalim in relaxing the rat bladder when IC_{50} values were compared. The low potency of minoxidil sulphate in the bladder prevented us from examining the effects of maximal relaxant concentration of this agent on ^{42}K efflux from strips of detrusor, and the concentration used did not produce a detectable increase in ^{42}K efflux (although in the same experiment it did stimulate ^{42}K efflux from the rat portal vein). The reason for the low potency of minoxidil sulphate in the bladder is not immediately obvious, but could be due to a relatively low density of the K-channels through which this agent exerts its effects. A low K^+ conductance has previously been proposed by Brading (1987) to explain the low resting membrane potential in bladder (based on published values in rabbit and guinea-pig bladders of approx. −37 mV; Callahan & Creed, 1981; 1985). However, the low potency of minoxidil sulphate in rat bladder is consistent with a similar low potency in opening K-channels in brain slices (Schmid-Antomarchi *et al.*, 1990). It is possible either that the type of K^+ channel opened by minoxidil sulphate in blood vessels is different from that in bladder or brain, or that the channel in portal vein possesses a different recognition site for minoxidil sulphate. In the rat bladder, the relaxant effects of minoxidil sulphate may be solely due to K-channel opening, since this compound relaxed contractions induced by 20 mM but not 80 mM KCl. However, in the rat portal vein, Newgreen *et al.* (1990) found that minoxidil sulphate produced relatively little membrane hyperpolarization

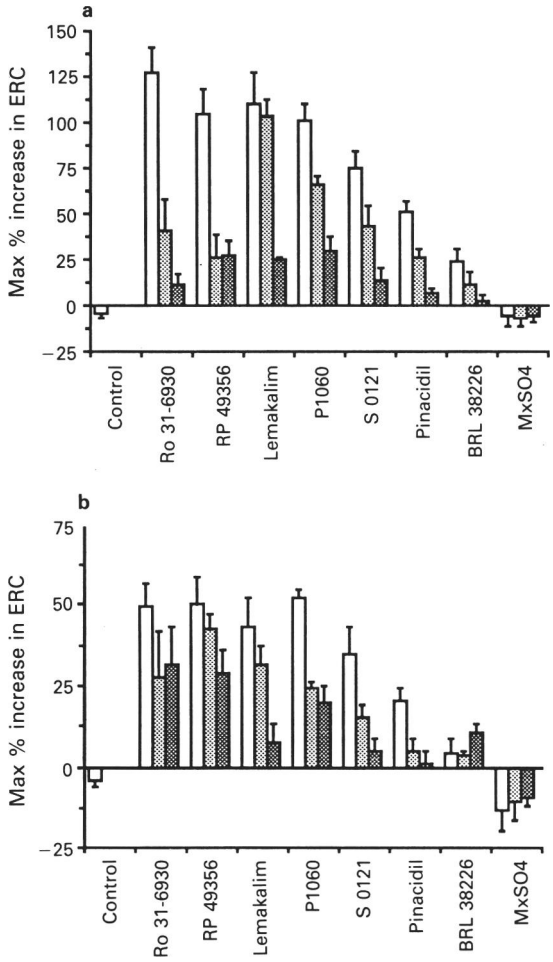

Figure 5 Rat detrusor: maximum increases in (a) ^{42}K or (b) ^{86}Rb efflux rate coefficients stimulated by K-channel openers (open columns) and antagonism by 1 μM (stippled columns) or 3 μM (dark stippled columns) glibenclamide. The concentration of each K-channel opener used was approximately 3 times that which produced a maximal relaxation of rat detrusor precontracted with 20 mM KCl. The concentrations used were: 3 μM Ro 31-6930, 30 μM RP 49356, 15 μM lemakalim, 15 μM P 1060, 30 μM pinacidil, 30 μM minoxidil sulphate ($MxSO_4$), 90 μM S 0121 and 100 μM BRL 38226. Each column represents the mean from 4–5 animals. Vertical bars indicate s.e.mean.

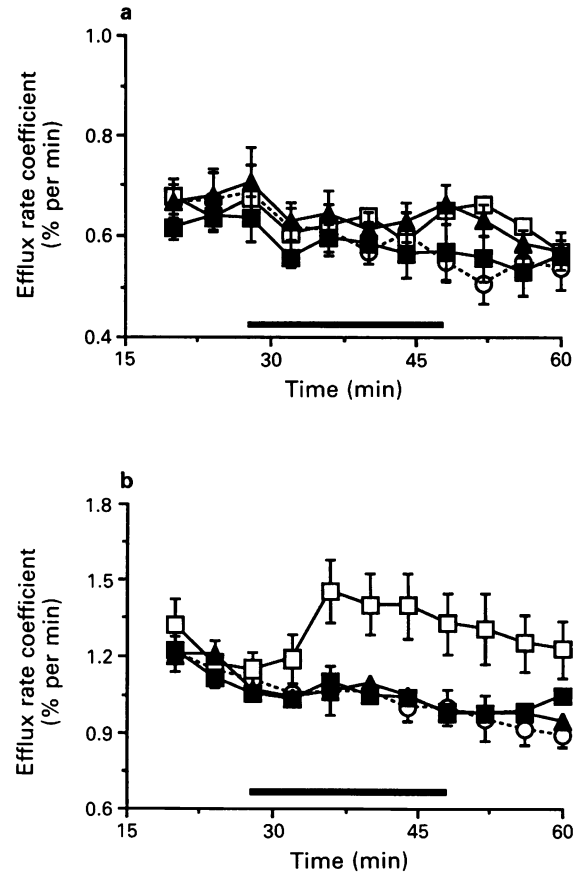

Figure 6 Minoxidil sulphate (30 μM, □) had no effect on the ^{42}K efflux rate coefficient in rat detrusor (a) but increased the efflux rate in rat portal vein (b). The effect of minoxidil sulphate on rat portal vein was fully antagonized by 1 μM (▲) or 3 μM (□) glibenclamide; (○) indicates vehicle controls. The horizontal bar indicates the time during which the tissues were exposed to minoxidil sulphate or vehicle. Glibenclamide was present for 28 min before and during the exposure to minoxidil sulphate. Each point represents the mean from 5 animals. Vertical bars indicate ± s.e.mean.

and proposed that the relaxant effects of this agent in this tissue might not be entirely due to the opening of potassium channels.

Pinacidil, in an equiactive mechano-inhibitory concentration to the other K-channel openers, stimulated efflux to a lesser extent. Moreover, in concentrations above 10 μM, pinacidil reduced the magnitude of contractions induced by 80 mM KCl. This is consistent with an earlier report of the effects of pinacidil on human detrusor (Fovaeus *et al.*, 1989) and strongly suggests that part of the relaxant effect is due to a mechanism other than K-channel opening. In the human bladder, the contractile effect of KCl can be explained solely by entry of Ca^{2+} through voltage-sensitive Ca^{2+} channels (Maggi *et al.*, 1989). Assuming an intracellular K^+ concentration for bladder muscle in the range 125–182 mM (Brading, 1981), the equilibrium potential for K^+ in depolarizing Krebs solution ($[K^+] = 86$ mM) would be within the range −10 mV to −20 mV which should be sufficient to ensure that voltage-sensitive Ca^{2+} channels are in an open condition (Bolton *et al.*, 1984). The inhibitory effect of pinacidil, and possibly high concentrations of other K-channel openers under such conditions could therefore be due to inhibition of Ca^{2+} entry. This is supported by the recent finding that pinacidil inhibits Ca^{2+} influx into canine mesenteric arteries (Masuzawa *et al.*, 1990).

In the present study, consistent with the findings of Bray *et al.* (1987) and Hof *et al.* (1988), BRL 38226 abolished spontaneous activity in the rat portal vein but was far less potent than its enantiomer lemakalim (BRL 38227). Only the highest concentration of BRL 38226 used in this study (30 μM) reduced 20 mM KCl-induced activity of rat detrusor. However, since the K-channel openers were approximately 8 times more potent in reducing spontaneous activity in the rat portal vein than in reducing 20 mM KCl-induced contractions in rat detrusor, concentrations of BRL 38226 less than 30 μM were not expected to be effective. The highest concentration of BRL 38226 which we were able to use (100 μM) had no effect on ^{86}Rb efflux, but produced a small increase in ^{42}K efflux. The relaxant effect of BRL 38226 is, therefore, likely to result from K-channel opening. In contrast, S 0121, which differs from BRL 38226 only by the addition of a methyl substituent at C5 on the lactam ring (see Edwards & Weston, 1990), fully relaxed 20 mM KCl-induced activity but had no effect on 80 mM KCl-contracted bladder strips. This, together with the ability of S 0121 to stimulate both ^{42}K and ^{86}Rb efflux, suggests that the relaxant effects of this compound are exerted via opening of K-channels. The greater potency of S 0121 in comparison to BRL 38226 was surprising and not predictable from current views on structure-activity relationships in the benzopyran K-channel openers (Edwards & Weston, 1990).

In the bladder, similar mean pA_2 values (range 6.3–6.6) were obtained for the antagonism by glibenclamide of responses to lemakalim, pinacidil, Ro 31-6930, P1060 and RP 49356, sug-

gesting that these K-channel openers may be acting at the same site. In this tissue these values were low in comparison to those derived from other tissues, for example guinea-pig pulmonary artery (7.17–7.22; Eltz, 1989a), rabbit vas deferens (7.17; Eltze, 1989b), canine mesenteric artery (7.12–7.16; Masuzawa *et al.*, 1990), rat aorta and portal vein (7.2; Newgreen *et al.*, 1990), and rat portal vein (6.8–7.2; this study). Furthermore, for the bladder, the slopes of the Schild plots were greater than unity, in contrast to the situation in these other tissues. This might suggest that the K-channel openers are acting on channels in bladder which differ in their recognition sites from those in other types of smooth muscle. However, the relative potencies of the compounds tested in this study were similar to those reported in other tissues. Such data suggest that the channel types involved in the actions of the K-channel openers may be identical but that it is inappropriate to assume that the interaction between glibenclamide and the K-channel openers is competitive (for which there is no definitive evidence).

The antihypertensive effects of K-channel openers in animal models is now established (e.g. Arrigoni-Martelli *et al.*, 1980; Buckingham *et al.*, 1986; Paciorek *et al.*, 1990a) and cromakalim has also been demonstrated to be effective in man (Singer *et al.*, 1989). However, these agents may also have potential in the treatment of other disorders such as bladder instability; cromakalim abolishes the spontaneous contractions of human unstable bladder (Foster *et al.*, 1989b). In the present study, a K-channel opener which was more selective for non-vascular than for vascular tissue was not identified. Thus, a possible disadvantage of using currently available K-channel openers to reduce smooth muscle activity in tissues other than the vasculature is that the higher doses of these agents which would be necessary could also reduce blood pressure in normotensive patients. Nevertheless, pinacidil and cromakalim are more potent *in vitro* in hypertrophied than in normal rat or pig bladder (Foster *et al.*, 1989b; Malmgren *et al.*, 1990). Furthermore, *in vivo*, both agents are effective inhibitors of the spontaneous contractions associated with bladder hypertrophy in rats and pigs (Foster *et al.*, 1989b; Malmgren *et al.*, 1990). In addtion, Williams *et al.* (1990) have shown cromakalim to be effective in reducing nocturnal asthma using doses which have no effect on blood pressure. Further studies, together with the development of bladder-selective agents are clearly indicated.

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References

ANDERSSON, K.-E. & MATTIASSON, A. (1988). Drug treatment of the overactive detrusor. *Drugs of Today*, **24**, 337–348.

ARRIGONI-MARTELLI, E., KAERGAARD-NIELSEN, C., BANG OLSEN, U. & PETERSEN, H.J. (1980). N″-cyano-N-4-pyridyl-N′-1,2,2-trimethylpropylguanidine, monohydrate (P1134): a new, potent vasodilator. *Experientia*, **36**, 445–447.

BOLTON, T.B., LANG, R.J. & TAKEWAKI, T. (1984). Mechanism of action of noradrenaline and carbachol on smooth muscle of guinea-pig anterior mesenteric artery. *J. Physiol.*, **351**, 549–572.

BRADING, A.F. (1981). Ionic distribution and mechanisms of transmembrane ion movement. In *Smooth Muscle: An Assessment of Current Knowledge*. ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. pp. 65–92. London: Edward Arnold.

BRADING, A.F. (1987). Physiology of bladder smooth muscle. In *The Physiology of the Lower Urinary Tract*. ed. Torrens, M. & Morrison, J.F.B. pp. 161–191. Berlin, Heidelberg: Springer-Verlag.

BRAY, K.M., NEWGREEN, D.T. & WESTON, A.H. (1987). Some effects of the enantiomers of the potassium channel openers, BRL 34915 and pinacidil, on rat blood vessels. *Br. J. Pharmacol.*, **91**, 357P.

BUCKINGHAM, R.E., CLAPHAM, J.C., HAMILTON, T.C., LONGMAN, S.D., NORTON, J. & POYSER, R.H. (1986). BRL 34915, a novel antihypertensive agent: comparison of effects on blood pressure and other haemodynamic parameters with those of nifedipine in animal models. *J. Cardiovasc. Pharmacol.*, **8**, 798–804.

CALLAHAN, S.M. & CREED, K.E. (1981). Electrical and mechanical activity in the isolated urinary tract of the guinea-pig. *Br. J. Pharmacol.*, **74**, 353–358.

CALLAHAN, S.M. & CREED, K.E. (1985). The effects of oestrogens on spontaneous activity and responses to phenylephrine of the mammalian urethra. *J. Physiol.*, **358**, 35–46.

ECKL, K.M. & GREB, W.H. (1987). Potassium channel activation in the smooth muscle cell membrane – a new mechanism of action in antihypertensive treatment – early clinical results with BRL34915. *Clin. Exp. Ther.*, **49**, 160.

EDWARDS, G. & WESTON, A.H. (1989). Potassium channel openers: their development and prospects. *Curr. Cardiovasc. Patents*, **1**, 1810–1822.

EDWARDS, G. & WESTON, A.H. (1990). Structure-activity relationships of K^+ channel openers. *Trends Pharmacol. Sci.*, **11**, 417–422.

ELTZE, M. (1989a). Glibenclamide is a competitive antagonist of cromakalim, pinacidil and RP 49356 in guinea-pig pulmonary artery. *Eur. J. Pharmacol.*, **165**, 231–239.

ELTZE, M. (1989b). Competitive antagonism by glibenclamide of cromakalim inhibition of twitch contractions in rabbit vas deferens. *Eur. J. Pharmacol.*, **161**, 103–106.

ESCANDE, D., THURINGER, D., LEGUERN, S. & CAVERO, I. (1988). The potassium channel opener cromakalim (BRL 34915) activates ATP-dependent K^+ channels in isolated cardiac myocytes. *Biochem. Biophys. Res. Commun.*, **154**, 620–625.

FOSTER, C.D., FUJII, K., KINGDON, J. & BRADING, A.F. (1989a). The effect of cromakalim on the smooth muscle of the guinea-pig urinary bladder. *Br. J. Pharmacol.*, **97**, 281–291.

FOSTER, C.D., SPEAKMAN, M.J., KINGDON, J. & BRADING, A.F. (1989b). The effects of cromakalim on the detrusor muscle of human and pig urinary bladder. *Br. J. Urol.*, **97**, 281–291.

FOVAEUS, M., ANDERSSON, K.-E. & HEDLUND, H. (1989). The action of pinacidil in the isolated human bladder. *J. Urol.*, **141**, 637–640.

FOX, J.S., WHITEHEAD, E. & SHANKS, R.G. (1987). Cardiovascular effects of the novel antihypertensive agent BRL34915 in normal volunteers. *Br. J. Clin. Pharmacol.*, **23**, 600P.

FRIEDEL, H.A. & BROGDEN, R.N. (1990). Pinacidil: a review of its pharmacodynamic and pharmacokinetic properties and therapeutic potential in the treatment of hypertension. *Drugs*, **39**, 929–967.

HAMILTON, T.C., WEIR, S.W. & WESTON, A.H. (1986). Comparison of the effects of BRL 34915 and verapamil on electrical and mechanical activity in rat portal vein. *Br. J. Pharmacol.*, **88**, 103–111.

HOF, R.P., QUAST, U., COOK, N.S. & BLARER, S. (1988). Mechanism of action and systemic and regional hemodynamics of the potassium channel activator BRL 34915 and its enantiomers. *Circ. Res.*, **62**, 679–686.

LEBLANC, N., WILDE, D.W., KEEFE, K.D. & HUME, J.R. (1989). Electrophysiological mechanisms of minoxidil sulphate-induced vasodilation of rabbit portal vein. *Circ. Res.*, **65**, 1102–1111.

LONGMORE, J., NEWGREEN, D.T. & WESTON, A.H. (1990). Effects of cromakalim, RP 49356, diazoxide, glibenclamide and galanin in rat portal vein. *Eur. J. Pharmacol.*, **190**, 75–84.

MAGGI, C.A., GIULIANI, S., PATACCHINI, TURINI, D., BARBANTI, G., GIACHETTI, A. & MELI, A. (1989). Multiple sources of calcium for contraction of the human urinary bladder muscle. *Br. J. Pharmacol.*, **98**, 1021–1031.

MALMGREN, A., ANDERSSON, K.-E., SJOGREN, C. & ANDERSSON, P.O. (1989). Effects of pinacidil and cromakalim (BRL 34915) on bladder function in rats with detrusor instability. *J. Urol.*, **142**, 1124–1138.

MALMGREN, A., ANDERSSON, K.-E., ANDERSSON, P.O., FOVAEUS, M. & SJOGREN, C. (1990). Effects of cromakalim (BRL 34915) and pinacidil on normal and hypertrophied rat detrusor *in vitro*. *J. Urol.*, **143**, 828–834.

MASUZAWA, K., MATSUDA, T. & ASANO, M. (1990). Evidence that pinacidil may promote the opening of ATP-sensitive K^+ channels yet inhibit the opening of Ca^{2+}-activated K^+ channels in K^+-contracted canine mesenteric artery. *Br. J. Pharmacol.*, **100**, 143–149.

McGUIRE, E.J. (1984). Detrusor response to outflow obstruction. *World J. Urol.*, **2**, 208–210.

MEISHERI, K.D., CIPKUS, L.A. & TAYLOR, C.J. (1988). Mechanism of action of minoxidil sulphate-induced vasodilation: a role for

increased K^+ permeability. *J. Pharmacol. Exp.* Ther., **245**, 751–760.

MUNDY, A.R. & STEPHENSON, T.P. (1984). The urge syndrome. In *Urodynamics: Principles, Practice and Application.* ed. Mundy, A.R., Stephenson, T.P. & Wein, A.J. pp. 212–228. Edinburgh: Churchill Livingstone.

NEWGREEN, D.T., BRAY, K.M., MCHARG, A.D., WESTON, A.H., DUTY, S., BROWN, B.S., KAY, P.B., EDWARDS, G., LONGMORE, J. & SOUTHERTON, J.S. (1990). The action of diazoxide and minoxidil sulphate on rat blood vessels: a comparison with cromakalim. *Br. J. Pharmacol.*, **100**, 605–613.

PACIOREK, P.M., BURDEN, D.T., BURKE, Y.M., COWLRICK, I.S., PERKINS, R.S., TAYLOR, J.C. & WATERFALL, J.F. (1990a). Preclinical pharmacology of Ro 31-6930, a new potassium channel opener. *J. Cardiovasc. Pharmacol.*, **15**, 188–197.

PACIOREK, P.M., COWLRICK, I.S., PERKINS, R.S., TAYLOR, J.C., WILKINSON, G.F. & WATERFALL, J.F. (1990b). Evaluation of the bronchodilator properties of Ro 31-6930, a novel potassium channel opener in the guinea-pig. *Br. J. Pharmacol.*, **100**, 289–294.

SCHMID-ANTOMARCHI, H., AMOROSO, S., FOSSET, M. & LAZDUNSKI, M. (1990). K^+ channel openers activate brain sulfonylurea-sensitive K^+ channels and block neurosecretion. *Proc. Natl. Acad. Sci., U.S.A.*, **87**, 3489–3492.

SINGER, D.R.J., MARKANDU, N.D., MILLER, M.A., SUGDEN, A.L. & MACGREGOR, G.A. (1989). Potassium channel stimulation in normal subjects and in patients with essential hypertension: an acute study with cromakalim (BRL34915). *J. Hypertens.*, **7**, 294–295.

WEIR, S.W. & WESTON, A.H. (1986). The effects of BRL 34915 and nicorandil on electrical and mechanical activity and on ^{86}Rb efflux in rat blood vessels. *Br. J. Pharmacol.*, **88**, 121–128.

WILLIAMS, A.J., LEE, T.H., COCHRANE, G.M., HOPKIRK, A., VYSE, T., CHIEW, F., LAVENDER, E., RICHARDS, R.H., OWEN, S., STONE, P., CHURCH, S. & WOODCOCK, A.A. (1990). Attenuation of nocturnal asthma by cromakalim. *Lancet*, **ii**, 334–336.

WINQUIST, R.J., HEANEY, L.A., WALLACE, A.A., BASKIN, E.P., STEIN, R.B., GARCIA, M.L. & KACZOROWSKI, G.J. (1989). Glyburide blocks the relaxation response to BRL 34915 (cromakalim), minoxidil sulfate and diazoxide in vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **248**, 149–156.

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Interference of BN 52021, an antagonist of PAF, with different forms of active anaphylaxis in the guinea-pig: importance of the booster injection

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1 BN 52021, an antagonist of platelet activating factor (PAF), was inactive against bronchoconstriction in guinea-pigs sensitized with low amounts of ovalbumin (OA) injected twice, at a 14 day interval and challenged i.v. 7 days later.

2 Serum IgG titers increased for 7 weeks after the booster injection at day 14 and returned to low levels at day 96.

3 Administered by the intratracheal (i.t.) route at 1 mg, BN 52021 failed to inhibit bronchoconstriction induced by the i.t. administration of OA to guinea-pigs tested 7, 28, 56 and 84 days after the booster injection, even when the titers of circulating IgG had declined with time. BN 52021 was also inactive against bronchoconstriction in guinea-pigs boosted at day 98 and tested 7 days later and against contractions and thromboxane (Tx) B_2 and histamine release induced by OA-challenged parenchymal lung strips from the boosted guinea-pigs.

4 Sensitized unboosted guinea-pigs displayed reduced IgG serum titers. Used 21 or 70 days after the sensitizing injection, they did develop bronchoconstriction upon the i.t. instillation of OA, which was blocked by BN 52021. The latter also inhibited OA-induced contractions of lung parenchymal strips from these unboosted guinea-pigs.

5 When boosted and non-boosted guinea-pigs received OA i.t. and bronchoalveolar lavage fluid was collected 10 min later, the number of eosinophils increased markedly in boosted, but not in non-boosted guinea-pigs.

6 The booster injection of antigen thus modifies the response of the lung and PAF appears to be relevant for antigen-induced bronchoconstriction in unboosted animals, but loses its major role following the booster injection.

Introduction

Bronchial asthma is refractory to antihistamines of the H_1 type and there are reasons to moderate the initial enthusiasm concerning the participation of peptido-leukotrienes in allergic asthma (Vargaftig *et al.*, 1985). The finding that platelet-activating factor (PAF) imitates the signs and symptoms of acute inflammation and immediate hypersensitivity (Pinckard *et al.*, 1979; Vargaftig *et al.*, 1980; Basran *et al.*, 1984; Braquet *et al.*, 1987) led to the hypothesis that it may display the role negated to histamine and to lipoxygenase metabolites.

The PAF antagonists available at present are useful tools to investigate the role of this mediator and its involvement in pathology and may offer potential therapeutic applications. One of them, ginkgolide B (BN 52021) (Braquet *et al.*, 1985; Braquet & Godfroid, 1987), selectively antagonizes the effects of PAF on the bronchopulmonary system and on the circulating blood cells of the guinea-pig (Touvay *et al.*, 1986a,b; Desquand *et al.*, 1986). BN 52021, which is not an antihistamine, was first reported to inhibit anaphylactic bronchoconstriction induced by the intravenous (i.v.) or intra-pulmonary aerosolization of ovalbumin (OA) to guinea-pigs passively sensitized with homologous (Cirino *et al.*, 1986; Vilain *et al.*, 1986; Desquand *et al.*, 1987; Lagente *et al.*, 1987) or with heterologous (Touvay *et al.*, 1985; 1986b; Desquand & Vargaftig, 1988) serum raised in animals immunized with OA. Overall, these results suggested that PAF contributes to passive anaphylaxis and led us to study the effect of BN 52021 on bronchoconstriction induced by active anaphylaxis. Active shock involves complex mechanisms (Detsouli *et al.*, 1985; Desquand & Vargaftig, 1988). Since in initial experiments different procedures of sensitization led to differences in the effectiveness of BN 52021 against anaphylactic bronchoconstriction, we studied the mechanisms which account for those differences, and particularly the role of the booster injection of antigen.

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Methods

Procedures of active sensitization

Hartley guinea-pigs of either sex (300–500 g) were actively sensitized at day 0 by the sub-cutaneous (s.c.) injection of 0.5 ml of 0.9% NaCl solution (saline) containing 10 μg of OA dispersed in 1 mg $Al(OH)_3$ (modified from Andersson, 1980). As seen in Table 1, when appropriate, those guinea-pigs were boosted with a similar injection of antigen at day 14. They were used 7, 28, 56 or 84 days after the booster injection (groups called respectively 14 + 7, 14 + 28, 14 + 56 or 14 + 84). In some experiments, the booster injection was omitted and the animals were used 14, 21, 70 or 96 days after the first injection (groups called respectively 0–14, 0–21, 0–70 or 0–96). Finally, another group of guinea-pigs was sensitized by a first injection on day 0, was boosted only on day 98 and used 7 days later. Under these conditions, the animals were either sensitized with a single or with two injections of OA, irrespective of the time of injection.

In vivo *preparation*

Guinea-pigs were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.p.) and prepared for the recording of bronchial resistance to inflation on a Beckman Dynograph R 511 (Schiller Park, Il., USA) as described by Lefort & Vargaftig (1978).

Spontaneous breathing was suppressed with pancuronium (2 mg, i.v.). A jugular vein was cannulated for drug injection and one carotid artery was cannulated for blood sampling; 0.5 ml of blood was collected to evaluate the IgG titer. The bronchial reactivity was assessed with 5-hydroxytryptamine (3 μg, i.v.) and experiments were initiated when reproducible responses were obtained.

In a first series of experiments, guinea-pigs actively sensitized with a booster injection of antigen (group called 14 + 7) were challenged with OA, either perfused i.v. at the dose of 1 mg kg^{-1} during 1 min or injected as a bolus at the dose of 30 μg kg^{-1}. When appropriate, BN 52021 was injected at 1 mg kg^{-1}, 3 min before OA, control animals being given the solvent.

In separate *in vivo* experiments, a tube was inserted into the tracheal cannula to a depth of 8 mm below the medium incision and 1 mg of BN 52021 was instilled in a volume of 100 μl, 30 min before PAF or OA, control animals receiving 100 μl of the solvent. On these occasions, PAF was given i.t. at 1 μg in a volume of 100 μl to non-immunized or to actively sensitized and boosted guinea-pigs of group 14 + 7 and OA was given i.t., at 100 μg or 1 mg to boosted or non-boosted animals, respectively, in a volume of 100 μl. In those guinea-pigs, both cervical vagi nerves were transected at the level of the neck. The i.t. instillation of Evans Blue dye was followed by a homogeneous bluing of the lungs, validating the procedure. The bronchopulmonary resistance to inflation following administration of the antigen was monitored and bronchoconstriction was expressed in cm^2 of the area of the tracing over the baseline for 20 min, a procedure which takes into account the increase in inflation pressure and the duration of the response. In addition, in those cases in which BN 52021 antagonized the anaphylactic bronchoconstriction, the peak intensity of the pulmonary response was evaluated (1–3 min after OA) as well as its intensity 20 min after OA administration. These parameters were expressed in cmH_2O.

Blood cell counts

Blood samples (200 μl) were collected from the carotid artery for the assessment of total leucocyte and platelet counts with a Coulter Counter ZBI. Blood samples were obtained before and 1, 3 and 6 min after the i.v. injection of OA and also before and then 1, 3, 10 and 30 min after the i.t. administration of PAF or of OA.

Determination of OA-specific IgG by enzyme-linked-immunosorbent-assay (ELISA)

A sample (0.5 ml) of blood was collected from the carotid artery of anaesthetized guinea-pigs before the beginning of the *in vivo* experiments for the determination of IgG titers by ELISA. Flat-bottomed polystyrene microtiter plates (Nunc) were coated overnight at 4°C with 100 μl of 1 μg ml^{-1} OA in 0.05 M sodium carbonate buffer, pH 9.5. The plates were washed twice in 0.15 M phosphate-buffered saline (PBS), pH 7.2 containing 0.1% Tween 20 (PBS-Tween) and drained. All washes were performed in this way, and subsequent incubations were performed for 2 h at 37°C in a moist chamber. All dilutions of sera and antisera were prepared in PBS-Tween. Each well was incubated successively with the following solutions with washes between each step: 200 μl of 1 μg ml^{-1} of bovine serum albumin (BSA); 100 μl of serum diluted 1/10 to 1/(2 × 10^6); 100 μl of peroxidase-labelled goat anti-guinea-pig IgG antiserum diluted 1/2000; 100 μl of the substrate solution at pH 4.0 containing 1 M citrate, 3% hydrogen peroxide (30% w/w) and 250 μg ml^{-1} of 2,2'azino-*bis*(3 ethyl benzothiozaline sulphonic acid (ABTS). The enzyme-substrate reaction was stopped after 30 min by adding to each well 50 μl of a 10% sodium dodecyl sulphate (SDS) solution in distilled water. Absorbances were measured at 450 nm with an automated microplate-reader (MR 700, Dynatech Laboratories Inc., Marnes La Coquette, France). Titers of OA-specific IgG were expressed as the dilution giving 50% of the maximum net absorbance after subtraction of the values obtained with control sera from non-immunized guinea-pigs.

Preparation of the isolated lung strips

Guinea-pigs actively sensitized with a booster injection (group 14 + 7) or without a booster injection (group 0–21) were anaesthetized and ventilated. A mid-thoracotomy was performed, the lungs were then removed and rinsed with 20 ml of Krebs solution injected through the pulmonary artery. Peripheral sub-pleural strips (2.5–3 cm long; 3–4 mm wide) were dissected and mounted under 2 g tension in an isolated organ bath containing 16 ml Krebs solution at 37°C and aerated with 95% O_2:5% CO_2. Each strip was connected to a Gould-Statham force displacement transducer (Lulich *et al.*, 1976). After approximately 1 h for equilibration, histamine was added cumulatively (1–100 μM) at 3 min intervals. After several rinses, 30 min later, the same cycle was renewed; the maximal contraction induced by cumulative concentrations of histamine was taken as a reference. Lung strips were stimulated by the addition of OA 1 μg ml^{-1} directly to the organ bath for lung strips from guinea-pigs of group 14 + 7 and with OA, 1 and 10 μg ml^{-1}, for lung strips from guinea-pigs of group 0–21. BN 52021 at a concentration of 0.3 mM was added to the organ baths 10 min before OA.

Contractile responses to cumulative doses of histamine were determined in the presence of BN 52021 (3 × 10^{-4} M) as a control for non-specific inhibition.

The intensities of the contractions were expressed as a percentage of the maximal contraction of the same lung strip induced by histamine. The intensity of the contraction of the treated lung strip as compared to that of the lung strip from the same animal and exposed to the drug solvent was used to express drug effectiveness.

Aliquots of 200 μl and of 1 ml of Krebs solution were removed from the organ bath before and 20 min after the stimulation by OA, to evaluate thromboxane B_2 (TxB_2) formation and histamine release.

Sampling of bronchoalveolar cells

The cell composition of the bronchoalveolar lavage fluid (BAL) from guinea-pigs of groups 14 + 7 and 0–21 and its modification following the instillation of OA were investigated. Guinea-pigs were challenged i.t. as described above with saline (controls) or with 100 μg of OA in the case of animals of group 14 + 7 and 1 mg for those of group 0–21. The only difference, as compared to the *in vivo* experiments, was that the animals were pretreated with 500 μg kg^{-1} of the antihistamine, mepyramine, to prevent intense bronchoconstriction and the increased vascular permeability resulting from the shock, which would prevent the cell collection. Five minutes after the OA instillation, 5 ml of sterile saline at room temperature was instilled through a polyethylene cannula introduced into the trachea, and recovered immediately. This was followed by five repeated lavages with 10 ml aliquots of sterile saline until 50 ml was obtained in a Corning tube. After centrifugation for 10 min at 200 *g* at room temperature, the cells were resuspended in 1 ml of sterile saline. The total leucocyte counts were evaluated with a Malassez haemacytometer and cell viability was estimated with the Trypan Blue exclusion test. The suspension containing 120 000 cells ml^{-1} was then centrifuged in a Shandon-Elliott cytometer, cells were counted and differentiated by May-Grunwald-Giemsa staining (Diff-Quick, American Scientific Products, McGaw park, IL., U.S.A.). Results are expressed as percentage of macrophages, eosinophils, lymphocytes and neutrophils in the cell population.

Radioimmunoassay of thromboxane B_2

TxB_2 was assayed according to the method of Sors *et al.* (1978). Briefly, aliquots (200 μl from the organ bath) were left

at room temperature for 60 min (to allow conversion of TxA_2 into its stable metabolite TxB_2) and were stored at −20°C. For the radioimmunoassay (RIA), the samples were thawed and incubated overnight at 4°C with ^{125}I-labelled TxB_2 and anti-TxB_2 antiserum in a phosphate buffer (10 mM, pH 7.4) containing bovine gamma globulin (0.3% w/w). The next day, bound and free fractions were separated by the addition of a solution of polyethyleneglycol 6000 (30% in distilled water) followed by centrifugation at 2000 *g* and 4°C for 10 min. The supernatant was decanted and the radioactivity present in the pellet, corresponding to the bound fraction was counted for 1 min in a Kontron Analytical gamma counter (model MDA 312).

Spectrofluorimetric assay of histamine

One ml of the collected fractions was mixed with 1 ml of perchloric acid (0.8 N). After centrifugation for 30 min at 3500 r.p.m. and at 4°C, the supernatants were stored at 4°C. An automatic fluorimetric assay for histamine was performed according to Shore *et al.* (1959) as modified by Lebel (1983).

Materials

The Krebs solution was composed of (mM): NaCl 118, KCl 4.7, $CaCl_2$, $2H_2O$ 2.5, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 5.6.

The PAF antagonist BN 52021 (9H-1,7a-(epoxymethano)-1H, 6aH-cyclopenta(c) furo(2,3-b)furo-3', 2':3,4)cyclopenta(1,2-d) furn-5,9,12-(4H)-trione,3-tert-butyl hexahydro-8-methyl) extracted from *Ginkgo biloba* leaves (IHB-IPSEN Research Laboratories, Le Plessis Robinsson, France) was in a sodic form (lactone open, titer 31%) and was dissolved in a solution prepared with 0.56 g of NaCl in 100 ml distilled H_2O.

PAF (1-0-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine, powder from Sigma) was dissolved and further diluted in saline containing 0.1% bovine serum albumin.

The other drugs were dissolved in saline and were obtained as indicated: sodium pentobarbitone (Nembutal, Sanofi, France); pancuronium bromide (Pavulon, Organon, France); mepyramine maleate (Rhône-Poulenc Santé, Vitry/Seine, France); 5-hydroxytryptamine (5-HT), histamine, 2,2'azino-*bis*(3 ethyl benzothiazoline sulphonic acid) (ABTS), sodium dodecyl sulphate (SDS) (Sigma Chemical Co., St. Louis, MO, U.S.A.); chicken OA (Miles, Naperville, IL, U.S.A.); $Al(OH)_3$, perchloric acid, polyethyleneglycol 6000 (Merck, Darmstrad, GFR); peroxidase-labelled goat anti-guinea-pig IgG (Biosis, Compiègne, France). The antibody radiolabelled ligands for the radioimmunoassay of TxB_2 were from the URIA (Unité Associée Institut Pasteur-INSERM no. 207).

Data analysis

The results are expressed as means ± s.e.mean. Statistical differences between two means of data were evaluated by the unpaired Student's *t* test for *in vivo* experiments or for the quantification of the cell population and with paired Student's *t* test for *in vitro* experiments performed with lung strips.

Results

Failure of BN 52021 to block anaphylactic bronchoconstriction induced by the injection of ovalbumin to guinea-pigs actively sensitized with a booster injection of antigen (group 14 + 7)

Pretreatment of the animals of group 14 + 7 with i.v. BN 52021 (1 mg kg^{-1}) failed to inhibit bronchoconstriction, the accompanying decrease in blood cell counts induced by OA infused at 1 mg kg^{-1} during 1 min and the moderate bronchoconstriction following a bolus injection of 30 μg kg^{-1} (Figure 1). The H_1-histamine receptor antagonist, mepyramine, injected i.v. at 200 μg kg^{-1} 10 min before the perfusion of OA, 1 mg kg^{-1}, to those guinea-pigs blocked significantly the anaphylactic bronchoconstriction (65.9% inhibition) but failed to reduce the decrease in cell counts (data not shown).

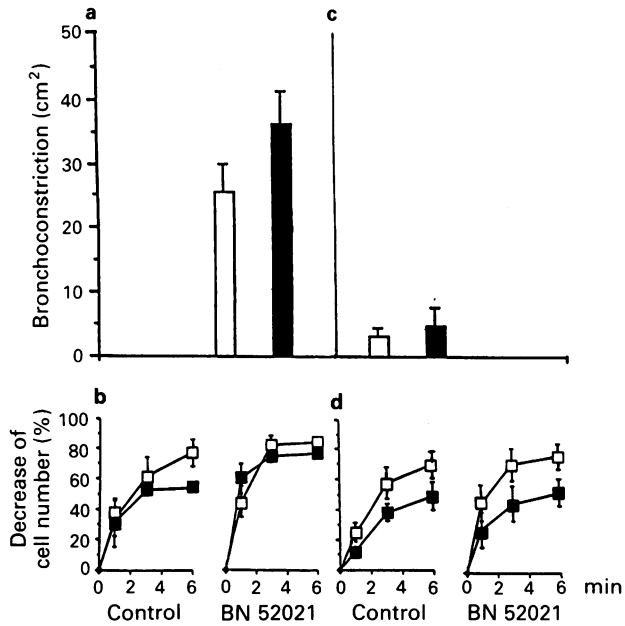

Figure 1 Interference of BN 52021 (1 mg kg^{-1}, i.v.) with the effects of the i.v. administration of ovalbumin (OA) to actively sensitized guinea-pigs of group 14 + 7. Bronchoconstriction, expressed in cm² and thrombocytopenia and leucopenia, expressed as % of the decrease in platelet (open symbols) and leucocyte (closed symbols) counts are represented in the upper and lower panel, respectively; s.e.means are shown by vertical bars. Open columns represent bronchoconstrictions of control untreated guinea-pigs and closed columns represent bronchoconstrictions of those that were treated with BN52021. (a) and (b) Show the effects on animals perfused with OA, 1 mg kg^{-1} and (c) and (d) on those injected with 30 μg kg^{-1}. Experiments with OA perfusion, n = 4–8; with bolus injection of OA, n = 7–13.

Bronchoconstriction, thrombocytopenia and leucopenia induced by the instillation of ovalbumin to guinea-pigs of group 14 + 7

The administration of 100 μg of OA into the guinea-pig trachea induced an intense and reversible bronchoconstriction (Figure 2), accompanied by a moderate thrombocytopenia and leucopenia (both were below 30% of basal values) during the 30 min following the challenge (data not shown).

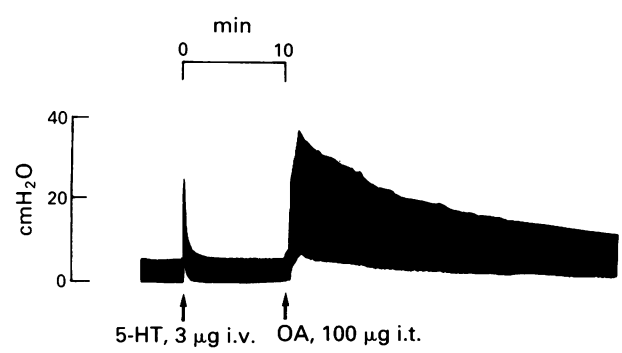

Figure 2 Representative tracing of anaphylactic bronchoconstriction induced by the instillation of 100 μg of ovalbumin (OA) to guinea-pigs actively sensitized of group 14 + 7. The bronchial reactivity was monitored with 5-hydroxytryptamine (5-HT, 3 μg, i.v.). Time scale: 10 min; vertical scale: increase in bronchial resistance to inflation, in cmH_2O, expressing bronchoconstriction.

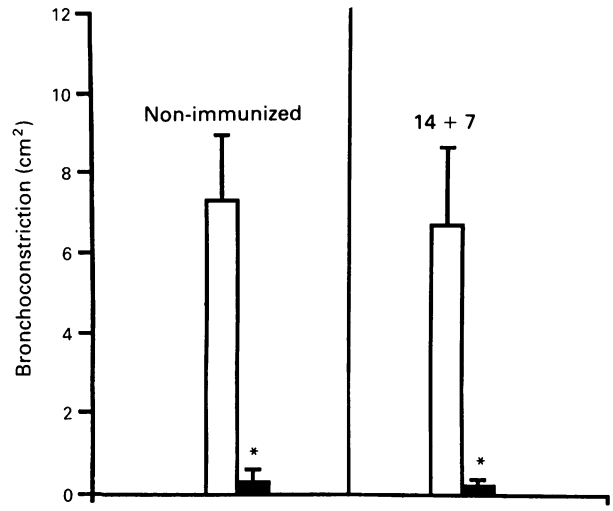

Figure 3 Interference of BN 52021 (1 mg, i.t.) with the intensity of bronchoconstriction (cm^2) induced by an instillation of 1 μg of PAF to guinea-pigs non-immunized or actively sensitized with a booster injection (group 14 + 7). Open columns represent bronchoconstrictions of control guinea-pigs and closed columns represent bronchoconstrictions of treated guinea-pigs, vertical bars shown s.e.means. Experiments with guinea-pigs non-immunized: $n = 5$; with guinea-pigs of group 14 + 7: $n = 6$–8. * $P < 0.05$.

Inhibition by BN 52021 of bronchoconstriction induced by the instillation of PAF to guinea-pigs non-immunized or actively sensitized with a booster injection (group 14 + 7)

BN 52021 maintained its ability to antagonize the effects of PAF administered by the i.t. route in non-immunized as well as in actively sensitized and boosted guinea-pigs of group 14 + 7. Indeed, the i.t. administration of 1 μg of PAF to both groups of animals induced bronchoconstrictions of the same intensity which were blocked by the i.t. instillation of 1 mg of BN 52021, 30 min beforehand (Figure 3). The instillation of PAF to both groups of animals induced a thrombocytopenia of 10–20% and a leucopenia of 20–30% which were not modified by BN 52021 (not shown).

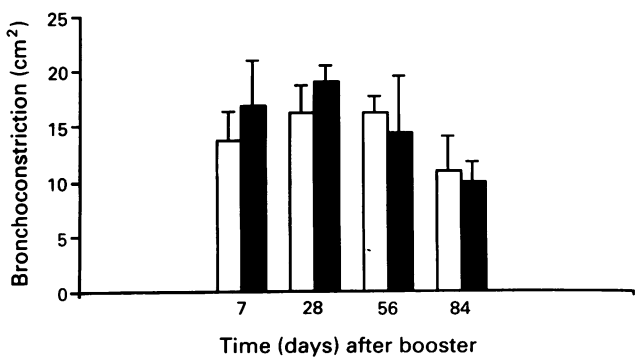

Figure 4 Interference of BN 52021 (1 mg, i.t.) with the intensity of bronchoconstriction (cm^2) induced by an instillation of 100 μg of ovalbumin (OA) to guinea-pigs actively sensitized with a booster injection and used 7, 28, 56 and 84 days after the booster injection. Open columns represent bronchoconstrictions of control guinea-pigs and closed columns represent bronchoconstrictions of treated guinea-pigs; vertical bars show s.e.means. Experiments with guinea-pigs of groups 14 + 7 and 14 + 56: $n = 5$; with guinea-pigs of groups 14 + 28 and 14 + 84: $n = 4$.

Failure of BN 52021 to interfere with bronchoconstriction induced by the instillation of ovalbumin to guinea-pigs actively sensitized with a booster injection (groups 14 + 7, 14 + 28, 14 + 56 and 14 + 84)

The administration of 100 μg of OA into the trachea of guinea-pigs actively sensitized and used 7, 28 or 56 days after the booster injection induced intense and comparable bronchoconstrictions, which were not modified by the i.t. administration of 1 mg of BN 52021 (Figure 4). Sera of these guinea-pigs had high titers of IgG (Table 1) and a marked capacity to sensitize passively isolated lung strips from non-immunized guinea-pigs (Desquand *et al.*, 1990). Both parameters were low when sera were provided by guinea-pigs bled 84 days after the booster injection. However, anaphylactic bronchoconstriction was also not modified by BN 52021 in this group of animals.

The instillation of OA to guinea-pigs sensitized by the different procedures involving a booster injection of antigen at day 14, induced a thrombocytopenia of 10–20% and a leucopenia of 20–30%, i.e., comparable to those induced in the guinea-pigs of group 14 + 7. These reductions of blood cell counts were not modified by BN 52021.

Failure of BN 52021 to interfere with contractions, formation of thromboxane B_2 and release of histamine from lung strips from guinea-pigs of group 14 + 7

BN 52021 at 0.3 mM, a concentration which was inactive against the contractions induced by the cumulative doses of histamine, also failed to inhibit the contractions of the lung strips by OA and the accompanying TxB_2 formation and histamine release (Figure 5). Increasing the concentration of BN 52021 to 1 mM was also inactive (control lung strips contractions: 143.0 ± 9.1 expressed as % of total contraction to histamine; treated lung strips contractions: 89.7 ± 30.4 as % of total contraction to histamine; $n = 4$).

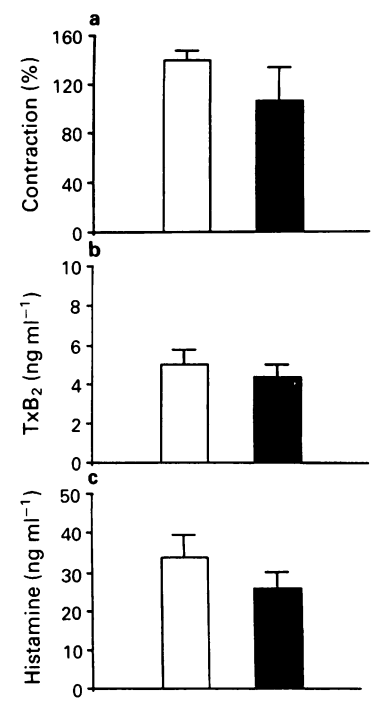

Figure 5 Interference of BN 52021 (0.3 mM) with the ovalbumin (OA)-induced effects on lung strips from actively sensitized guinea-pigs of group 14 + 7. The contractions (a, in % of the maximal effect of histamine) and the liberation (ng ml^{-1}) of thromboxane B_2 (TxB_2) (b) and histamine (c) are represented by open columns for control lung strips and by solid columns for treated lung strips; vertical bars show s.e.means. OA was used at 1 μg ml^{-1} ($n = 8$).

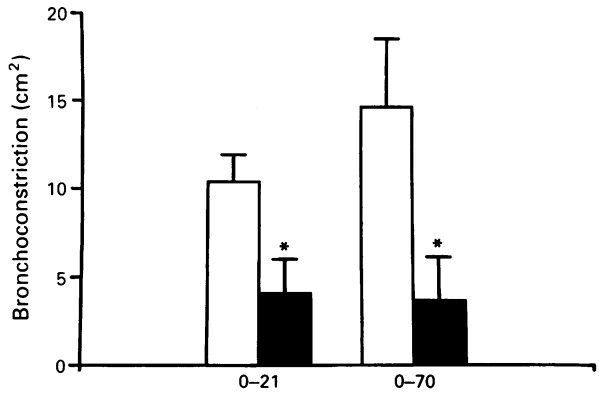

Figure 6 Interference of BN 52021 (1 mg, i.t.) with the intensity of bronchoconstriction (cm^2) induced by an instillation of 1 mg of ovalbumin (OA) to guinea-pigs actively sensitized without a booster injection (groups 0–21 and 0–70). Open columns represent bronchoconstrictions of control guinea-pigs and solid columns represent bronchoconstrictions of treated animals; vertical bars show s.e.means. Experiments with guinea-pigs of group 0–21: $n = 5$–7; with guinea-pigs of group 0–70: $n = 5$. * $P < 0.05$.

Inhibition by BN 52021 of bronchoconstriction induced by the instillation of ovalbumin to guinea-pigs actively sensitized without a booster injection (groups 0–21 and 0–70)

Sera of guinea-pigs which were sensitized without a booster injection and were bled at days 21 or 70 contained very low amounts of IgG (Table 1), and failed to sensitize passively lung strips from non-sensitized guinea-pigs (Desquand *et al.*, 1990). Nevertheless, as shown in Figure 5, these guinea-pigs did develop anaphylactic bronchoconstriction to OA administered i.t. at 1 mg, which was of the same intensity as that observed after the instillation of 100 μg of OA to the boosted guinea-pigs. This anaphylactic bronchoconstriction was blocked by 1 mg of i.t. BN 52021 (Figure 6). In addition, the peak intensities of bronchoconstriction induced by the i.t. administration of OA to guinea-pigs of groups 0–21 and 0–70 were of 27.3 $\pm$ 4.7 and 28.5 $\pm$ 5.2 cmH_2O, respectively. After BN 52021, these values were reduced to 8.3 $\pm$ 3.5 and 10.7 $\pm$ 4.2 cmH_2O, respectively ($P < 0.05$). BN 52021 also blocked the duration of bronchoconstriction, since the intensity 20 min after the OA administration to guinea-pigs of groups 0–21 and 0–70, which were of 10.3 $\pm$ 1.9 and 18.6 $\pm$ 5.4 cmH_2O, respectively in controls, were reduced to 2.6 $\pm$ 1.1 and 4.6 $\pm$ 2.5 cmH_2O, respectively, after BN 52021 ($P < 0.05$). Leucopenia and thrombocytopenia were not modified by BN 52021 (not shown).

Interference of BN 52021 with contractions and formation of thromboxane B_2 and release of histamine from lung strips from guinea-pigs of group 0–21

OA was added at 1 and 10 $\mu g\,ml^{-1}$ to the organ baths containing lung strips from guinea-pigs of group 0–21. OA, 1 $\mu g\,ml^{-1}$, was used to stimulate the strips of guinea-pigs of group 14 + 7 and 10 $\mu g\,ml^{-1}$ was used since, in *in vivo* experiments, the amounts of OA had to be increased by ten fold when instilled to guinea-pigs of group 0–21, in order to match the responses of guinea-pigs of group 14 + 7. As shown in Figure 7, OA at 1 $\mu g\,ml^{-1}$ induced a contraction of lung strips accompanied by the formation of TxB_2 and by the release of histamine, which were less intense than when 10 $\mu g\,ml^{-1}$ of OA was used. In this case, the contractions and the release of mediators were as intense as when lung strips from guinea-pigs of group 14 + 7 were stimulated with OA, 1 $\mu g\,ml^{-1}$. Nevertheless, under both conditions, BN 52021, added at 0.3 mM to the organ baths, reduced significantly the contractions of lung strips to OA, without modifying the accompanying formation of TxB_2 and release of histamine.

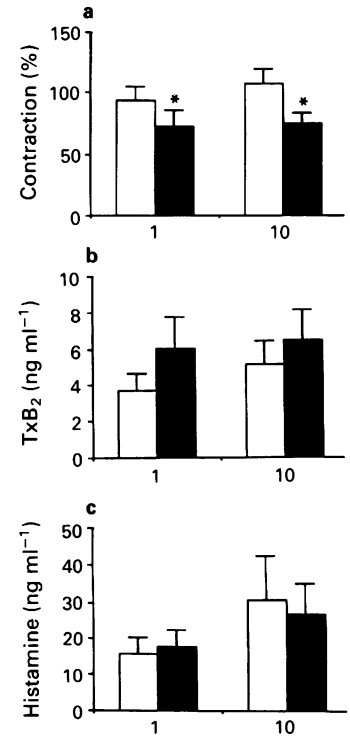

Figure 7 Interference of BN 52021 (0.3 mM) with the effects of the stimulation of lung strips from actively sensitized guinea-pigs without booster injection of group 0–21 with ovalbumin (1 or 10 $\mu g\,ml^{-1}$). The contractions ((a) in % of the maximal effect of histamine) and liberation ($ng\,ml^{-1}$) of thromboxane B_2 (TxB_2) (b) and histamine (c) are represented by open columns for control lung strips and by solid columns for treated strips ($n = 7$); vertical bars show s.e.mean. * $P < 0.05$.

Interference by BN 52021 with bronchoconstriction induced by the instillation of ovalbumin to guinea-pigs actively sensitized with a booster injection at day 98

Sera of guinea-pigs which were sensitized without a booster and were bled at day 98 contained low amounts of IgG (Table 1). The instillation of 1 mg of OA to those guinea-pigs induced bronchoconstriction of variable intensities (2.8 $\pm$ 1.7 cm^2; $n = 5$). Nevertheless, if a booster injection of antigen was performed at day 98, the amounts of circulating IgG detected (Table 1) and the intensities of the bronchoconstriction (17.0 $\pm$ 1.1 cm^2, $n = 5$) induced by the instillation of 100 μg of OA 7 days later, were of the same order of magnitude as those of guinea-pigs of group 14 + 7. After 1 mg of BN 52021 i.t., the intensity of bronchoconstriction was of 11.5 $\pm$ 2.8 cm^2 ($n = 6$). Those values were not statistically different i.e., BN 52021 became inactive. BN 52021 also failed to inhibit thrombocytopenia and leucopenia in these guinea-pigs (not shown).

Basal content in inflammatory cells of the BAL from guinea-pigs actively sensitized with (group 14 + 7) or without a booster injection (group 0–21): effects of antigen instillation

BAL of control guinea-pigs instilled with 100 μl of saline contained 15.9 $\pm$ 4.3 $\times$ 10^6 cells in group 14 + 7 and 15.5 $\pm$ 2.9 $\times$ 10^6 cells in group 0–21. These values were not statistically different. When animals were instilled with OA, the number of cells recovered in the BAL of guinea-pigs of group 14 + 7 was 6.9 $\pm$ 3.6 $\times$ 10^6 cells and 6.3 $\pm$ 1.8 $\times$ 10^6 cells for group 0–21, i.e., were lower than those of guinea-pigs

Table 1 Titers of IgG in sera of guinea-pigs bled at different times after the sensitization

Procedure of active sensitization	*Titers of anti OA circulating IgG*
With booster	
14 + 7	1696.7 ± 320.7
14 + 28	1535.0 ± 674.2
14 + 56	1900.1 ± 984.6
14 + 84	106.5 ± 27.7
98 + 7	2285.0 ± 864.1
Without booster	
0–21	211.7 ± 43.6
0–70	248.3 ± 64.6
0–98	412.4 ± 372.1

The titers of IgG in the sera of guinea-pigs bled 7, 28, 56 or 84 days after the booster injection at day 14, in the sera of guinea-pigs bled 7 days after the booster injection at day 98 and in the sera of guinea-pigs which were not boosted but were bled at days 21, 70 or 98 after sensitization, are expressed by the reciprocal of the maximal dilution giving 50% of the absorbance by ELISA, as shown in the table (n = 3–6).

instilled with saline ($P < 0.05$). The percentage of dead cells was always below 10% of the total cell count. As seen in Figure 8, no difference in the percentage of alveolar macrophages, eosinophils or neutrophils recovered from saline-instilled guinea-pigs of groups 14 + 7 or 0–21 was observed, whereas the percentage of lymphocytes was increased when the animals were boosted during immunization. When both groups of animals were instilled with OA, a statistically significant increase of the percentage of eosinophils was observed in the BAL of guinea-pigs of group 14 + 7, but not group 0–21.

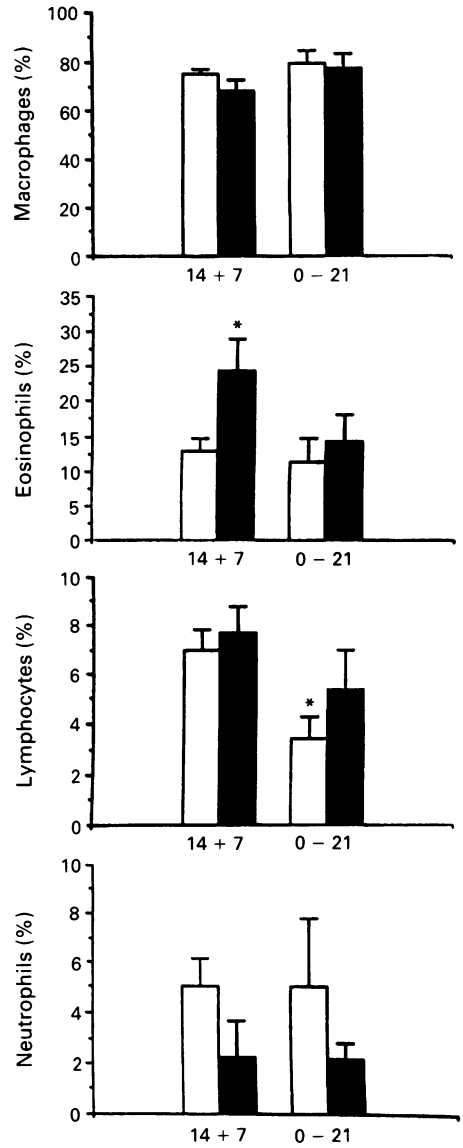

Figure 8 Basal content in inflammatory cells of the bronchoalveolar lavage fluid (BAL) from guinea-pigs actively sensitized with (group 14 + 7) or without a booster injection (group 0–21). Effects of antigen instillation. The percentages of alveolar macrophages, eosinophils, lymphocytes and neutrophils in the BAL from guinea-pigs of group 14 + 7 or 0–21 were evaluated after instillation with saline or with ovalbumin (n = 5–6). * $P < 0.05$.

Discussion

Our aim in this study was to evaluate the effect of BN 52021 on antigen-induced bronchoconstriction in actively sensitized guinea-pigs. When guinea-pigs were sensitized with low amounts of OA injected twice at a 14 day interval and were challenged with i.v. OA 7 days later, BN 52021 was inactive against bronchoconstriction triggered by bolus injections of OA or by its infusion, under conditions where the anti-histamine mepyramine is effective, indicating a major role for histamine. The failure of BN 52021 to block antigen-induced bronchoconstriction in those actively sensitized guinea-pigs might result from the multiplicity of target cells and mediators concerned when antigen is given i.v., from the different types and amounts of immunoglobulins involved or finally, from an hypothesized role of the booster injection. These three possibilities were investigated.

Different targets and a sudden and massive release of inflammatory mediators participate in anaphylaxis following the systemic administration of antigen. OA given i.v., may thus induce lung responses involving mediators released from extrapulmonary organs such as the liver, a rich source of histamine. Under those conditions, the effects of i.v. OA are probably accounted for by both PAF-dependent and independent mechanisms, which may explain the failure of BN 52021 to inhibit bronchoconstriction. Lagente *et al.* (1988) suggested that local bronchopulmonary anaphylaxis is highly dependent upon the *in situ* generation of PAF and indeed, Fitzgerald *et al.* (1985) demonstrated that large amounts of PAF are formed when antigen is given directly into the trachea of perfused lungs, which is much less apparent when the vascular route is used. For these reasons, we delivered BN 52021 as well as antigen i.t., to reach the bronchopulmonary system directly. Here, antigen induced an immediate bronchoconstriction, accompanied by a moderate thrombocytopenia and leucopenia. These mild reductions in cell counts, which probably result from the leakage of OA to the systemic circulation under the conditions of the antigen-induced increased local vascular permeability, confirm that this model involves more directly the bronchopulmonary system, a situation more relevant to human asthma. Bronchoconstriction induced by the i.t. administration of 100 μg of OA to actively sensitized and boosted guinea-pigs of group 14 + 7 is significantly antagonized by mepyramine (Vannier *et al.*, personal communication), indicating an important role for histamine under those conditions as well. At first, we verified that BN 52021 blocks bronchoconstriction induced by an i.t. administration of PAF to non-immunized guinea-pigs and then that it keeps this property when guinea-pigs are actively sensitized and boosted. This indicates that BN 52021 still interacts with the receptor for PAF in the boosted guinea-pigs, in contrast to the results obtained *in vitro* in the model of perfused lungs where PAF antagonists such as BN 52021, which inhibit PAF-induced effects on isolated lungs from non-immunized or from immunized but non-boosted guinea-pigs of group 0–21,

fail to do so on lungs from boosted guinea-pigs of group 14 + 7 (Pretolani *et al.*, 1989).

The i.t. administration of BN 52021 to guinea-pigs of group 14 + 7, 30 min before the instillation of OA failed to inhibit bronchoconstriction. In addition, BN 52021 also failed here to reduce the contractions and the TxB_2 formation and histamine liberation by OA-challenged parenchymal lung strips (present results), as well as the release of mediators from perfused lungs provided by boosted guinea-pigs of group 14 + 7 (Lagente *et al.*, 1985). It is thus clear that BN 52021 is inactive against anaphylaxis of guinea-pigs of group 14 + 7, irrespective of the lung preparation used and of the route of administration of the antigen.

The sensitization procedure used by us involves the formation of a mixture of IgE and IgG (Andersson, 1980) and we recently demonstrated that the latter are more important for anaphylactic bronchoconstriction than the former (Desquand *et al.*, 1991). To determine the possible relationship between the intensity of the immune response or the sub-class of immunoglobulins formed and the lack of effects of BN 52021, anaphylactic bronchoconstriction and the amounts of circulating IgG were studied over a period of time. Neither bronchoconstriction nor IgG were detected up to 14 days after the sensitizing injection of OA. If the antigen booster was administered at day 14, the amounts of IgG increased for 7 weeks and returned at day 96, back to titers similar to those of non-boosted animals (see below). Here again, i.t. BN 52021 failed to inhibit bronchoconstriction induced by the instillation of 100 μg of OA to guinea-pigs used at different intervals after the booster injection (groups 14 + 28, 14 + 56 and 14 + 84). In all these cases, bronchoconstriction was practically of the same intensity and was refractory to BN 52021, even for the animals of the group 14 + 84, which contained very low titers of IgG in their sera, as compared to the other groups. Thus, refractoriness of anaphylactic bronchoconstriction to BN 52021 probably does not involve differences in the IgG titers. It is noteworthy that the IgE titers have a time-course identical to that of IgG (Desquand *et al.*, 1990) and accordingly, sensitivity of anaphylactic bronchoconstriction to BN 52021 does also not result from the selective involvement of one type of immunoglobulin. It can nevertheless be argued that the booster injection of antigen may induce formation of antibody of a higher affinity than that produced during the primary response and that this could account for the refractoriness of anaphylactic bronchoconstriction of boosted guinea-pigs to BN 52021.

The guinea-pigs which developed an anaphylactic bronchoconstriction refractory to the PAF antagonist had been sensitized twice. We thus decided to study the possible role of the booster injection in determining whether BN 52021 would or would not behave as an antagonist. Even though 21 or 70 days after a single OA injection at day 0 guinea-pigs showed reduced IgG titers, both groups developed an anaphylactic bronchoconstriction with 1 mg of OA which was of the same intensity as that observed after the instillation of 100 μg to boosted guinea-pigs. This indicates that immunoglobulins are associated with the targeted inflammatory cells before their levels become detectable in the serum. In contrast to its failure in the case of boosted guinea-pigs, BN 52021 blocked the intensity and the duration of the bronchoconstriction in the single-sensitized animals. The efficacy of BN 52021 against local anaphylaxis after antigen instillation to non-boosted guinea-pigs of group 0–21 indicates that it interferes with an intrapulmonary target shared by the antigen. Failure of BN 52021 to block the slight leucopenia and thrombocytopenia after i.t. antigen administration is not surprising, since the intensity of these systemic effects is very low, which in fact is one of the interests of this model.

The *in vivo* effects agree with those on isolated lung strips, where BN 52021 reduced significantly the OA-induced contractions, even though it failed to prevent the accompanying liberation of TxB_2 and histamine. Nevertheless, lung strips may not be an adequate model for studying anti-allergic substances. Indeed, using antagonists of the different mediators involved, we failed to suppress antigen-induced activation of lungs strips provided by passively (Desquand *et al.*, 1991), or by actively sensitized guinea-pigs (Detsouli *et al.*, 1985; Pretolani *et al.*, 1987). As discussed below, inflammatory cell invasion of the lungs following the booster may account for the differences in sensitivity to PAF antagonists. It is thus not surprising that BN 52021 is not fully effective against anaphylactic contractions of parenchymal lung strips.

At this stage, it became clear that bronchoconstriction in guinea-pigs actively sensitized without a booster injection is suppressed by BN 52021 and becomes refractory to the PAF antagonist after the booster. In support, when guinea-pigs were sensitized with a single dose of a high amount of OA, both Berti *et al.* (1986) and we (data not shown) observed that BN 52021 inhibits bronchoconstriction induced by the i.v. administration of OA to those unboosted animals 21 days after the sensitization injection, as well as the antigen-induced formation of TxB_2 and release of histamine from isolated perfused lungs (Berti *et al.*, 1988).

Another argument in favour of a central role of the booster, is provided by experiments with a group of guinea-pigs sensitized once, boosted at day 98 and used 7 days later; the booster induced an increase in the titers of circulating IgG, which became comparable to those of guinea-pigs of the group 14 + 7. Here also, BN 52021 failed to inhibit bronchoconstriction.

We suggested that the effectiveness of BN 52021 against anaphylaxis may not only be accounted for by its anti-PAF effects, but may also result from uncharacterized additional anti-anaphylactic properties (Desquand *et al.*, 1988). Nevertheless, in our hands, WEB 2086, a PAF antagonist structurally unrelated to BN 52021 (Casals-Stenzel, 1987) was also inactive against the anaphylactic bronchoconstriction by i.v. OA in guinea-pigs actively sensitized and boosted (Pretolani *et al.*, 1987) whereas WEB 2086 was effective when used up to four days after the booster (Desquand *et al.*, 1990). It is thus likely that the booster injection modifies the lung responsiveness and that this modification is completed a few days later. In addition, it is clear that the subtle differences in the effectiveness of PAF antagonists against anaphylaxis are neither specific for BN 52021 nor result from the use of the i.t. route of administration.

Our claim that the booster injection determines whether the anaphylactic bronchoconstriction is or is not suppressed by BN 52021 explains the initial disappointing results obtained with PAF antagonists used against active anaphylactic bronchoconstriction and negates previous conclusions that PAF is not involved in active anaphylaxis (Detsouli *et al.*, 1985; Lagente *et al.*, 1985; Danko *et al.*, 1988). This finding can probably be generalized. Indeed, Pretolani *et al.* (1988) reported that in the *in vitro* model of perfused lungs, the pharmacological modulation of the bronchopulmonary and secretory effect of PAF changes when the lungs are obtained from boosted or non-boosted guinea-pigs, since the booster injection triggers important modifications of the lung reactivity to the phospholipid mediator.

Finally, the present observations that the booster injection modifies the sensitivity of the anaphylactic bronchoconstriction to BN 52021 may be related to the modification of the content in inflammatory cells of the BAL as well as to their recruitment. Indeed, under our conditions, the cell content of the BAL of the animals sensitized with and without a booster injection of antigen was similar for macrophages, eosinophils and neutrophils, but was different with respect to lymphocytes, since their number practically doubled in boosted as compared to non-boosted animals. An eosinophil accumulation following allergen provocation is reported to be associated with the late asthmatic responses (Hutson *et al.*, 1988). Under our conditions, even though the percentage of eosinophils in basal conditions was similar in boosted and non-boosted guinea-pigs, their number increased markedly 5 min after antigen instillation to boosted guinea-pigs and was

not modified in non-boosted animals. These modifications, in clear association with the booster injection may be important for the pharmacological differences we now describe.

In summary, our results show that the booster injection of antigen to a pre-sensitized animal determines the relative efficacy of a PAF antagonist on subsequent antigen challenge.

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References

ANDERSSON, P. (1980). Antigen-induced bronchial anaphylaxis in actively sensitized guinea-pigs. Pattern of response in relation to immunization regimen. *Allergy*, **35**, 65–71.

BASRAN, G.S., PAGE, C.P., PAUL, W. & MORLEY, J. (1984). Platelet-activating factor: a possible role in the dual response to allergen? *Clin. Allergy*, **14**, 75–79.

BERTI, F., OMINI, C., ROSSINI, G. & BRAQUET, P. (1986). Protection by two ginkgolides, BN 52020 and BN 52021, against guinea-pig lung anaphylaxis. *Pharmacol. Res. Commun.*, **18**, 775–793.

BERTI, F., GALLI, G., MAGNI, F., ROSSINI, G., VILLA, L., PUGLISI, L., PARSARGIKLIAN, R. & BRAQUET, P. (1988). The PAF-acether receptor antagonist BN 52021 inhibits mediator release during guinea-pig active lung anaphylaxis. *Pharmacol. Res. Commun.*, **12**, 1047–1059.

BRAQUET, P., SPINNEWYN, B., BRAQUET, M., BOURGAIN, R.H., TAYLOR, J.E., NUNEZ, D. & DRIEU, K. (1985). BN 52021 and related compounds: A new series of highly specific PAF-acether antagonists isolated from *Ginkgo biloba*. *Blood Vessels*, **16**, 559–572.

BRAQUET, P. & GODFROID, J.J. (1987). PAF-acether specific binding sites: 2. Design of specific antagonists. *Trends Pharmacol. Sci.*, **7**, 397–403.

BRAQUET, P., TOUQUI, L., SHEN, T.Y. & VARGAFTIG, B.B. (1987). Perspectives in platelet-activating factor research. *Pharmacol. Rev.*, **39**, 97–143.

CASALS-STENZEL, J. (1987). Effects of WEB 2086, a novel antagonist of platelet-activating factor, in active and passive anaphylaxis. *Immunopharmacol.*, **13**, 117–124.

CIRINO, M., LAGENTE, V., LEFORT, J. & VARGAFTIG, B.B. (1986). A study with BN 52021 demonstrates the involvement of PAF-acether in IgE-dependent anaphylactic bronchoconstriction. *Prostaglandins*, **32**, 121–127.

DANKO, G., SHERWOOD, J.E., GRISSOM, B., KREUTNER, W. & CHAPMAN, R.W. (1988). Effect of the PAF antagonists, CV-3988 and L-652,731 on the pulmonary and haematological responses to guinea-pig anaphylaxis. *Pharmacol. Res. Commun.*, **20**, 785–798.

DESQUAND, S., LEFORT, J., DUMAMEY, C. & VARGAFTIG, B.B. (1990). The booster injection of antigen during active sensitization modifies the anti-anaphylactic activity of the PAF antagonist WEB 2086. *Br. J. Pharmacol.*, **100**, 217–222.

DESQUAND, S., ROTHHUT, B. & VARGAFTIG, B.B. (1991). Role of immunoglobulins G1 and G2 for the anaphylactic shock in the guinea-pig. *Int. Arch. Allergy Appl. Immunol.*, (in press).

DESQUAND, S., TOUVAY, C., RANDON, J., VILAIN, B., MARIDONNEAU-PARINI, I., ETIENNE, A., LEFORT, J., BRAQUET, P. & VARGAFTIG, B.B. (1986). Interference of BN 52021 (ginkgolide B) with the bronchopulmonary effects of PAF-acether in the guinea-pig. *Eur. J. Pharmacol.*, **127**, 83–95.

DESQUAND, S. & VARGAFTIG, B.B. (1988). Interference of the PAF-acether antagonist BN 52021 with bronchopulmonary anaphylaxis. Can a case be made for PAF-acether in bronchopulmonary anaphylaxis in the guinea-pig? In *Ginkgolides. Chemistry, Biology, Pharmacology and Clinical Perspectives*, ed. Braquet, P., Vol. 1, pp. 271–281. S.A., J.R. Prous Sciences Publishers.

DESQUAND, S., VILAIN, B., LAGENTE, V., RANDON, J., TOUVAY, C., LEFORT, J., BRAQUET, P. & VARGAFTIG, B.B. (1987). Interference of PAF-acether antagonist BN 52021 with anaphylaxis in the guinea-pig. In *Advances in Prostaglandin, Thromboxane and Leukotriene Research*, ed. Samuelson, B., Paoletti, R. & Ramwell, P.W., Vol. 17, pp. 814–817. New York: Raven Press.

DETSOULI, A., LEFORT, J. & VARGAFTIG, B.B. (1985). Histamine and leukotriene-independent guinea-pig anaphylactic shock unaccounted for by Paf-acether. *Br. J. Pharmacol.*, **84**, 801–810.

FITZGERALD, M., MONCADA, S. & PARENTE, L. (1985). The release of PAF-acether and lyso-PAF from sensitized guinea-pig lungs. *Br. J. Pharmacol.*, **86**, 573.

HUTSON, P.A., HOLGATE, S.T. & CHURCH, M.K. (1988). Inhibition by nedocromil sodium of early and late phase bronchoconstriction and airway cellular infiltration provoked by ovalbumin inhalation in conscious sensitized guinea-pigs. *Br. J. Pharmacol.*, **94**, 6–8.

LAGENTE, V., DESQUAND, S., RANDON, J., LEFORT, J. & VARGAFTIG, B.B. (1985). Interference of PAF-acether antagonists with the effects of PAF itself and the anaphylactic shock *in vitro* and *in vivo* in guinea-pig bronchopulmonary preparations. *Prostaglandins*, **30**, 703.

LAGENTE, V., TOUVAY, C., RANDON, J., DESQUAND, S., CIRINO, M., VILAIN, B., LEFORT, J., BRAQUET, P. & VARGAFTIG, B.B. (1987). Interference of the PAF-acether antagonist BN 52021 with passive anaphylaxis in the guinea-pig. *Prostaglandins*, **33**, 265–274.

LAGENTE, V., DESQUAND, S., HADVARY, P., CIRINO, M., LELLOUCH-TUBIANA, A., LEFORT, J. & VARGAFTIG, B.B. (1988). Interference of the PAF antagonist Ro 19-3704 with PAF and antigen-induced bronchoconstriction in the guinea-pig. *Br. J. Pharmacol.*, **94**, 27–36.

LEBEL, B. (1983). A high-sampling-rate automated continuous-flow fluorimetric technique for the analysis of nanogram levels of histamine in biological samples. *Anal. Biochem.*, **133**, 16.

LEFORT, J. & VARGAFTIG, B.B. (1978). Role of platelets in aspirin-sensitive bronchoconstriction in the guinea-pig; interactions with salicylic acid. *Br. J. Pharmacol.*, **6**, 35–42.

LULICH, K.M., MITCHELL, H.W. & SPARROW, M.P. (1976). The cat lung strip as an *in vitro* preparation of peripheral airways: a comparison of β-adrenoceptor agonists, autacoids and anaphylactic challenge on the lung strip and trachea. *Br. J. Pharmacol.*, **58**, 71–78.

PINCKARD, R.N., FARR, R.S. & HANAHAN, D.J. (1979). Physicochemical and functional identity of rabbit platelet-activating factor (PAF) released *in vitro* from IgE sensitized basophils. *J. Immunol.*, **123**, 1847–1857.

PRETOLANI, M., LEFORT, J., MALENCHÈRE, E. & VARGAFTIG, B.B. (1987). Interference of the novel PAF-acether antagonist WEB 2086 with the bronchopulmonary responses to PAF-acether and to active and passive shock in the guinea-pig. *Eur. J. Pharmacol.*, **140**, 311–321.

PRETOLANI, M., LEFORT, J. & VARGAFTIG, B.B. (1988). Active sensitization induces lung hyperresponsiveness in the guinea-pigs: pharmacological modulation and triggering role of the booster injection. *Am. Rev. Respir. Dis.*, **138**, 1572–1578.

PRETOLANI, M., LEFORT, J. & VARGAFTIG, B.B. (1989). Limited interference of specific PAF-acether antagonists with hyperresponsiveness to PAF-acether itself of lungs from actively sensitized guinea-pigs. *Br. J. Pharmacol.*, **97**, 433–442.

SHORE, P.A., BURKHALTER, A. & COHN, V.H. (1959). A method for the fluorimetric assay of histamine in tissues. *J. Pharmacol. Exp. Ther.*, **127**, 182–186.

SORS, H., PRADELLES, P. & DRAY, F. (1978). Analytic methods for thromboxane B_2 measurement and validation on radioimmunoassay by gas liquid chromatography-mass spectrometry. *Prostaglandins*, **16**, 277–290.

TOUVAY, C., ETIENNE, A. & BRAQUET, P. (1985). Inhibition of antigen-induced lung anaphylaxis in the guinea-pig by BN 52021, a new specific PAF-acether receptor antagonist isolated from Ginkgo biloba. *Agents Actions*, **17**, 371–372.

TOUVAY, C., VILAIN, B., ETIENNE, A., SIROIS, P., BORGEAT, P. & BRAQUET, P. (1986a). Characterization of platelet-activating factor (PAF-acether)-induced contractions of guinea-pig lung strips by selected inhibitors of arachidonic acid metabolism and by PAF antagonists. *Immunopharmacol.*, **16**, 97–104.

TOUVAY, C., VILAIN, B., TAYLOR, J.E., ETIENNE, A. & BRAQUET, P. (1986b). Proof of the involvement of platelet-activating factor (PAF-acether) in pulmonary complex immune systems using a specific PAF-acether receptor antagonist: BN 52021. *Prog. Lipid Res.*, **26**, 277–288.

VARGAFTIG, B.B., BORGEAT, P., BRAQUET, P., BRAQUET, M., BROCKLEHURST, W.E., DAHLEN, S.E., DRAZEN, J.M., ETIENNE, A., FITZPATRICK, F.A., HENSON, P.M., HOLGATE, S.T., ISRAEL, E.,

MENCIA-HUERTA, J.M., MURPHY, R.C., ROBINSON, C. & SHORE, S. (1985). Leukotrienes and immediate hypersensitivity. *Ann. Inst. Pasteur, Immunol.*, **136D**, 175–228.

VARGAFTIG, B.B., LEFORT, J., CHIGNARD, M. & BENVENISTE, J. (1980). Platelet-activating factor induces a platelet-dependent bronchoconstriction unrelated to the formation of prostaglandin derivatives. *Eur. J. Pharmacol.*, **65**, 185–192.

VILAIN, B., LAGENTE, V., TOUVAY, C., DESQUAND, S., RANDON, J., LEFORT, J., BRAQUET, P. & VARGAFTIG, B.B. (1986). Pharmacological control of the *in vivo* passive anaphylactic shock by the PAF-acether antagonist compound BN 52021. *Pharmacol. Res. Commun.*, **18**(Suppl), 119–126.

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Anti-arrhythmic activities of opioid agonists and antagonists and their stereoisomers

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1 A series of opioid agonists, antagonists and their (+)-stereoisomers were tested for antiarrhythmic activity in the rat coronary artery occlusion model.

2 Naloxone (0.01–2 mg kg^{-1}) significantly reduced the incidence and severity of cardiac arrhythmias, in accordance with previous published studies.

3 The non-opioid stereoisomer, (+)-naloxone, was equipotent with naloxone against occlusion-induced arrhythmia.

4 Similar non-stereospecific antiarrhythmic effects were induced by another opioid antagonist, Win 44,441-3 and its stereoisomer Win 44,441-2.

5 The opioid agonists, morphine and levorphanol, protected against occlusion-induced arrhythmia as did the opioid antagonists, and the (+)-stereoisomer, dextrorphan, was equipotent to levorphanol.

6 It is concluded that the antiarrhythmic effects of opioid drugs are not mediated by opioid receptors. A direct effect on ionic currents in cardiac muscle is suggested as the mechanism of opioid antiarrhythmic activity.

Introduction

The opioid antagonist, naloxone, has been shown previously to reduce the incidence and severity of cardiac arrhythmias resulting from coronary ligation in rats (Fagbemi *et al.*, 1982). Similar antiarrhythmic activity of naloxone was later described in other species with various experimental models (Huang *et al.*, 1986; Lee *et al.*, 1986). The accepted explanation for this action of naloxone is the blockade of opioid receptors which prevents the putative disturbing effects of endogenous opioid peptides. We recently found, however, that the non-opioid stereoisomer (+)-naloxone is as effective as (−)-naloxone in abolishing arrhythmias, indicating that the antiarrhythmic activity of the drug is not mediated by opioid receptors (Sarne *et al.*, 1988). Our conclusions contradicted the findings of Parratt & Sitsapesan (1986), who showed that two other opioid antagonists, Win 44,441 and Mr 1452, protect against cardiac arrhythmia in a stereospecific manner. The stereospecificity, however, was much lower than expected for opioid receptors (see Discussion for details).

A major difference between these two aforementioned studies is the model of arrhythmia: we studied adrenaline-induced arrhythmia while Parratt & Sitsapersan (1986) used coronary artery occlusion. In order to compare the two studies, and to elucidate the mechanism by which opiates exert their antiarrhythmic activity, we tested the effects of various opioid agonists and antagonists in a model of coronary occlusion-induced arrhythmia. The results support our hypothesis that the antiarrhythmic action of opiates does not involve opioid receptors.

Methods

Charles River descendent male rats (400–500 g) were anaesthetized intraperitoneally with pentobarbitone (30 mg kg^{-1}). The femoral vein was cannulated for administration of drugs, and the electrocardiogram (lead II) was recorded by limb electrodes. Coronary artery occlusion was carried out as described by Clark and co-workers (1980).

Drugs were administered intravenously in 0.1–1.0 ml of dextrose, followed, 2 min later, by the ligation of the cardiac artery. The electrocardiogram was monitored for 90 min, or until death of the animal.

The dysrhythmias identified in the electrocardiogram included ventricular ectopic beats (VEBs), ventricular tachycardia (VT) and ventricular fibrillation (VF). Arrhythmia was defined as more than 10 VEBs or a dysrhythmic incidence (VT or VF) of more than 10 s duration.

The following drugs were used: the opioid antagonists naloxone and Win 44,441-3 (guadazocine) and their stereoisomers (+)-naloxone and Win 44,441-2; the opioid agonists morphine and levorphanol and the (+)-stereoisomer dextrorphan. Each experiment consisted of 6 groups of 5–8 animals each: the control group received dextrose, and the other 5 groups received various drugs in different doses. The experiments were carried out double blind and drugs code was revealed only after the electrocardiogram had been analyzed.

The Chi Square and Fisher Exact Probability tests were used for comparing proportions, and the Mann-Whitney test provided non-parametric quantitative comparisons (Siegel, 1956).

Results

Coronary artery occlusion elicited severe arrhythmias in 38 of 51 control rats (74%); 17 of these rats (44%) died before the end of the experiment (90 min). The number of VEBs in control animals during the first 10 min following occlusion was 41.1 ± 37 (Table 1).

Administration of 2 mg kg^{-1} naloxone 2 min before ligation reduced the incidence and severity of cardiac arrhythmia: only 7 out of 22 (32%) showed severe arrhythmia, with 4 rats (18%) dying in 90 min. The number of VEBs in the naloxone-treated group was 2.5 ± 5. All the examined parameters were significantly lower in the naloxone-treated group than in control animals. A lower dose of naloxone, 0.01 mg kg^{-1}, was still effective in protecting rats from arrhythmia, while 0.001 mg kg^{-1} naloxone offered no protection (Table 1). The non-opiate isomer (+)-naloxone showed similar protective effects and dose-dependency (Table 1).

Moderate doses (0.1 mg kg^{-1}) of the opioid antagonist Win 44,441-3 and its stereoisomer Win 44,441-2 were similarly effective in preventing arrhythmias (Table 1). Higher doses of these drugs (1–4 mg kg^{-1}) were not effective and sometimes induced arrhythmias and caused death even without ligation

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Table 1 The effect of opioid agonists, antagonists and stereoisomers on coronary occlusion-induced arrhythmias

Drug	*Dose* ($mg\,kg^{-1}$)	n	*Arrhythmic rats* (%)	*VEBs in 10 min*
Control	—	51	74	41.1
Naloxone	2	22	32**	2.5**
	0.01	8	37*	5.9*
	0.001	5	60	78.8
(+)-Naloxone	2	7	14**	4.0**
	0.01	6	16**	4.5*
Win 44,441-3	2	9	100	45.9
	0.1	11	27*	3.2**
	0.01	10	50	53.4
Win 44,441-2	0.1	7	14**	4.3**
	0.01	5	40	51.0
Levorphanol	0.2	6	66	79.5
	0.05	5	20*	5.4*
	0.005	5	20*	0.4**
Dextrorphan	0.05	5	40	15.0
	0.005	5	20*	0.8**
Morphine	4	7	29*	6.7
	0.2	12	83	19.5

VEBs: ventricular ectopic beats.
Significantly less than control: * $P < 0.05$, ** $P < 0.01$.

of the coronary artery. These arrhythmogenic effects of Win 44,441 were associated with a fall in blood pressure and led to death in a few animals, similar to the lethal side-effects of high doses of other known antiarrhythmic drugs (Bigger & Hoffman, 1990).

The opioid agonist, levorphanol, was also an effective antiarrhythmic agent: $0.005\,mg\,kg^{-1}$ reduced the incidence of severe arrhythmias to 20%, compared to 74% in controls, and the number of VEBs to 0.4, compared to 41.1 in controls. Higher doses ($0.05\,mg\,kg^{-1}$) were somewhat less effective, while a dose of $0.2\,mg\,kg^{-1}$ elicited arrhythmias even without coronary occlusion. Similar results were obtained with the non-opioid stereoisomer dextrorphan.

The classical opioid agonist, morphine, exhibited similar antiarrhythmic activity, although a significant effect could be obtained only at relatively high doses ($2–4\,mg\,kg^{-1}$).

Discussion

The present study confirms the original findings of Fagbemi *et al.* (1982) on the antiarrhythmic activity of naloxone. The pharmacological effects of naloxone are commonly attributed to the blockade of opioid receptors which prevents the action of endogenous opioid peptides, an explanation supported by Parratt & Sitsapesan (1986) who showed that the (+)-isomers Win 44,441-2 and Mr 1453 were less effective than their (−)-opioid antagonist counterparts. Quantitative examination of their results, however, reveals that Mr 1453 was only 2–10 times less potent than its opioid stereoisomer Mr 1452. The expected ratio of potency between these two isomers at the opioid receptor is 300 (Merz *et al.*, 1974).

An alternative explanation for the antiarrhythmic action of naloxone is a direct, non-opioid effect on the cardiac muscle. This hypothesis was supported by our finding (Sarne *et al.*, 1988) that (+)-naloxone, which is 1000–10,000 less potent as an opioid antagonist (Iijima *et al.*, 1978) is equipotent as an antiarrhythmic agent. We decided to verify the results of our previous study which was carried out on adrenaline-induced arrhythmia, by testing it with the more conventional, coronary occlusion model used by Parrat & Sitsapesan. The present study confirms our earlier findings and supports our hypothesis that the antiarrthymic effect of naloxone does not involve opioid receptors. Furthermore, similar results were obtained in the present study with another pair of opioid antagonist isomers, Win 44,441-3 and Win 44,441-2.

This hypothesis gains additional support from our finding that opioid agonists are also effective antiarrhythmic agents. Both morphine and levorphanol protected the animals from ligation-induced arrhythmia, similar to the opioid antagonists. This effect was also not stereospecific with the non-opioid (+)-isomer, dextrorphan, equipotent to levorphanol.

While our results seem to contradict previous findings by Parratt & Sitsapesan (1986), it must be remembered that the two studies differ in several ways, including strains of rats, timing of drug administration and severity of the induced arrhythmias. In our present model, as well as in our previous studies on adrenaline-induced arrhythmia (Sarne *et al.*, 1988) and ouabain-induced arrhythmia (Sarne *et al.*, unpublished observations), the antiarrhythmic activity of opiates was not stereospecific. Parratt & Sitsapesan (1986), on the other hand, observed some stereospecificity, albeit much lower than expected for opioid receptors. It is feasible that two different mechanisms mediate the antiarrhythmic activity of opiates, one that involves opioid receptors and another non-opioid in nature. The latter mechanism predominates in our model, while both mechanisms are involved in Parratt & Sitsapesan's model, leading to partial opioid stereospecificity.

The mechanism by which opioid agonists and antagonists exert their non-opioid antiarrhythmic activity can be explained by our *in vitro* studies on cardiac muscle (Sarne, 1989) in which opiates were found to suppress excitability of isolated strips of muscles in a non-stereospecific fashion. These findings are in accord with previous studies on the suppression of sodium and potassium currents by opioid drugs (Frazier *et al.*, 1973; Carratu & Mitolo-Chieppa, 1982; Brasch, 1986). The molecular basis for this effect is currently under investigation in our laboratory. Preliminary results with radiolabelled markers indicate that opioid agonists as well as opioid antagonists interact with voltage-dependent sodium channels, and that this interaction is not mediated by opioid receptors. This non-opioid action of opiates should be taken into consideration when interpreting the pharmacological effects of opioid drugs.

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References

BIGGER, J.T. & HOFFMAN, B.F. (1990). Antiarrhythmic drugs. In *Goodman and Gilman's The Pharmacological Basis of Therapeutics.* ed. Gilman, A., Rall, T.W., Nies, A.S. & Taylor, P. 8th edition, New York: Pergamon Press.

BRASCH, H. (1986). Influence of optical isomers (+)- and (−)-naloxone on beating frequency, contractile force and action potential of guinea-pig isolated cardiac preparations. *Br. J. Pharmacol.*, **88**, 733–740.

CARRATU, M.R. & MITOLO-CHIEPPA, D. (1982). Inhibition of ionic currents in frog node of Ranvier treated with naloxone. *Br. J. Pharmacol.*, **77**, 115–119.

CLARK, C., FOREMAN, M.I., KANE, K.A., MCDONALD, F.M., PARRATT, J.R. (1980). Coronary artery ligation in anesthetized rats as a method for production of experimental dysrhythmias and for the determination of infarct size. *J. Pharmacol. Methods*, **3**, 357–368.

FAGBEMI, O., LEPRAN, I., PARRATT, J.R. & SZEKERES, L. (1982). Naloxone inhibits early arrhythmias resulting from acute coronary ligation. *Br. J. Pharmacol.*, **76**, 504–506.

FRAZIER, D.T., OHTA, M. & NARAHASHI, T. (1973). Nature of the morphine receptor present in the squid axon. *Proc. Soc. Exp. Biol. Med.*, **142**, 1209–1214.

HUANG, X.D., LEE, A.Y.S., WONG, T.M., ZHAN, C.Y. & ZHAO, Y.Y. (1986). Naloxone inhibits arrhythmias induced by coronary artery occlusion and reperfusion in anaesthetized dogs. *Br. J. Pharmacol.*, **87**, 475–477.

IIJIMA, I., MINAMIKAWA, A.J., JACOBSON, A.E., BROSSI, A., RICE, K.C. & KLEE, W.A. (1978). Studies in the (+)-morphinan series. 5'. Synthesis and biological properties of (+)-naloxone. *J. Med. Chem.*, **21**, 398–400.

LEE, A.Y., UNANG, T.W.K. & WONG, T.M. (1986). Prevention and reversal of ouabain-induced cardiotoxicity by naloxone in the guinea-pig. *Clin. Exp. Pharmacol. Physiol.*, **13**, 55–58.

MERZ, M., LANGBEIN, A., STOCKHAUS, K., WALTHER, G. & WICK, H. (1974). Structure-activity relationships in narcotic antagonists with N-furylmethyl substituents. *Adv. Biochem. Psychopharm.*, **8**, 91–107.

PARRATT, J.R. & SITSAPESAN, R. (1986). Stereospecific antiarrhythmic effect of opioid receptor antagonists in myocardial ischaemia. *Br. J. Pharmacol.*, **87**, 621–622.

SARNE, Y., HOCHMAN, I., ESHED, M. & OPPENHEIMER, E. (1988). Antiarrhythmic action of naloxone: Direct, non-opiate effect on the rat heart. *Life Sci.*, **43**, 859–864.

SARNE, Y. (1989). Non opiate effects of opioid agonists and antagonists on cardiac muscle. *Adv. Biosci.*, **75**, 551–554.

SIEGEL, S. (1956). *Nonparametric Statistics.* Kogakusha: McGraw-Hill.

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Effects of antagonists on quisqualate and nicotinic receptor-mediated currents of midbrain neurones in culture

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1 The action of non-N-methyl-D-aspartate (non-NMDA) and nicotinic antagonists on excitatory postsynaptic currents (e.p.s.cs) and on quisqualate (Quis)- and nicotine-gated currents was studied by use of whole-cell recording in dissociated culture of the rat midbrain.

2 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX; 0.1 μM) and kynurenic acid (0.1 mM) attenuated network-generated and miniature e.p.s.cs while mecamylamine (100 μM) and hexamethonium (400 μM) had no effect. Acetylcholine (ACh) enhanced or suppressed e.p.s.cs. The suppressing effect of ACh was blocked by atropine (0.1–10 μM).

3 ACh (50–1000 μM) and quisqualate (Quis, 0.1–20 μM) induced inward currents with the same reversal potential as e.p.s.cs.

4 Application of Quis and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) in a low concentration (0.5 and 5 μM, respectively) evoked a maintained current which was attenuated by CNQX (1 μM) and kynurenic acid (0.5 mM) but not by mecamylamine (100 μM).

5 Higher concentrations of Quis (5–20 μM) and AMPA (50–100 μM) evoked a transient and a maintained current component. Kynurenic acid (1 mM) reduced the transient but not the maintained component. CNQX (5–10 μM) increased the maintained component without reducing the transient one; 20 μM CNQX reduced both components.

6 ACh-induced transient current was mimicked by nicotine and reversibly and dose-dependently blocked by mecamylamine. Atropine (10 μM), hexamethonium (400 μM) as well as CNQX (100 μM) and kynurenic acid (1 mM) did not affect the current.

7 Hexamethonium (50–400 μM) voltage-dependently depressed the maintained current elicited by both Quis and ACh.

8 In conclusion, although the antagonists examined here seem to discriminate between non-NMDA and nicotinic receptor-mediated e.p.s.cs, they vary considerably in respect of their mode of action when tested on Quis, AMPA and ACh-induced currents.

Introduction

Both nicotinic and non-N-methyl-D-aspartate (non-NMDA) receptors have been shown to mediate fast synaptic excitation in the mammalian CNS (for a review of the literature see Nicoll *et al.*, 1990). Identity of action of excitatory amino acids (EAA) and the endogenously released transmitter and their pharmacological identity, i.e. the selective inhibitory effect of antagonists on both synaptic and exogenously induced events, had led to the conclusion that fast excitatory postsynaptic potentials (e.p.s.ps) in the mammalian CNS are generated by EAA. Recently a growing body of experimental evidence has pointed to the presence of functional nicotinic receptors in the brain (Egan & North, 1986; Lipton *et al.*, 1987; McCormick & Prince, 1987; Vidal & Changeux, 1989). Since nicotinic and non-NMDA agonists elicit an identical conductance change, it is important to determine the extent to which the selectivity of non-NMDA and nicotinic receptor antagonists pertains to the appropriate receptor type. To do this, we investigated the selectivity of the two competitive EAA antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Honoré *et al.*, 1988) and kynurenic acid (Perkins & Stone, 1982; Ganong *et al.*, 1983) and the two nicotinic receptor antagonists, mecamylamine and hexamethonium. The antagonists were tested for acetylcholine (ACh)- and quisqualate (Quis)-induced currents and spontaneous synaptic currents in midbrain cultures by use of whole-cell recording. Midbrain neurones have been shown to express functional nicotinic receptors (Clarke *et al.*, 1985; Calabresi *et al.*, 1989), and *in situ* hybridization has revealed several genes of the nicotinic ACh receptor in this area (Goldman *et al.*, 1986; 1987).

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Methods

Cell culture

Ventral tegmental tissue from 14 day old rat embryos was mechanically dissociated and plated on a primary culture of glial cells from the same area (Misgeld & Swandulla, 1989; Swandulla & Misgeld, 1990). Cells were cultivated in Eagles basal medium (BME, Gibco) supplemented with 10% heat-inactivated horse serum (Gibco), 0.3% glucose, 2 mM glutamine, penicillin (25 iu ml^{-1}, Sigma) and streptomycin (25 μg ml^{-1}, Sigma). After 3 days in culture, horse serum was reduced to 2% and 10 μM cytosine arabinoside (Ara-C Sigma) was added for 68 h to inhibit growth of mitotic cells.

Recording conditions

The whole-cell configuration (Hamill *et al.*, 1981) was used to record with a single electrode- (for specification of the clamp see Misgeld *et al.*, 1989) or a EPC-7-patch clamp amplifier. All currents were recorded at −80 mV holding potential unless otherwise stated. During electrophysiological experiments cells were bathed in a solution containing (in mM): NaCl 145, KCl 5, $CaCl_2$ 5, $MgCl_2$ 2, glucose 15, HEPES 10 at pH 7.3 and room temperature. Tetrodotoxin (TTX, 0.3 μM) and picrotoxin (20 μM) were added to the extracellular solution except while recording spontaneous synaptic potentials. Recording

electrodes (3–5 MΩ), pulled from borosilicate glass (Clark, UK), were filled with (in mM): NaCl 10, CsCl 100, EGTA 5, $CaCl_2$ 0.25, glucose 10, HEPES 10 (pH 7.3). KCl was used instead of CsCl while recording synaptic activity. In some experiments K-gluconate replaced KCl in the intracellular solution. Data were stored on a chart recorder and on a PC.

Drugs were dissolved in extracellular solution (pH 7.3) and applied by superfusion from a wide bore (tip diameter 100 μm) 3-barrelled pipette positioned 100–200 μm from the recorded cell (Carbone & Lux, 1987). By this method, a quickly inactivating response to Quis application can be seen (Tang *et al.*, 1989) which is not detected if slower perfusion systems were used. Between drug applications, the cell was continuously washed with extracellular solution. Antagonists were tested by comparison with the control response before and after simultaneous application of agonist and antagonist. Results are expressed as mean ± s.e.mean with n equal to the number of cells studied.

Drugs

The following drugs were used: acetylcholine chloride (ACh), hexamethonium chloride, kynurenic acid, mecamylamine hydrochloride, picrotoxin, quisqualic acid (Quis), tetrodotoxin (TTX), all obtained from Sigma, F.R.G. α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), D-α-amino-5-phosphonopentanoic acid (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were from Tocris, England; nicotine salicylate was from Roth, F.R.G.

Results

Spontaneous synaptic activity

Cells were recorded between 3 and 8 weeks after plating. They exhibited spontaneous synaptic activity which was reduced by low Ca- (1 mM) and high Mg- (6 mM) containing extracellular solution (Figure 1a) and by TTX (0.3 μM). Some synaptic potentials were reversed around −10 mV, when CsCl electrodes were used, and around −60 to −50 mV while recording with gluconate filled electrodes. The reversal potential of these synaptic events, the shift of their reversal potential with changes in intracellular Cl concentration and their sensitivity to picrotoxin (20 μM) (Figure 1b) suggest that they are due to Cl conductance increase following the activation of $GABA_A$ receptors.

In the presence of picrotoxin, synaptic activity shifted from irregular activity to rhythmically occurring clusters of e.p.s.ps (Figure 1b). These e.p.s.ps reversed at potentials positive to +5 mV and were attenuated by the excitatory amino acid receptor antagonists CNQX (1 μM; Figure 1c,d) and kynurenic acid (0.5 mM; Figure 1c), while they were not reduced by α-amino-5-phosphonopentanoic acid (APV, 20–50 μM; $n = 4$) in a voltage range between −80 and −20 mV. This suggests the generation of e.p.s.ps through a non-NMDA receptor type. In the presence of picrotoxin (20 μM) and TTX (0.3 μM), under voltage clamp, spontaneous miniature e.p.s.cs (Jarolimek & Misgeld, 1991) were observed (Figure 2b(ii)). They also reversed at holding potentials positive to +5 mV. Miniature e.p.s.cs were reduced by kynurenic acid (0.1–1.0 mM; $n = 11$; Figure 2a) and CNQX (0.1–10 μM; $n = 11$; Figure 2b), but were neither affected by the nicotinic receptor antagonists mecamylamine (up to 100 μM) and hexamethonium (up to 400 μM) nor by the muscarinic antagonist atropine (1 μM). ACh altered excitatory synaptic activity (in the presence of picrotoxin), even in cells in which it did not induce the inward current described below. In cells, in which a barrage of synaptic potentials followed the application of ACh (Figure 3a(i), b(i)), atropine was ineffective. However, atropine (0.1–10 μM) reversed the effect of ACh in cells in which ACh reduced network or miniature e.p.s.cs (Figure 3: b(ii),(iii); c(i),(ii)).

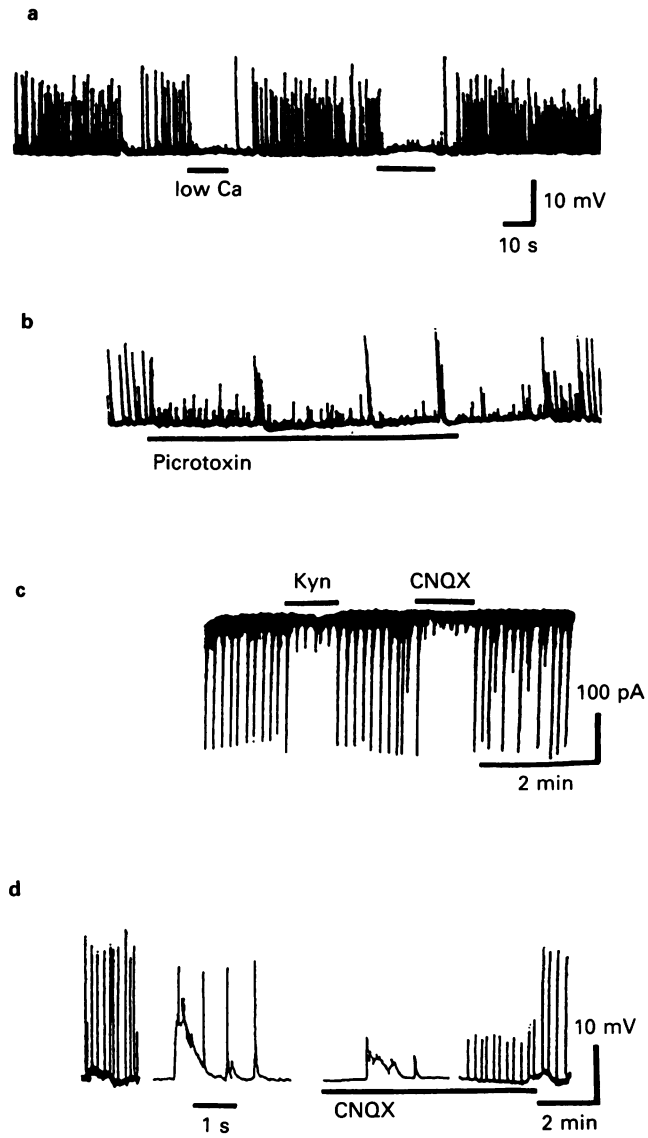

Figure 1 Synaptic activity in the midbrain culture. (a) Spontaneous network activity was reduced by low Ca- (1 mM), high Mg- (6 mM) solution. (b) Effect of picrotoxin (20 μM) on spontaneous activity in the same neurone as in (a). $V_H = -70$ mV. Calibration in (a) applies also to (b). (c) Network activity in the presence of picrotoxin under voltage clamp and its reduction by kynurenic acid (Kyn 0.5 mM) and 6-cyano-7-nitroquinoxaline- 2,3-dione (CNQX, 1 μM). (d) Effect of 1 μM CNQX on e.p.s.p. clusters occurring in the presence of picrotoxin. Drug application periods in this and subsequent figures are indicated by horizontal bars. In current clamp recordings, spikes truncated by the frequency characteristics of the chart recorder. Under voltage clamp, holding potential was −80 mV in this and all other figures, unless stated otherwise.

Acetylcholine- and quisqualate-induced currents

ACh (50–1000 μM) and quisqualate (Quis; 0.5–20 μM) elicited inward currents at negative holding potentials. While Quis-induced current was stable with subsequent applications throughout the recording, the ACh-induced response sometimes declined after the first application (Figure 5b). The voltage-dependence of the ACh-induced current and the effect of antagonists were examined in cells displaying a reliably stable response to ACh.

Both Quis- and ACh-induced currents showed concentration-dependence (Figure 4). As has been previously reported (Mayer & Vyklicky, 1989; Tang *et al.*, 1989), low doses of Quis (0.5–1 μM) evoked a current which was maintained during drug application, while higher concentrations of Quis induced a fast desensitizing, transient current which was

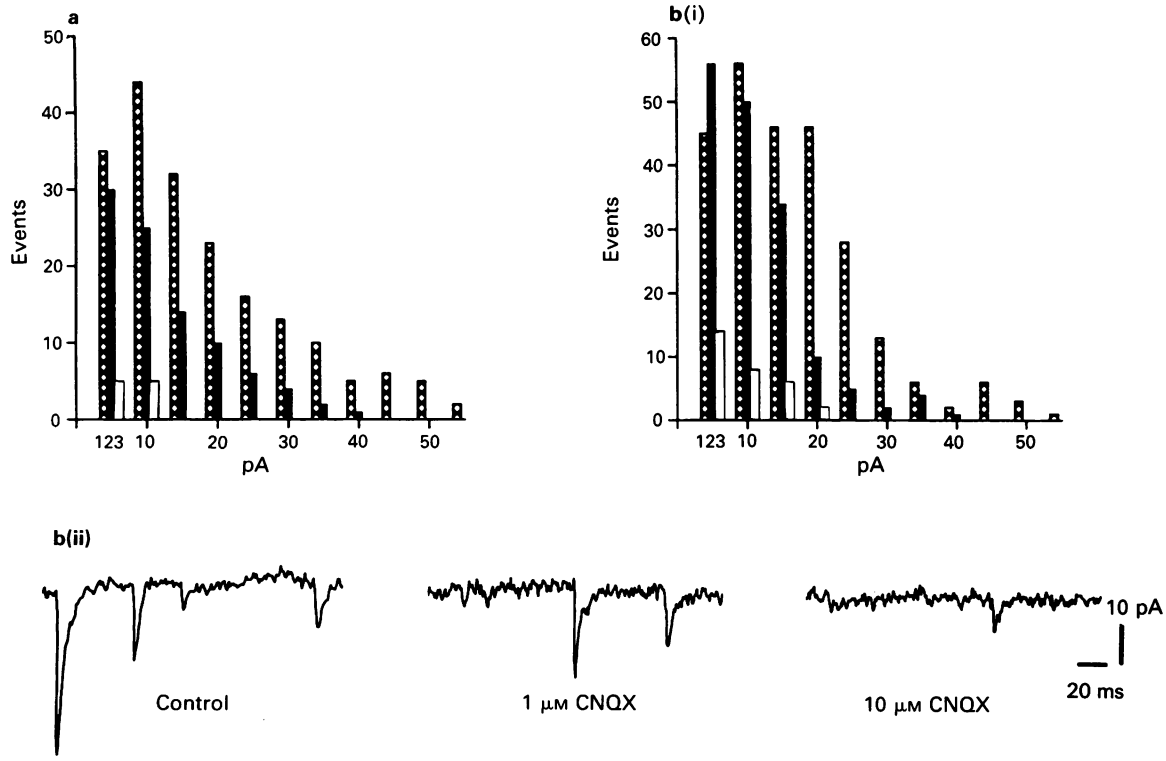

Figure 2 Effects of kynurenic acid and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) on miniature e.p.s.cs. Miniature e.p.s.cs recorded in picrotoxin (20 μM), tetrodotoxin (0.3 μM) and Cd^{2+} (20 μM) of which examples are shown in b(ii) (control) were strongly reduced by 0.1 mM kynurenic acid (Kyn) or 1 μM CNQX and nearly blocked by 1 mM Kyn or 10 μM CNQX.

The effects of the drugs are shown by histograms (a, b(i)) for miniature e.p.s.cs recorded in a single cell before, during and after drug application. Only cells with full recovery were considered. Sampling rate was 10 s at a frequency of 2 kHz. Analysis was done in 5 pA bins, minimum being twice the noise level. In (a) column 1 (hatched) control; column 2 (solid) 0.1 mM Kyn; column 3 (open) 1 mM Kyn. In (b(i)), column 1 (hatched) control; column 2 (solid) 1 μM CNQX; column 3 (open) 10 μM CNQX.

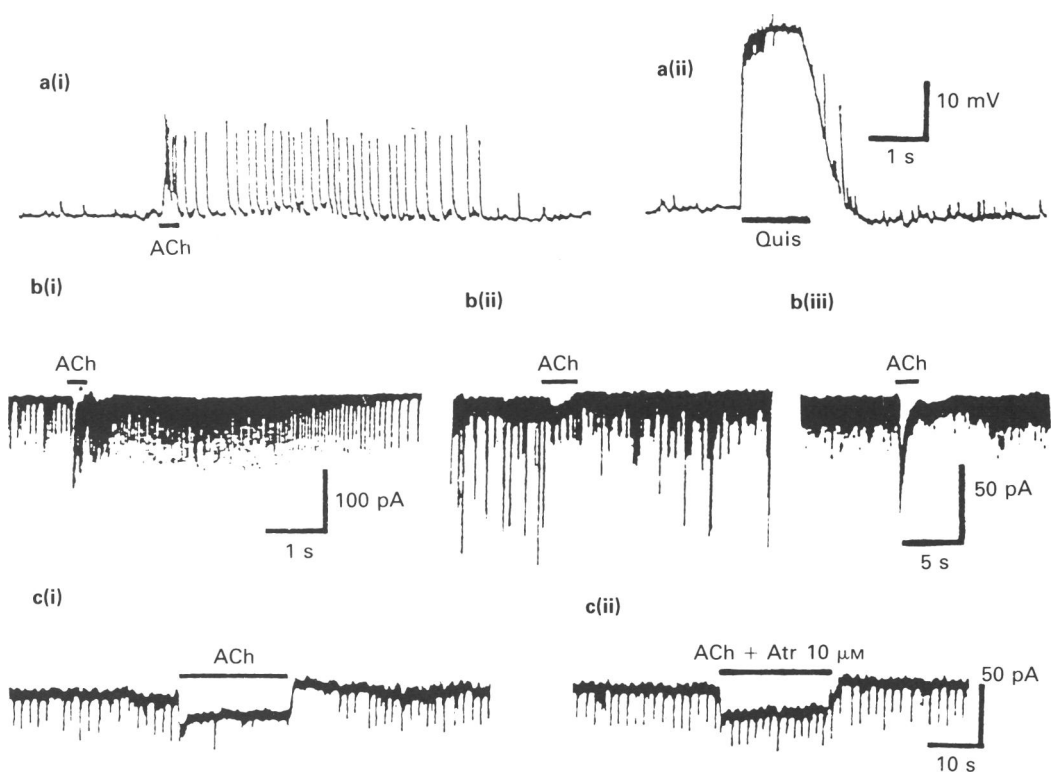

Figure 3 Effects of acetylcholine (ACh, 100 μM) on synaptic activity. (a) In picrotoxin, ACh (100 μM) induced a barrage of e.p.s.ps without depolarizing the cell (i), while quisqualate (Quis 10 μM) depolarized the same cell (ii). (b) Effects of ACh (100 μM) on e.p.s.cs recorded in the presence of picrotoxin in three different cells: (i) following the ACh-induced inward current, a barrage of e.p.s.cs is elicited; (ii) in the absence of a significant inward current, ongoing synaptic activity is reduced by ACh; (iii) in tetrodotoxin and picrotoxin, miniature e.p.s.cs are blocked after the ACh-induced inward current. (c) During and after the ACh-induced maintained inward current synaptic activity was reduced (i); this effect of ACh was antagonized by atropine (Atr) (ii).

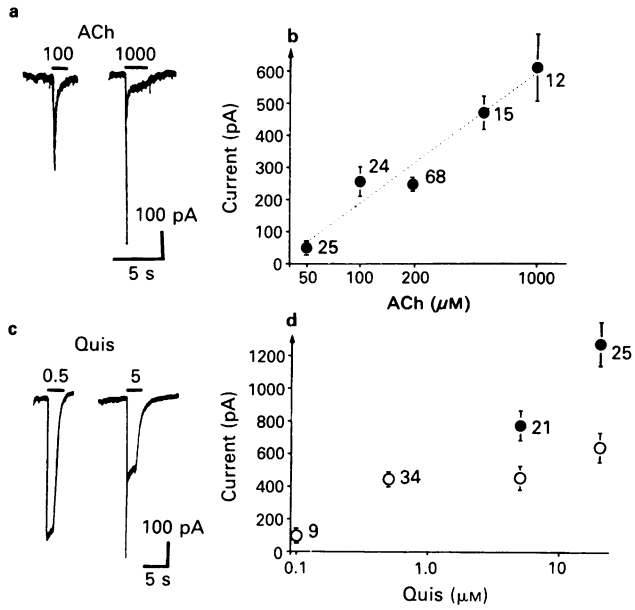

Figure 4 (a) Acetylcholine (ACh) in 100 μM concentration evoked a transient current while, at higher concentrations, the transient current was followed, in the same cell, by a maintained component. (c) A low concentration of quisqualate (Quis) induced only a maintained current while, in the same cell, a higher concentration induced both a transient and a maintained current component. (b,d) Concentration-effect curves for ACh- and Quis-induced currents. In (d) concentration-effect is presented for both components: (●) transient current; (○) maintained current. Concentrations given in (a,c) are in μM. Concentration-effect curves (b,d) are presented as mean (s.e.mean shown by vertical bars) from the number of cells indicated in the plot. Currents in this and all subsequent figures were recorded in the presence of tetrodotoxin.

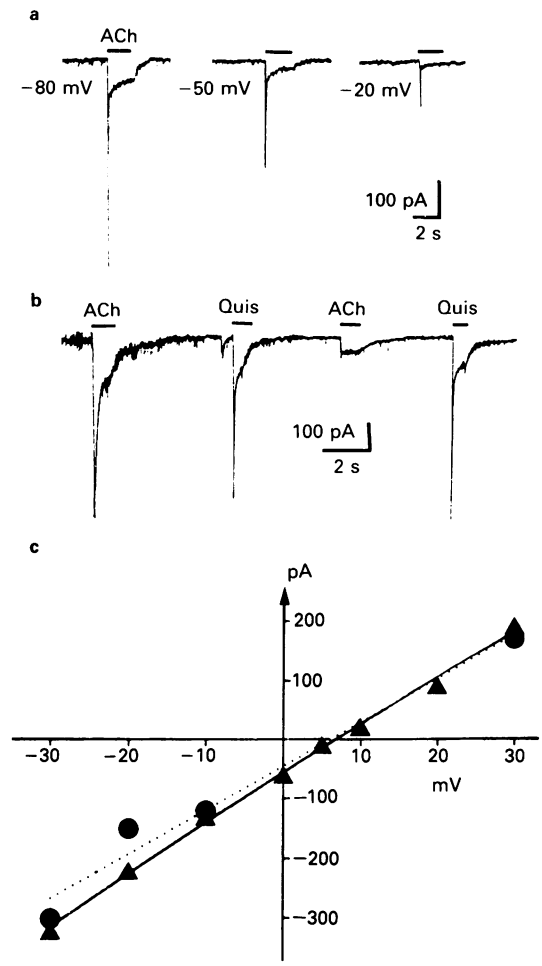

Figure 5 Characteristics of acetylcholine (ACh)- and quisqualate (Quis)-induced currents. (a) Transient and maintained inward currents elicited by ACh (1 mM) and recorded at the different holding potentials indicated. (b) Rapid rundown of an inward current induced by ACh (100 μM) with repeated application, while a similar response evoked by Quis (10 μM) remained stable in the same cell. (c) I/V plot for currents induced by ACh (●, 100 μM) and Quis (▲, 10 μM) in the same cell. ACh-evoked current amplitude normalised to that of Quis at −80 mV. Solid line is the regression line connecting Quis-induced currents; broken line is that connecting the ACh-induced currents.

followed by a slowly desensitizing, maintained current (Figure 4c). ACh elicited mostly a fast transient current, the rate of desensitization of which increased with increasing ACh concentration (Figure 4a). In the higher concentration range of ACh (0.5–1.0 mM) an additional, maintained component was sometimes apparent. In a minority of cells, ACh induced only a slow, maintained inward current.

Both transient and maintained components of ACh- and Quis-induced currents showed the same I/V characteristics (Figure 5c). The inward currents activated by ACh and Quis increased with voltage (Figure 5a,c), and currents reversed around +10 mV i.e. close to the calculated equilibrium potential of +8 mV for a nonselective cationic conductance (for ACh-evoked current the mean reversal determined from 10 cells was +10.1 ± 1.9 mV, Quis-induced current reversed at +8.6 ± 1.5 mV, $n = 6$).

ACh-evoked current could be mimicked by nicotine (50–500 μM), the two responses showing similar I/V characteristics (Figure 6c). However, because currents induced by nicotine rapidly faded, ACh was chosen as the agonist for detailed testing. Atropine (1–10 μM) did not diminish the current induced by ACh (100–500 μM; $n = 6$). However, in some instances (e.g. Figure 6a,b), the current induced by ACh in the presence of atropine (1 μM) differed with respect to the slope of its I/V curve from that without atropine suggesting interference by a muscarinic component.

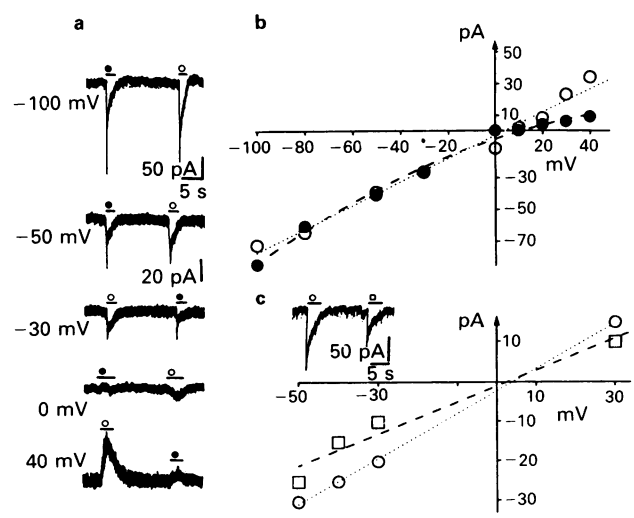

Figure 6 Acetylcholine (ACh)-induced nicotinic receptor-mediated current (a,b). In the presence of atropine (1 μM) the ACh (200 μM)-induced current (○) reversed linearly, but not the current induced by ACh alone (●) in the same cell. (c) Voltage-dependence of the nicotine (100 μM, □)- and ACh plus atropine (○)-evoked peak currents are similar when recorded in the same cell.

Effects of antagonists on quisqualate-induced current

CNQX (1 and 5 μM), an excitatory amino acid antagonist for non-NMDA receptors, reduced maintained currents induced by low doses of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA, 5–20 μM, $n = 14$; Figure 7a(i)) and Quis (0.1–1 μM, $n = 12$; Figure 7b(i)). Biphasic currents induced by 50–100 μM AMPA ($n = 14$, Figure 7a(ii) and by 5–20 μM Quis

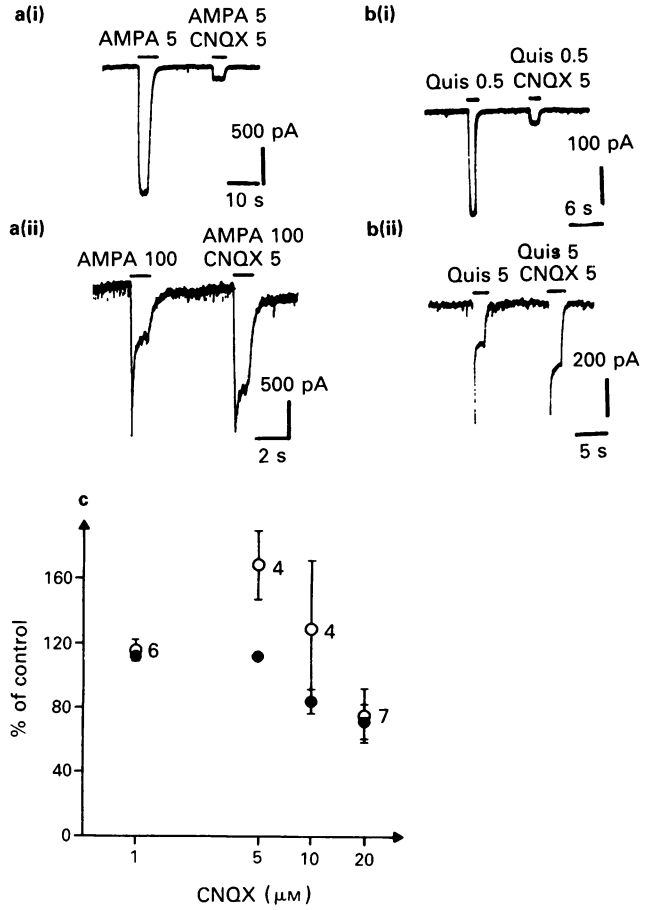

Figure 7 Effects of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) on α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and quisqualate (Quis) induced currents. (a(i)) CNQX (5 μM) reduced the amplitude of the maintained current induced by 5 μM AMPA. (a(ii)) CNQX (5 μM) rather unexpectedly enhanced the amplitude of the maintained current which followed the transient current induced by 100 μM AMPA. (b(i)(ii)) The same effects of CNQX were observed if 0.5 or 5 μM Quis were applied. (c) Dose-response curve for inhibition by CNQX of the transient (●) and maintained (○) currents elicited by 20 μM Quis. Each cell served as its own control, control set to 100%. Only cells in which current amplitude elicited by Quis before and after CNQX application were of the same size were considered. Number of cells indicated, bars represent s.e.mean.

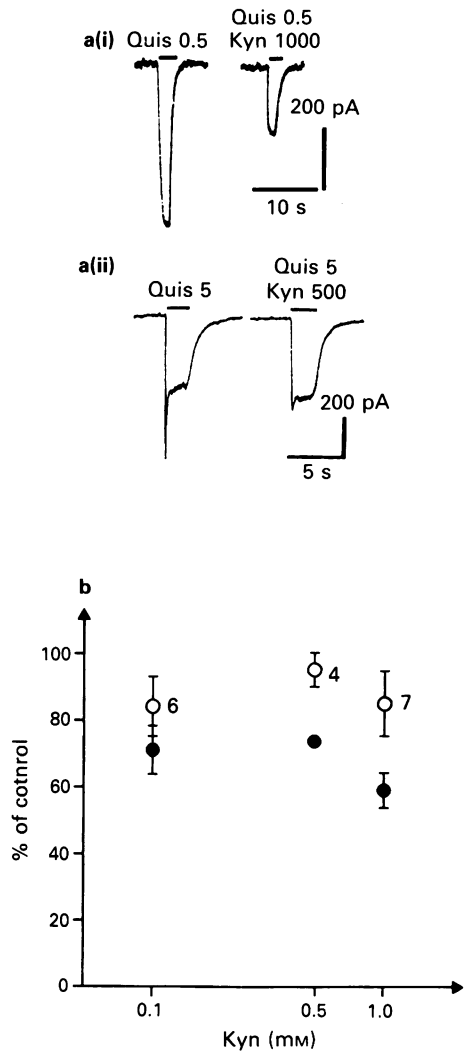

Figure 8 Effect of kynurenic acid (Kyn) on quisqualate. (Quis) induced currents. (a(i)) Kyn (1 mM) reduced the amplitude of the maintained current induced by 0.5 μM Quis. (a(ii)) Kyn (0.5 mM) reduced the transient component of the current induced by Quis (5 μM) leaving the maintained component unaffected. (b) Dose-response curve for inhibition by Kyn of the transient (●) and maintained (○) currents elicited by 5 μM Quis. Same protocol as in Figure 7c.

(n = 12, Figure 7b(ii), c) were not reduced by CNQX (1–5 μM). On the contrary, CNQX increased the amplitude of the maintained component induced by 50–100 μM AMPA and 5–20 μM Quis (Figure 7a(ii), b(ii)). After increasing the CNQX concentration (20 μM) both current components were reduced (Figure 7c). APV (20 μM, n = 3) had no effect on the transient or on the plateau current. Kynurenic acid (0.1–1 mM, n = 12) also reduced maintained currents induced by low doses of quisqualate (Figure 8a(i)). However, only the transient component of the biphasic current induced by 20 μM Quis was diminished, while maintained currents were not affected (Figure 8a(ii)). ACh-(100 μM) induced current was not affected by CNQX (20 μM; n = 8 and 100 μM; n = 3) or by kynurenic acid (1 mM; n = 4).

Effects of antagonists on nicotine-induced current

Mecamylamine, a ganglionic nicotinic antagonist, suppressed ACh-induced currents in a dose-dependent manner. The effect was reversible and did not show voltage-dependence (Figure 9). The same effect of mecamylamine (100 μM) was observed when tested against various ACh concentrations (200–500 μM, n = 11). Currents induced by 0.5 μM Quis were unaffected by mecamylamine (100 μM; n = 6).

Hexamethonium (up to 400 μM) had in most instances no effect on ACh-induced currents (50–500 μM, n = 12). Only in two cells, in which the ACh-induced current was of the slow-maintained type, was a voltage-dependent blockade by hexa-

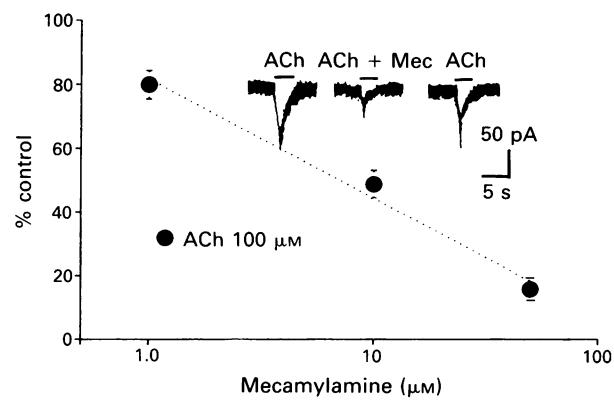

Figure 9 Current induced by acetylcholine (ACh) is reversibly and dose-dependently reduced by mecamylamine (inset for 100 μM ACh and 10 μM mecamylamine (Mec)). Each point is the mean from 4 cells; bars show s.e.mean.

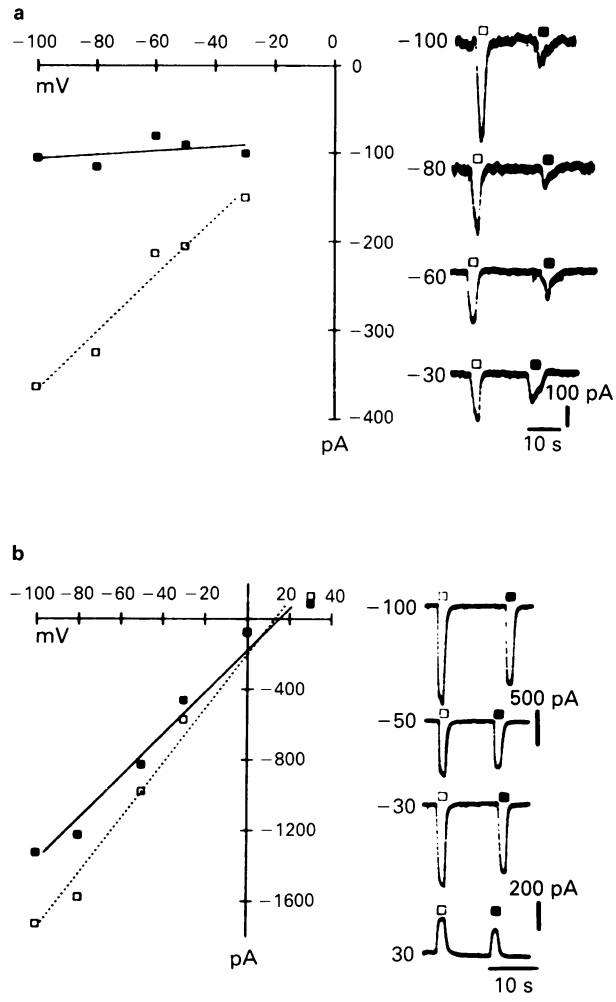

Figure 10 Voltage-dependent reduction by hexamethonium of acetylcholine (ACh)- and quisqualate Quis-induced currents. (a) I/V plot for currents induced by ACh (100 μM (□) and ACh (100 μM) simultaneously applied with hexamethonium (50 μM) (■). (b) I/V plot for Qui (0.5 μM)-induced current (□) and the current induced by simultaneous application of Quis (0.5 μM) and hexamethonium (400 μM) (■). In (a) and (b) actual recordings of the cells plotted are shown to the right. Membrane potential indicated at the beginning of each trace.

methonium (50 μM) observed (Figure 10a). Hexamethonium also attenuated maintained currents evoked by a low concentration of Quis (0.5 μM). This effect was concentration-dependent; 50 μM hexamethonium diminished the Quis-current by $23 \pm 5.6\%$ ($n = 4$) and 400 μM decreased the current by $65 \pm 10\%$ ($n = 4$) at a holding potential of -80 mV. Hexamethonium-induced blockade of the Quis current was voltage-dependent ($n = 3$, Figure 10b). Currents resulting from application of higher Quis concentrations, consisting of a transient and a maintained component, were not affected by hexamethonium ($n = 4$).

Discussion

The non-NMDA receptor antagonists CNQX and kynurenic acid, but not the NMDA antagonist APV and the nicotinic antagonists mecamylamine and hexamethonium, reduce network generated e.p.s.cs and miniature e.p.s.cs in the midbrain culture, suggesting that e.p.s.cs are mediated by an EAA. Despite the apparent ability of the antagonists to discriminate non-NMDA receptor-mediated e.p.s.cs from nicotinic receptor-mediated e.p.s.cs, their interaction with their respective agonist is rather complicated and unforseen.

Identification of receptors involved in the generation of e.p.s.ps in mammalian CNS is based primarily on their blockade by antagonists such as CNQX and kynurenic acid and the ineffectiveness of nicotinic receptor antagonists. This approach requires selectivity and specificity of the antagonists. Strictly speaking, suitability of an antagonist should be evaluated while testing its effect on the signals induced by various synaptically released transmitters. Since in midbrain culture there was no evidence of nicotinic receptor-mediated spontaneous e.p.s.cs, this approach was not available. Instead, we tested the effects of antagonists on responses produced by the exogenously applied agonists, Quis and ACh. By comparison with AMPA-induced currents, it was ensured that Quis effects under study were mediated by the so-called AMPA-receptor type.

Both e.p.s.cs and Quis and nicotinic receptor-induced currents reversed at the same membrane potential. Currents evoked by Quis and ACh contained a desensitizing component, which is considered characteristic of ACh and EAA receptors mediating synaptic responses (Katz & Thesleff, 1957; Trussel *et al.*, 1988; Mayer & Vyklicky, 1989; Tang *et al.*, 1989). However, Quis- and ACh-induced currents differed in that ACh induced much smaller currents than Quis. Not all cells responding to Quis and exhibiting e.p.s.cs responded to ACh.

The difficulty in comparing synaptically and exogenously induced currents becomes apparent when considering the transient and maintained components of the Quis-induced current. The transient component, induced by activation of a high-conductance channel-coupled receptor, is elicited by Quis concentrations above 1 μM (Tang *et al.*, 1989). However, CNQX and kynurenic acid reduced the transient current only at concentrations much higher than those required to reduce spontaneous e.p.s.cs. Since both CNQX and kynurenic acid are considered competitive antagonists (Blake *et al.*, 1988; 1989; Bertolino *et al.*, 1989) this difference may indicate a lower concentration, a lower affinity or a faster removal of the synaptically released ligand compared with exogenous ligands. The comparison of antagonists' actions on synaptic and evoked responses is further complicated by their differential effects on transient and maintained components of the evoked current. CNQX eventually enhanced the maintained current without affecting the transient current, while kynurenic acid reduced the transient but not the maintained component. It has been suggested that the ratio between transient and maintained components of Quis-current is governed by desensitization, which increases with an increase in Quis or AMPA concentration (Mayer & Vyklicky, 1989). Because desensitization of glutamate receptors may be fast (Franke *et al.*, 1987) and receptors for glutamate are primarily located on dendrites (Swandulla & Misgeld, 1990), in whole cell recording some of the current may be entirely lost by desensitization. Therefore, effects of CNQX may reflect in part removal of desensitization. Such an action may be in line with a competitive antagonism. By the same token, kynurenic acid, which did not increase the maintained current, may not be a purely competitive antagonist.

Since nicotinic receptor-mediated responses did not have their synaptic counterpart in our experimental model, characterization of the nicotinic antagonists could not be complete, and the possibility of the blockade of nicotinic e.p.s.ps by CNQX and kynurenic acid cannot be excluded. However, our study showed that high concentrations of non-NMDA antagonists did not affect the small current induced by low concentrations of ACh. Likewise, high concentrations of the nicotinic antagonist mecamylamine did not affect the response to low concentrations of Quis and had no effect on e.p.s.cs.

The effect of hexamethonium raises another issue. Hexamethonium did not reduce transient nicotinic and Quis currents, but reduced in a voltage-dependent manner maintained responses to both agonists. Hexamethonium is known as a partial channel blocking agent (Ascher *et al.*, 1979). Therefore, its ability to reduce Quis- and nicotine-induced currents may

be due to a similarity of Quis- and nicotinic-ionic channels. Despite this apparent lack of selectivity, hexamethonium is a ganglionic blocking agent which does not seem to reduce EAA-mediated e.p.s.ps in central neurones.

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References

ASCHER, P., LARGE, W.A. & RANG, H.P. (1979). Studies on the mechanism of action of acetylcholine antagonists on rat parasympathetic ganglion cells. *J. Phsyiol.*, **295**, 139–170.

BERTOLINO, M., VICINI, S. & COSTA, E. (1989). Kynurenic acid inhibits the activation of kainic and N-methyl-D-aspartic acid-sensitive ionotropic receptors by a different mechanism. *Neuropharmacol.*, **28**, 453–457.

BLAKE, J.F., BROWN, M.W. & COLLINGRIDGE, G.L. (1988). CNQX blocks acidic amino acid induced depolarizations and synaptic components mediated by non-NMDA receptors in rat hippocampal slices. *Neurosci. Lett.*, **89**, 182–186.

BLAKE, J.F., YATES, R.G., BROWN, M.W. & COLLINGRIDGE, G.L. (1989). 6-Cyano-7-nitroquinoxaline-2,3-dione as an excitatory amino acid antagonist in area CA1 of rat hippocampus. *Br. J. Pharmacol.*, **97**, 71–76.

CALABRESI, P., LACEY, M.G. & NORTH, R.A. (1989). Nicotinic excitation of rat ventral tegmental neurones *in vitro* studied by intracellular recording. *Br. J. Pharmacol.*, **98**, 135–140.

CARBONE, E. & LUX, H.D. (1987). Kinetics and selectivity of a low-voltage-activated calcium current in chick and rat sensory neurones. *J. Physiol.*, **386**, 547–570.

CLARKE, P.B.S., HOMMER, D.W., PERT, A. & SKIRBOLL, L.R. (1985). Electrophysiological actions of nicotine on substantia nigra single units. *Br. J. Pharmacol.*, **85**, 827–835.

EGAN, T.M. & NORTH, R.A. (1986). Actions of acetylcholine and nicotine on rat locus coeruleus neurons *in vitro*. *Neuroscience*, **19**, 565–571.

FRANKE, C., HATT, H. & DUDEL, J. (1987). Liquid filament switch for ultra-fast exchanges of solutions at excised patches of synaptic membrane of crayfish muscle. *Neurosci. Lett.*, **77**, 199–204.

GANONG, A.H., LANTHORN, T.H. & COTMAN, C.W. (1983). Kynurenic acid inhibits synaptic and acidic amino acid-induced responses in the rat hippocampus and spinal cord. *Brain Res.*, **273**, 170–174.

GOLDMAN, D., SIMMONS, D., SWANSON, L.W., PATRICK, J. & HEINEMANN, S. (1986). Mapping of brain areas expressing RNA homologous to two different acetylcholine receptor α-subunit cDNAs. *Proc. Natl. Acad. Sci., U.S.A.*, **83**, 4076–4080.

GOLDMAN, D., DENERIS, E., LUYTEN, W., KOCHHAR, A., PATRICK, J. & HEINEMANN, S. (1987). Members of nicotinic acetylcholine receptor gene family are expressed in different regions of the mammalian central nervous system. *Cell*, **48**, 965–973.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HONORÉ, T., DAVIES, S.N., DREJER, J., FLETCHER, E.J., JACOBSEN, P., LODGE, D. & NIELSEN, F.E. (1988). Quinoxalinediones: Potent competitive non-NMDA glutamate receptor antagonists. *Science*, **241**, 701–703.

JAROLIMEK, W. & MISGELD, U. (1991). Reduction of $GABA_A$ receptor-mediated inhibition by the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline- 2,3-dione in cultured neurons of rat brain. *Neurosci. Lett.*, (in press).

KATZ, B. & THESLEFF, S. (1957). A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J. Physiol.*, **138**, 63–80.

LIPTON, S.A., AIZENMAN, E. & LORING, R.H. (1987). Neural nicotinic acetylcholine responses in solitary mammalian retinal ganglion cells. *Pflügers Arch.*, **410**, 37–43.

MAYER, M.L. & VYKLICKY, L. JR. (1989). Concanavalin A selectively reduces desensitization of mammalian neuronal quisqualate receptors. *Proc. Natl. Acad. Sci., U.S.A.*, **86**, 1411–1415.

McCORMICK, D.A. & PRINCE, D.A. (1987). Actions of acetylcholine in the guinea-pig and cat medial and lateral geniculate nuclei, *in vitro*. *J. Physiol.*, **392**, 147–165.

MISGELD, U., MÜLLER, W. & POLDER, H.R. (1989). Potentiation and suppression by eserine of muscarinic synaptic transmission in the guinea-pig hippocampal slice. *J. Physiol.*, **409**, 191–206.

MISGELD, U. & SWANDULLA, D. (1989). Quisqualate receptor-mediated rhythmic bursting of rat hypothalamic neurons in dissociated cell culture. *Neurosci. Lett.*, **98**, 291–296.

NICOLL, R.A., MALENKA, R.C. & KAUER, J.A. (1990). Functional comparison of neurotransmitter receptor subtypes in mammalian central nervous system. *Physiol. Rev.*, **70**, 513–565.

PERKINS, M.N. & STONE, T.W. (1982). An iontophoretic investigation of the actions of convulsant kynurenines and their interaction with the endogenous excitant quinolinic acid. *Brain Res.*, **247**, 184–187.

SWANDULLA, D. & MISGELD, U. (1990). Development and properties of synaptic mechanisms in a network of rat hypothalamic neurons grown in culture. *J. Neurophysiol.*, **64**, 215–226.

TANG, C.-M., DICHTER, M. & MORAD, M. (1989). Quisqualate activates a rapidly inactivating high conductance ionic channel in hippocampal neurons. *Science*, **243**, 1474–1477.

TRUSSELL, L.O., THIO, L.L., ZORUMSKI, C.F. & FISCHBACH, G.D. (1988). Rapid desensitization of glutamate receptors in vertebrate central neurons. *Proc. Natl. Acad. Sci., U.S.A.*, **85**, 4562–4566.

VIDAL, C. & CHANGEUX, J.-P. (1989). Pharmacological profile of nicotinic acetylcholine receptors in the rat prefrontal cortex: An electrophysiological study in a slice preparation. *Neuroscience*, **29**, 261–270.

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Pharmacological evidence for the possible involvement of repetitive action potentials in facilitation by GABA of catecholamine secretion in bovine adrenal chromaffin cells

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1 γ-Aminobutyric acid (GABA) evokes catecholamine (CA) secretion and enhances the stimulation-evoked CA secretion via facilitation of Ca^{2+} entry in a Cl^--dependent manner. The present study was designed to investigate further the ionic mechanism of modulation by GABA of CA secretion from adrenal medulla, using a primary culture of bovine chromaffin cells.

2 Tetrodotoxin (TTX), a voltage-sensitive Na^+ channel blocker, reduced GABA-evoked CA secretion.

3 Inhibition of the sodium pump by ouabain or removal of extracellular K^+ enhanced GABA-evoked CA secretion in a TTX-sensitive manner.

4 Tetraethylammonium (TEA) and cesium, which are known to block some types of K^+ channels, markedly enhanced GABA-evoked CA secretion in a concentration-related fashion. TEA-induced enhancement of the GABA-evoked CA secretion was attenuated by TTX or replacement of extracellular Na^+ by choline. On the other hand, ouabain accelerated the effect of TEA.

5 TEA and ouabain also enhanced GABA-induced Ca^{2+} influx and accumulation of cytosolic Ca^{2+}, assessed with $^{45}Ca^{2+}$ uptake and quin2 fluorescence.

6 Veratridine increased accumulation of cytosolic Ca^{2+} in a TTX-sensitive manner. GABA facilitated the veratridine-induced elevation of cytosolic Ca^{2+} even when the GABA-induced rise of cytosolic Ca^{2+} levelled off.

7 These results suggest the involvement of repetitive action potentials in modulation of GABA by Ca^{2+} mobilization and, as a consequence, of the CA secretion in chromaffin cells.

Introduction

Adrenal medulla chromaffin cells possess γ-aminobutyric acid (GABA)ergic system: GABA is synthesized and stored, released in response to depolarizing stimulation and taken up again (see Kitayama & Tsujimoto, 1986; Alho *et al.*, 1986 for review). Numerous attempts have been made to investigate the functional role of GABA in the adrenal medulla, and its possible involvement in the regulation of the catecholamine (CA) secretion. GABA itself evokes CA secretion in isolated perfused adrenal glands of the cow (Sangiah *et al.*, 1974; Kitayama & Tsujimoto, 1986) and dog (Kitayama *et al.*, 1984; Kataoka *et al.*, 1986), bovine isolated adrenal chromaffin cells (Castro *et al.*, 1989) and cultured bovine adrenal chromaffin cells (Kitayama *et al.*, 1986; Kataoka *et al.*, 1988). However, the results demonstrated in the literature are contradictory with regard to the effect of GABA on CA secretion evoked by nicotinic acetylcholine (ACh) receptor stimulation. Furthermore, the mechanism by which GABA modulates CA secretion is not fully understood.

There is much evidence from electrophysiological studies to show that GABA depolarizes the cell body of sympathetic ganglion via changes in Cl^- permeability (DeGroat, 1970; Bowery & Brown, 1974; Adams & Brown, 1975) and that chromaffin cells possess GABA-induced Cl^- currents (Bormann & Clapham, 1985; Cottrell *et al.*, 1985). Therefore, it may be assumed that GABA depolarizes chromaffin cells, resulting in an activation of voltage-sensitive ion channels and, as a consequence, a modulation of Ca^{2+} homeostasis influencing CA secretion. We have recently demonstrated that $GABA_A$ receptor stimulation causes Ca^{2+} influx via a Cl^--dependent mechanism, resulting in the facilitatory modulation of basal and stimulation (e.g. ACh, excess K^+ or veratridine)-evoked CA secretion in cultured bovine adrenal chromaffin cells (Kitayama *et al.*, 1990a,b). In addition, GABA caused a depolarization measured by the bis-oxonol fluorescence method and an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) via $GABA_A$ receptors in a cell population maintained in suspension culture (Kitayama *et al.*, 1990c,d). Based on these results, it is concluded that GABA causes a rapid elevation of $[Ca^{2+}]_i$ preceding CA secretion, one source of which may be the Ca^{2+} influx through voltage-sensitive Ca^{2+} channels.

On the other hand, the presence of voltage-sensitive Na^+ channels and their importance in CA secretion in adrenal chromaffin cells has been well documented (for review see Unger & Phillips, 1983; Kidokoro, 1985). In the first report of our series of investigations concerning the role of GABA in adrenal CA secretion, we showed that GABA-evoked secretion was not affected by tetrodotoxin (TTX) in isolated perfused adrenal glands of the dog (Kitayama *et al.*, 1984). We therefore concluded that there was no involvement of voltage-sensitive Na^+ channels in the action of GABA. However, we used only a high concentration of GABA which causes an almost maximal response. Under these conditions, the activity of voltage-sensitive Na^+ channels might have only a minor influence on the action of GABA, as has been suggested for ACh (Unger & Phillips, 1983; Kidokoro, 1985).

Recently, Peters *et al.* (1989) found that ionophoretic application of GABA markedly increased the rate of spontaneous occurrence of action potentials in cell-attached patches of bovine chromaffin cells. Consequently, we have investigated further the ionic mechanism of the facilitatory modulation by GABA of CA secretion, in order to answer the question whether or not discharge of action potentials evoked by GABA participates in the CA secretion.

Methods

Cell culture

Fresh bovine adrenal glands were obtained at a local slaughterhouse. Chromaffin cells were isolated enzymatically according to the procedure described by Fenwick *et al.* (1978) with

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some modifications (Morita *et al.*, 1987). For monolayer culture, cells were plated at densities of 10^6 cells/dish on 35 mm diameter tissue culture Petri dishes in 2 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, penicillin 100 units ml^{-1}, streptomycin 100 $\mu g\,ml^{-1}$, ascorbate 0.1 mM and HEPES 5 mM, and cultured for 5–7 days at 37°C in 5% CO_2:95% air. For suspension culture, cells suspended in DMEM were placed in Erlenmeyer plastic flasks (Corning) at densities of 5×10^5 cells ml^{-1}, and cultured for 24–72 h.

Catecholamine secretion and $^{45}Ca^{2+}$ uptake

To minimize basal secretion of CA, plated cells were kept for 2 h at room temperature after washing 3 times with phosphate-buffered Krebs Ringer solution composed of (mM): NaCl 154, KCl 5.6, $MgSO_4$ 1.1, $CaCl_2$ 1.3, Na_2HPO_4 2.15, NaH_2PO_4 0.85 and glucose 10 (pH 7.4 ± 0.1). Cells were then preincubated at 37°C for 6 min in 1 ml of fresh medium, the medium was aspirated, and the cells were further incubated at 37°C for 6 min in 1 ml of fresh medium with or without stimulant. At the end of incubation, the medium was immediately separated from the cells, an aliquot (0.5 ml) was placed in a test tube containing 50 μl of 1 N HCl for subsequent CA assay. When Na^+ was removed from the incubation medium, NaCl was substituted by the osmotically equivalent amount of choline Cl and the medium was buffered by HEPES (5 mM) instead of phosphate. CA was measured fluorometrically by the method of von Euler & Lishajko (1961) with (−)-adrenaline as the standard.

Measurement of $^{45}Ca^{2+}$ uptake was performed as described previously (Kitayama *et al.*, 1986). Briefly, cells were incubated at 37°C for 6 min with $^{45}CaCl_2$ (74 kBq ml^{-1}) in the presence or absence of compounds under investigation. At the end of incubation, cells were immediately separated from the incubation medium and washed four times on ice with ice-cold fresh medium within 30 s. The radioactivity remaining in the cells was extracted with 1 N NaOH and measured by liquid scintillation counting.

$[Ca^{2+}]_i$ measurement

Cells maintained in suspension culture for 24–72 h were used for assay. The washing medium and following suspension medium contained (in mM): NaCl 150, KCl 5, $MgSO_4$ 1, $CaCl_2$ 2.2, glucose 5, HEPES-Tris buffer 10 and bovine serum albumin 0.5% (pH 7.4 ± 0.1). $[Ca^+]_i$ was measured in the suspension of cells (2×10^6 ml^{-1}) loaded with fluorescence indicator quin2 according to the procedure of Tsien *et al.* (1982) and Kao & Schneider (1986), as described previously (Kitayama *et al.*, 1990c). Cell suspensions were incubated at 37°C for 30 min with 20 μM quin2/AM, followed by addition of 3 volumes of prewarmed medium and further incubated for 30 min, which gave a loading of 1.85 ± 0.20 nmol of quin2/10^6 cells (mean ± s.e.mean, $n = 3$).

Analysis of data

Values are expressed as mean ± s.e.mean. When the difference between two groups was determined, the s.e.mean was calculated as $(s.e._1^2 + s.e._2^2)^{1/2}$ where $s.e._1$ and $s.e._2$ are the standard errors of the mean for each group. Differences between means of groups were treated for significance with Student's t test. Data from quin2 experiments are representative examples of experiments performed with very similar results in at least three different batches of cells.

Materials

Drugs used were: bovine calf serum, DNaseI (Boehringer Manheim GmbH, FRG), collagenase type I, soybean trypsin inhibitor (Cooper Biomedical, Malvern, PA, U.S.A.), DMEM (Nissui Pharmaceutical, Tokyo, Japan), penicillin G, streptomycin (Meiji Seika, Tokyo, Japan), adrenaline, ouabain (Merck, Darmstadt, FRG), quin2/AM (Dojindo Laboratories, Kumamoto, Japan), tetrodotoxin (Sankyo, Tokyo, Japan), GABA (Katayama Chemical, Osaka, Japan), tetraethylammonium chloride, digitonin (Nakarai Tesque, Kyoto, Japan), veratridine, quin2 (Sigma, St. Louis, MO, U.S.A.). $^{45}CaCl_2$ (910.2 MBq mg^{-1}) was purchased from NEM, Boston, MA., U.S.A. Stock solutions of drugs were prepared in water, ethanol or dimethyl sulphoxide and diluted into assay medium just before use. Controls received the solvents only (maximum concentration, 1%).

Results

Catecholamine secretion

To investigate the possible involvement of repetitive action potentials in the stimulatory action of GABA on CA secretion as mentioned in the Introduction, we initially investigated the effect of tetrodotoxin (TTX), an antagonist of voltage-sensitive Na^+ channel, on GABA-evoked CA secretion. TTX markedly reduced the GABA-evoked CA secretion (Figure 1).

Table 1 shows the effect of Na^+ pump inhibition by ouabain or removal of extracellular K^+ on GABA-evoked CA secretion. Both treatments markedly enhanced the GABA-evoked CA secretion. TTX attenuated the effect of ouabain or K^+ removal on the action of GABA.

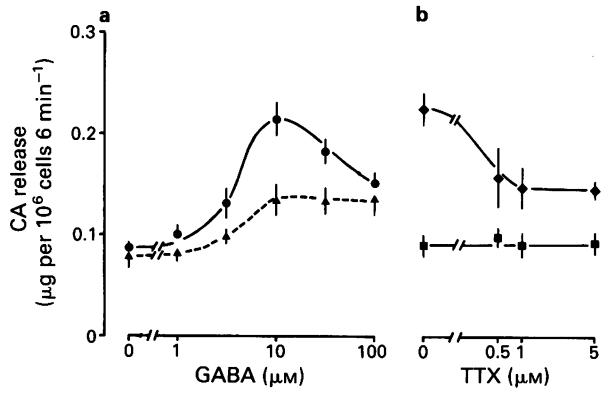

Figure 1 Effects of tetrodotoxin (TTX) on catecholamine (CA) secretion evoked by γ-aminobutyric acid (GABA) in cultured bovine adrenal chromaffin cells. (a) Cells were incubated with various concentrations of GABA in the absence (●) or presence (▲) of 1 μM TTX. (b) Effects of various concentrations of TTX on basal (■) and GABA (10 μM, ◆)-evoked CA secretion. Treatment with TTX was started from preincubation period. See Methods for details. Values represent mean (n = 3–4) with s.e.mean shown by vertical lines.

Table 1 Effects of tetrodotoxin (TTX) on the enhancement of γ-aminobutyric acid (GABA)-evoked catecholamine (CA) secretion by Na^+ pump inhibition in cultured bovine adrenal chromaffin cells

		CA release (μg per 10^6 cells 6 min^{-1})	
Treatment	*GABA-stimulation*	*In the absence of TTX*	*In the presence of TTX*
—	−	0.080 ± 0.015	0.079 ± 0.012
	+	0.218 ± 0.009	0.142 ± 0.008
Ouabain	−	0.117 ± 0.011	0.087 ± 0.007
	+	0.372 ± 0.013	0.195 ± 0.014
K^+-free	−	0.201 ± 0.055	0.157 ± 0.048
	+	0.614 ± 0.101	0.312 ± 0.059

Treatment with ouabain or K^+-free medium was started from preincubation period. See methods for details. Values represent mean ± s.e.mean, $n = 3$.

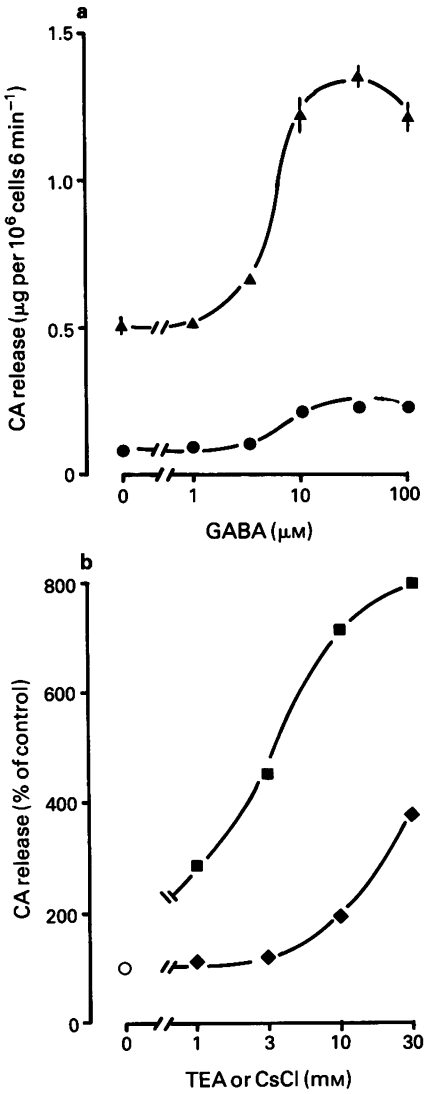

Figure 2 Effects of tetraethylammonium (TEA) and CsCl on γ-aminobutyric acid (GABA)-evoked catecholamine (CA) secretion in cultured bovine adrenal chromaffin cells. (a) Effects of TEA on concentration-response curve for GABA. Cells were incubated with various concentrations of GABA in the presence (▲) or absence (●) of TEA (10 mM). (b) Concentration-response curves for the effects of TEA (■) and CsCl (◆) on GABA-evoked CA secretion. TEA or CsCl was added at the same time as GABA (10 μM). In (b), basal and TEA- or CsCl-induced CA release has been subtracted from each response to the combination with GABA, thus reflecting control and altered GABA-evoked CA secretion. Values represent mean (n = 3–5) with s.e.mean shown by vertical bars.

Figure 2 shows the effects of the K^+ channel blockers, tetraethylammonium (TEA) and CsCl on GABA-evoked CA secretion. Both TEA and CsCl markedly enhanced the action of GABA in a concentration-dependent manner. Enhancement by TEA of the action of GABA was also sensitive to TTX (Table 2). A similar effect was observed when extracellular Na^+ was removed. Removal of extracellular Na^+ by itself did not affect GABA-evoked CA secretion. Table 2 also shows that ouabain accelerated the effect of TEA on GABA-evoked CA secretion.

$^{45}Ca^{2+}$ uptake

Figure 3 shows the effects of TTX and TEA on GABA-evoked $^{45}Ca^{2+}$ uptake into cultured bovine chromaffin cells. GABA and TEA caused a significant increase in $^{45}Ca^{2+}$ uptake. Simultaneous application of GABA and TEA induced a more than additive increase in $^{45}Ca^{2+}$ uptake. Although TTX failed

Table 2 Facilitation or attenuation of the tetraethylammonium (TEA)-induced enhancement of γ-aminobutyric acid (GABA)-evoked catecholamine (CA) secretion from cultured bovine adrenal chromaffin cells by tetrodotoxin (TTX), Na^+-free medium or ouabain

		CA release (μg per 10^6 cells 6 min^{-1})	
Treatment	*GABA-stimulation*	*In the absence of TEA*	*In the presence of TEA*
—	−	0.091 ± 0.013	0.238 ± 0.025
	+	0.194 ± 0.019	0.874 ± 0.056
TTX	−	0.083 ± 0.017	0.131 ± 0.010
	+	0.109 ± 0.008	0.439 ± 0.046
Na^+-free	−	0.142 ± 0.017	0.293 ± 0.032
	+	0.284 ± 0.035	0.664 ± 0.072
Ouabain	−	0.133 ± 0.012	0.418 ± 0.054
	+	0.337 ± 0.048	1.424 ± 0.029

Treatment with TTX, ouabain or Na^+-free medium was started from preincubation period. See methods for details. Values are means ± s.e.mean, n = 3.

to reduce the GABA- or TEA-induced increase in $^{45}Ca^{2+}$, it significantly reduced $^{45}Ca^{2+}$ uptake evoked by the combination of GABA and TEA.

$[Ca^{2+}]_i$

We further analysed the effects of TEA and ouabain on the GABA-induced change in $[Ca^{2+}]_i$ measured by quin2 fluorescence. GABA caused a rapid increase of $[Ca^{2+}]_i$, followed by a gradual decrease to basal level. The mechanism of the latter process is unclear at present; however, our previous observation suggested that it may be due to a desensitization of voltage-sensitive Ca^{2+} channels and/or internal buffering of $[Ca^{2+}]_i$ rather than a desensitization of $GABA_A$ receptor (Kitayama *et al.*, 1990c). TTX slightly reduced the GABA-induced rise of $[Ca^{2+}]_i$ (Figure 4). TEA markedly potentiated GABA-evoked rise of $[Ca^{2+}]_i$, although TEA by itself did not affect quin2 fluorescence in contrast to the $^{45}Ca^{2+}$ experiment. On the other hand, ouabain caused an increase of $[Ca^{2+}]_i$ and subsequent application of GABA induced an

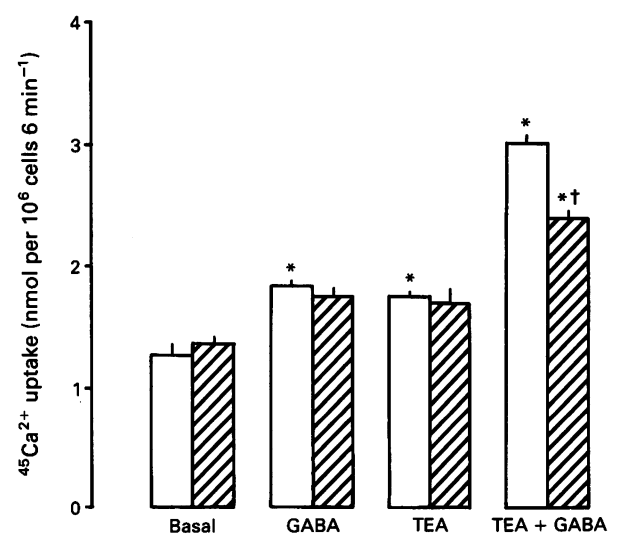

Figure 3 Effects of tetraethylammonium (TEA) and tetrodotoxin (TTX) on γ-aminobutyric acid (GABA)-induced $^{45}Ca^{2+}$ uptake in cultured bovine adrenal chromaffin cells. Cells were incubated in the medium containing 74 kBq ml^{-1} of $^{45}CaCl_2$ with or without compounds under investigation; GABA (10 μM), TEA (10 mM) or GABA (10 μM) plus TEA (10 mM) in the presence (hatched column) or absence (open column) of TTX (1 μM). Columns represent mean (n = 3–6) with s.e.mean shown by vertical lines. * $P < 0.05$ vs basal uptake in the presence or absence of TTX, respectively. † $P < 0.01$ vs corresponding control (absence of TTX).

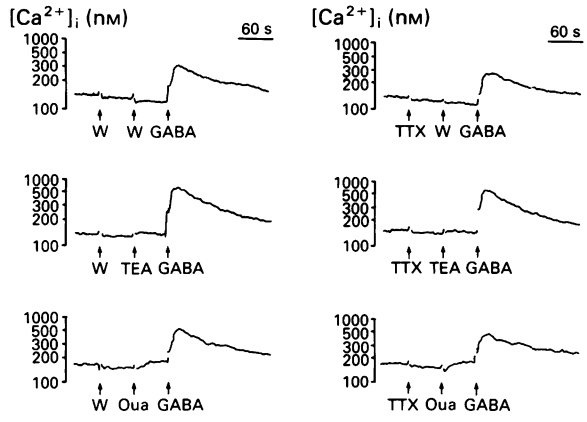

Figure 4 Effects of tetraethylammonium (TEA), ouabain and tetrodotoxin (TTX) on γ-aminobutyric acid (GABA)-induced rise of cytosolic Ca^{2+} in cultured bovine adrenal chromaffin cells. Cells received test compounds or solvent (distilled water, W) at a time point indicated. GABA, 100 μM; TTX, 1 μM; TEA, 10 mM; ouabain (Oua), 50 μM in final concentration. These and the following experiments are representative examples of experiments performed with very similar results in at least three different batches of cells.

additive increase of $[Ca^{2+}]_i$. TTX had little effect on the change of $[Ca^{2+}]_i$ caused by both drugs.

Figure 5 shows the interaction of GABA and veratridine in changes of $[Ca^{2+}]_i$. Veratridine caused a gradual rise of $[Ca^{2+}]_i$, which could be reversed by TTX (Figure 5a). GABA enhanced the veratridine-evoked rise of $[Ca^{2+}]_i$, even after the GABA-evoked rise of $[Ca^{2+}]_i$ levelled off (Figure 5b). This result also suggests that the desensitization mentioned above occurred down-stream of GABA-induced Cl^- currents, leading to depolarization. Simultaneous application of GABA

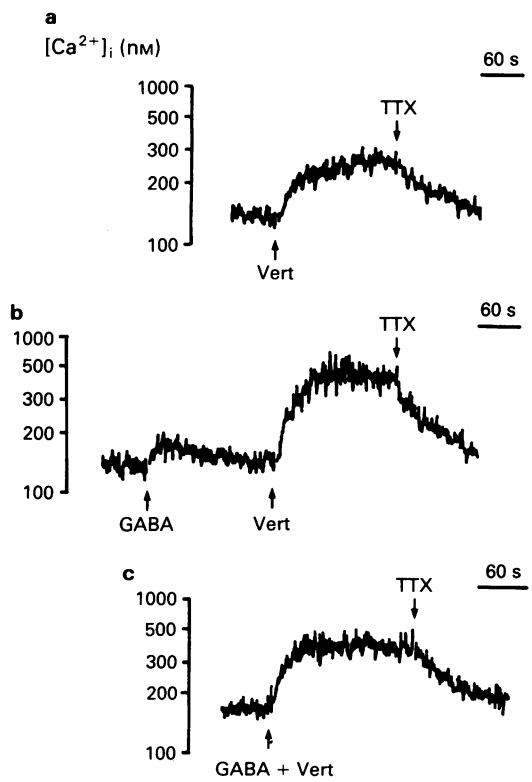

Figure 5 Interaction of γ-aminobutyric acid (GABA) and veratridine on $[Ca^{2+}]_i$ in cultured bovine adrenal chromaffin cells. GABA, veratridine (Vert) or tetrodotoxin (TTX) was added at the time point indicated to give a final concentration of 100 μM, 50 μM or 1 μM, respectively.

and veratridine caused a greater increase of $[Ca^{2+}]_i$ (Figure 5c).

Discussion

In the light of the concept of stimulus secretion coupling in the adrenal medulla, it is considered that stimulation of the nicotinic ACh receptor initiates depolarization by Na^+ entry through the receptor channels, followed by activation of voltage-sensitive Na^+ and Ca^{2+} channels, consequently resulting in Ca^{2+} entry which is the initial step in CA secretion (see Kidokoro, 1985, for review). Depolarization could be brought about by another process during stimulation with GABA: when $GABA_A$ receptor-linked Cl^- channels are activated, Cl^- leaves the cell, producing an inward current (Bormann & Clapham, 1985; Cottrell *et al.*, 1985). Therefore, we assumed that GABA-induced depolarization through changes in Cl^- permeability modifies the activities of voltage-sensitive Na^+ channels, as well as voltage-sensitive Ca^{2+} channels which it has been suggested may participate in the GABA-induced facilitation of CA secretion (Kitayama *et al.*, 1990a–c). Based on this hypothesis, the present study was designed to elucidate biochemically a possible interaction of GABA receptor ion channels and voltage-sensitive Na^+ channels in CA secretion.

Results of the present study show: (a) TTX reduced GABA-evoked CA secretion; (b) K^+ channel blockade by TEA or CsCl, Na^+ pump inhibition by ouabain or removal of extracellular K^+ faciliated GABA-evoked CA secretion associated with an increase in Ca^{2+} influx and $[Ca^{2+}]_i$ rise; (c) enhancement by TEA of the action of GABA was inhibited by TTX or removal of extracellular Na^+, and accelerated by ouabain; (d) synergy of the effects of GABA and veratridine on $[Ca^{2+}]_i$. These results suggest the involvement of Na^+ influx in GABA-evoked CA secretion, which may be through voltage-sensitive Na^+ channels.

An alternative explanation of the inhibitory or facilitatory actions of various agents used here on GABA-evoked CA secretion is as follows. The inward movement of Ca^{2+} across the plasma membrane required for CA secretion is initiated by the depolarization caused by Cl^- efflux through $GABA_A$ receptor-operated Cl^- channels. This model does not implicate external Na^+. In support of such a view, removal of extracellular Na^+ failed to affect the GABA-evoked CA secretion (Table 2). In normal medium, however, TTX did reduce the GABA-evoked CA secretion. Further, GABA markedly potentiated the veratridine-evoked $^{45}Ca^{2+}$ uptake (Kitayama *et al.*, 1990b), $[Ca^{2+}]_i$ rise (the present results) and CA secretion (Kitayama *et al.*, 1989a,b; 1990a). Thus we assume that GABA-induced depolarization also permits the involvement of voltage-sensitive Na^+ channels, resulting in the increase in Ca^{2+} entry. Findings from the electrophysiological study by Peters *et al.* (1989) indicating an excitatory action of GABA on the discharge of action potentials are in good agreement with this interpretation.

Experiments with TEA and ouabain provide additional support for this hypothesis. Both Na^+ and Ca^{2+} influx are opposed by the outward movement of K^+. An electrophysiological study demonstrated the presence of several types of K^+ channels in chromaffin cells and its inhibition by TEA (Marty & Neher, 1985). Further, Kidokoro & Ritchie (1980) explained the stimulatory action of TEA on KCl-induced CA secretion with regard to the action potential, and suggested that TEA is only effective on the portion of CA secretion which is mediated by spike activity. The present study demonstrated that the enhancement by TEA of GABA-evoked CA secretion was inhibited by TTX and removal of extracellular Na^+. Further, ouabain potentiated GABA-evoked CA secretion and accelerated the effect of TEA on the action of GABA. It has been reported that ouabain enhanced the carbachol- or veratridine-induced cellular accumulation of $^{22}Na^+$ which was associated with the increase of CA secretion (Wada *et al.*,

1985; 1986). Thus, the potentiating effect of TEA and ouabain on GABA-evoked CA release may be explained by assuming that stimulation of chromaffin cell with GABA accompanies the changes in Na^+ fluxes.

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References

ADAMS, P.R. & BROWN, D.A. (1975). Actions of γ-aminobutyric acid on sympathetic ganglion cell. *J. Physiol.*, **250**, 85–120.

ALHO, H., FUJIMOTO, M., GUIDOTTI, A., HANBAUER, I., KATAOKA, Y. & COSTA, E. (1986). γ-Aminobutyric acid (GABA) in the adrenal medulla: location, pharmacology, and function. In *Neurohistochemistry – Modern Methods and Applications*. ed. Panula, P., Paivarinta, H. & Soinila, S. pp. 453–464. New York: Alan R. Liss Inc.

BORMANN, J. & CLAPHAM, D.E. (1985). γ-Aminobutyric acid receptor channels in adrenal chromaffin cells: A patch-clamp study. *Proc. Natl. Acad. Sci., U.S.A.*, **82**, 2168–2172.

BOWERY, N.G. & BROWN, D.A. (1974). Depolarizing actions of γ-aminobutyric acid and related compounds on rat superior cervical ganglia *in vitro*. *Br. J. Pharmacol.*, **50**, 205–218.

CASTRO, E., OSET-GASQUE, M.J. & GONZÁLEZ, M.P. (1989). $GABA_A$ and $GABA_B$ receptors are functionally active in the regulation of catecholamine secretion by bovine chromaffin cells. *J. Neurosci. Res.*, **23**, 290–296.

COTTRELL, G.A., LAMBERT, J.J. & PETERS, J.A. (1985). Chloride currents activated by GABA in cultured bovine chromaffin cells. *J. Physiol.*, **365**, 90P.

DEGROAT, W.C. (1970). The actions of γ-aminobutyric acid and related amino acids on mammalian autonomic ganglia. *J. Pharmacol. Exp. Ther.*, **172**, 384–396.

FENWICK, E.M., FAJDIGA, P.B., HOWE, N.B.S. & LIVETT, B.G. (1978). Functional and morphological characterization of isolated bovine adrenal medullary cells. *J. Cell. Biol.*, **76**, 12–30.

KAO, L.-S. & SCHNEIDER, A.S. (1986). Calcium mobilization and catecholamine secretion in adrenal chromaffin cells – a quin2 fluorescence study. *J. Biol. Chem.*, **261**, 4881–4888.

KATAOKA, Y., FUJIMOTO, M., ALHO, A., GUIDOTTI, A., GEFFARD, M., KELLY, G.D. & HANBAUER, I. (1986). Intrinsic gamma aminobutyric acid receptor modulate the release of catecholamine from canine adrenal gland *in situ*. *J. Pharmacol. Exp. Ther.*, **239**, 584–590.

KATAOKA, Y., OHARA-IMAIZUMI, M., UEKI, S. & KUMAKURA, K. (1988). Stimulatory action of γ-aminobutyric acid on catecholamine secretion from bovine adrenal chromaffin cells measured by a real-time monitoring system. *J. Neurochem.*, **50**, 1765–1768.

KIDOKORO, Y. (1985). Electrophysiology of adrenal chromaffin cells. In *The Electrophysiology of the Secretory Cell*. ed. Poisner, A.M. & Trifaró, J. pp. 195–218. Amsterdam: Elsevier.

KIDOKORO, Y. & RITCHIE, A.K. (1980). Chromaffin cell action potential and their possible role in adrenaline secretion from rat adrenal medulla. *J. Physiol.*, **307**, 199–216.

KITAYAMA, S., KŌYAMA, Y., MORITA, K., DOHI, T. & TSUJIMOTO, A. (1986). Increase in catecholamine release and $^{45}Ca^{2+}$ uptake induced by GABA in cultured bovine adrenal chromaffin cells. *Eur. J. Pharmacol.*, **131**, 145–147.

KITAYAMA, S., MORITA, K., DOHI, T. & TSUJIMOTO, A. (1984). The nature of the stimulatory action of γ-aminobutyric acid in the isolated perfused dog adrenals. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **326**, 106–110.

KITAYAMA, S., MORITA, K., DOHI, T. & TSUJIMOTO, A. (1990a). Enhancement by GABA of the stimulation-evoked catecholamine release from cultured bovine adrenal chromaffin cells. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, 414–418.

KITAYAMA, S., MORITA, K., DOHI, T. & TSUJIMOTO, A. (1990b). GABAergic modulation of catecholamine release from cultured bovine adrenal chromaffin cells. Evidence for the involvement of Cl^--dependent Ca^{2+} entry. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, 419–424.

KITAYAMA, S., MORITA, K., DOHI, T. & TSUJIMOTO, A. (1990c). $GABA_A$ receptor-mediated increase of cytosolic Ca^{2+} in isolated bovine adrenal chromaffin cells. *Biochem. Biophys. Acta*, **1053**, 189–194.

KITAYAMA, S., MORITA, K., DOHI, T. & TSUJIMOTO, A. (1989a). Benzodiazepines facilitate the stimulatory action of γ-aminobutyric acid (GABA) on basal and veratridine-evoked catecholamine release from cultured bovine adrenal chromaffin cells. *Arch. Int. Pharmacodyn. Ther.*, **300**, 254–264.

KITAYAMA, S., OHTSUKI, H., MORITA, K., DOHI, T. & TSUJIMOTO, A. (1989b). GABAergic modulation of plasma membrane potential, cytosolic Ca^{2+} and catecholamine secretion in bovine adrenal chromaffin cells. *7th International Conference on Cyclic Nucleotide, Calcium and Protein Phosphorylation*, Kobe, Japan, Oct 8–13, Abst. no. 164.

KITAYAMA, S., OHTSUKI, H., MORITA, K., DOHI, T. & TSUJIMOTO, A. (1990d). Bis-oxonol experiment on plasma membrane potentials of bovine adrenal chromaffin cells: depolarizing stimuli and their possible interaction. *Neurosci. Lett.*, **116**, 275–279.

KITAYAMA, S. & TSUJIMOTO, A. (1986). Involvement of GABAergic mechanisms in the catecholamine secretion from adrenal medulla. In *GABAergic Mechanisms in the Mammalian Periphery*. ed. Erdö, S.L. & Bowery, N.G. pp. 249–259. New York: Raven Press.

MARTY, A. & NEHER, E. (1985). Potassium channels in cultured bovine adrenal chromaffin cells. *J. Physiol.*, **367**, 117–141.

MORITA, K., DOHI, T., KITAYAMA, S., KŌYAMA, Y. & TSUJIMOTO, A. (1987). Enhancement of stimulation-evoked catecholamine release from cultured bovine adrenal chromaffin cells by forskolin. *J. Neurochem.*, **48**, 243–247.

PETERS, J.A., LAMBERT, J.J. & COTTRELL, G.A. (1989). An electrophysiological investigation of the characteristics and function of $GABA_A$ receptors on bovine adrenomedullary chromaffin cells. *Pflügers Arch.*, **415**, 95–103.

SANGIAH, S., BOROWITZ, J.L. & YIM, G.K.W. (1974). Actions of GABA, picrotoxin and bicuculline on adrenal medulla. *Eur. J. Pharmacol.*, **27**, 130–135.

TSIEN, R.Y., POZZAN, T. & RINK, T.J. (1982). Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *J. Cell. Biol.*, **94**, 325–334.

UNGER, A. & PHILLIPS, J.H. (1983). Regulation of the adrenal medulla. *Physiol. Rev.*, **63**, 787–843.

VON EULER, U.W. & LISHAJKO, F. (1961). Improved technique for the fluorometric estimation of catecholamines. *Acta Physiol. Scand.*, **51**, 348–356.

WADA, A., TAKARA, H., YANAGIHARA, N., KOBAYASHI, H. & IZUMI, F. (1986). Inhibition of Na^+ pump enhances carbachol-induced influx of $^{45}Ca^{2+}$ and secretion of catecholamines by elevation of cellular accumulation of $^{22}Na^+$ in cultured bovine adrenal medullary cells. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **332**, 351–356.

WADA, A., IZUMI, F., YANAGIHARA, N. & KOBAYASHI, H. (1985). Modulation by ouabain and diphenylhydantoin of veratridine-induced ^{22}Na influx and its relation of ^{45}Ca influx and the secretion of catecholamines in cultured bovine adrenal medullary cells. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **328**, 273–278.

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Changes in neurotransmitter sensitivity in the mouse neocortical slice following propranolol and theophylline administration

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1 The mouse neocortical slice has been used to examine the sensitivity of neurones to isoprenaline, 5-hydroxytryptamine (5-HT) and adenosine acutely and following chronic treatment of animals with propranolol or theophylline.

2 While having little effect alone, all three agonists enhanced the d.c. depolarizing potential produced by N-methyl-D-aspartate (NMDA). The effect of (−)-isoprenaline (0.2 μM) was shared by (+)-isoprenaline at the much higher concentration of 10 μM.

3 Superfusion of slices with theophylline or 8-phenyltheophylline blocked responses to adenosine with evidence of selectivity. A single injection of theophylline 24 h before slice preparation did not alter agonist sensitivity, but when administered daily at 100 mg kg^{-1} for 14 days, the xanthine caused a loss of sensitivity to adenosine and (−)-isoprenaline but not 5-HT. The lower dose of theophylline, 10 mg kg^{-1} daily, also led to a loss of adenosine responses but no change of sensitivity to the amines.

4 Following the 14 day treatment with theophylline at 100 mg kg^{-1} daily in two groups of mice, responses to adenosine recovered to control levels after 20 days.

5 Propranolol superfusion blocked responses to both isomers of isoprenaline and 5-HT but did not affect sensitivity to adenosine.

6 Chronic treatment with propranolol at 25 mg kg^{-1} daily for 14 days induced a loss of sensitivity to (−)-isoprenaline and 5-HT but not adenosine. A lower dose of 5 mg kg^{-1} daily caused no change in responses to adenosine or 5-HT, but yielded an increased sensitivity to (−)-isoprenaline.

7 The results are discussed with respect to reports of receptor up-regulation in binding studies; caution is clearly required in extrapolating from such work to receptor activity in a functional system, especially in the case of theophylline and adenosine.

Introduction

Xanthines, including the caffeine and theophylline found in common beverages are among the most widely used drugs in the world. Large intakes of theophylline have been associated with enuresis, headache and seizures (Rall, 1985). Such symptoms may be particularly distressing for patients taking high therapeutic doses of theophylline or related preparations (Defreitas & Schwartz, 1979; Brumback *et al.*, 1984; Furukawa *et al.*, 1984). It is also clear that withdrawal from these compounds following their chronic usage can lead to a well-defined constellation of symptoms which include headache and confusion beginning, on average, 19 h after the last intake (Griffiths *et al.*, 1986). In order to determine the neuronal mechanisms which may underlie some of these phenomena we have become interested in the adaptive changes which may occur in the animal central nervous system during the chronic administration of theophylline.

It is now widely recognised that among the major pharmacological actions of theophylline, responsible for its behavioural stimulant properties, is the antagonism of endogenous adenosine (Daly *et al.*, 1981; Snyder *et al.*, 1981; Spealman, 1988; Choi *et al.*, 1988). This antagonistic action of theophylline was first described by Sattin & Rall (1970) on an adenylate cyclase system in guinea-pig neocortex but has since been demonstrated at a wide variety of adenosine receptors located presynaptically or postsynaptically (Stone, 1981; 1989; Williams, 1987). The concentrations of xanthines which induce behavioural effects are sufficient to block such adenosine receptors at far lower concentrations than are needed, for example, to inhibit phosphodiesterase or nucleotidase (Fredholm *et al.*, 1978; Rall, 1982; Fredholm & Lindgren, 1983).

One view of receptor regulation would predict that the chronic administration of theophylline would lead to an up-regulation of adenosine receptor density and function and that increased receptor activity would mediate the adverse effects seen during theophylline withdrawal. The present project was therefore initiated partly to determine whether any change in adenosine receptor function does indeed result from the chronic treatment of mice with theophylline.

In addition low doses of theophylline have recently been found to be of value in the treatment of essential tremor (Mally, 1989) being associated with fewer unwanted effects than the commonly accepted drug for this disorder, propranolol. The latter drug has also found a clinical use in the treatment of migraine (Anthony, 1978; Weber & Reinmuth, 1972) and anxiety states (Heiser & DeFrancisco, 1976; Linken, 1971). It remains unclear, however, whether any of these effects of propranolol are due to actions at β-adrenoceptors or whether other receptor populations are involved with an intermediate situation on pathways involved in the control of tremor and cerebrovascular tone. While it has been proposed that antagonism of β-adrenoceptors is involved in the anti-tremor activity (Young *et al.*, 1975; Taylor *et al.*, 1981; Kuroda *et al.*, 1988) an interference with 5-HT systems may be important (Hallberg, 1987).

The effects of chronic treatment with propranolol and theophylline have therefore been compared in order to ascertain both whether any changes of transmitter sensitivity could be found and whether any such changes of transmitter sensitivity are common to the two agents. Such a demonstration might provide clues as to why both drugs are effective in essential tremor (Mally, 1989).

In order to obtain the large amount of quantitative data required in this study the cortical wedge preparation was used. First described for rat tissue (Harrison & Simmonds, 1985) this preparation was subsequently developed for mouse neocortex by Burton *et al.* (1987), this species yielding a higher proportion of successful slices presumably by virtue of the more densely organised callosal projection. In preliminary experiments few of the compounds of interest to the present

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work had effects by themselves on these slices, but several were found to modify responses to the excitatory amino acid N-methyl-D-aspartate (NMDA). NMDA is a relatively selective agonist for one of three glutamate receptor subtypes (Watkins & Evans, 1981; Stone & Burton, 1988). The pharmacology of depolarizations induced by NMDA in mouse neocortex is comparable with the pharmacology in other species (Burton *et al.*, 1987).

Methods

Male mice were killed by cervical dislocation and the brain removed into cold bicarbonate buffered medium of the following composition (mM): NaCl 115, KCl 2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 10. Sections of the brain were cut at 500 μm with a vibratome, in a coronal plane to include regions of frontoparietal and cingulate cortex as described in detail previously (Burton *et al.*, 1987). These slices were then trimmed and transferred to two-compartment chambers where they were positioned across a greased slot in the dividing wall so that most of the grey matter lay on one side while the white matter extended into the second chamber.

Silver/silver chloride electrodes in contact with the solutions on the two sides of the dividing wall were used to record d.c. potentials which were displayed on digital oscilloscopes and chart recorders. The amplitude of induced d.c. potentials was used to quantify the drug effects. Both compartments were superfused with the bicarbonate buffer. N-methyl-D-aspartate (NMDA) was added into the fluid superfusing the pial side of the slice for 1 min periods. Other agonists were applied for 2 min periods beginning 1 min before NMDA. After obtaining control responses to agonists, antagonists were superfused for at least 20 min before further applications of agonists. Statistical comparisons were made by Student's *t* test.

For chronic administration, theophylline was dissolved fresh in dilute sodium hydroxide (pH 5.0) and injected immediately. Propranolol was dissolved in saline. Injections were given i.p.

Results

When applied alone to neocortical slices, adenosine 20 μM, 5-HT 10 μM, noradrenaline 10 μM and (±)-isoprenaline 10 μM had no discernible effects. We therefore elected to examine the possible modulation of sensitivity to the depolarizing amino acid NMDA as in our previous characterization of the mouse neocortical preparation (Burton *et al.*, 1987).

When superfused over the slices for periods of 1 or 2 min, NMDA induced depolarizations which have been discussed and illustrated extensively (Burton *et al.*, 1987; 1988) and which could be readily quantified. The concentration used throughout the present work was 20 μM, an amount that we have previously found to yield a response which is approximately 50% of the maximum attainable in this system.

A concentration-response curve to adenosine was obtained with concentrations between 0.2 and 250 μM. The curve was bell-shaped, with a dose-related enhancement of NMDA depolarization being seen up to 50 μM adenosine but with smaller enhancements at concentrations of 100 and 250 μM (Figure 1). A concentration of 20 μM was therefore routinely used, being a sub-maximal concentration on the ascending limb of the curve.

As well as adenosine, the effects of isoprenaline and 5-HT were examined so as to compare the effects of theophylline on other receptors and to determine whether any changes of receptor function were selective. The (−)-isomer of isoprenaline produced an increase in the size of NMDA responses and a concentration of 200 nM was used in further experiments since it induced an enhancement of NMDA depolarization by 38.5% ± 9.1 (n = 7) similar to that of 20 μM adenosine (Figure 2). This effect was shared by the (+)-isomer although a much higher concentration (10 μM) was required in order to produce a similar increase (33.0% ± 11.72, n = 4).

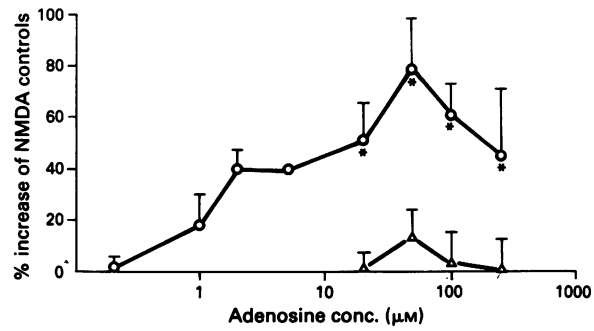

Figure 1 Concentration-response curves for adenosine potentiation of N-methyl-D-aspartate (NMDA) induced depolarization of neocortical slices. Results are shown for adenosine in control slices (○) and after 14 days pretreatment with theophylline 100 mg kg^{-1} daily (△). The points are the mean and vertical bars show 1 s.e.mean (n = 6). * $P < 0.05$ for pretreated compared with naive preparations.

Lower concentrations of (+)-isoprenaline (200 nM and 1 μM) had no significant effect on the slices.

5-HT at 10 μM but not 1 μM also potentiated significantly responses to NMDA (Figure 2). The potentiation seen at the higher concentration, 48.4% ± 8.8 (n = 7) was again comparable with that seen with 20 μM adenosine.

Effect of theophylline superfusion

Superfusion of the slices with theophylline at 10 μM and 100 μM had no effect on the size of control depolarizing

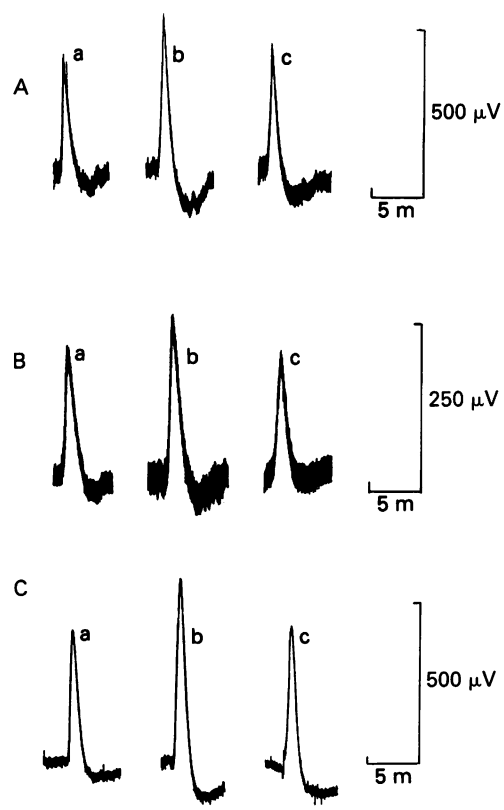

Figure 2 Records of cortical slice depolarization. Each panel shows a control response to 20 μM N-methyl-D-aspartate (NMDA) (a), a response in the presence of test agent (b) and recovery (c). Group A illustrates the enhancement of NMDA-induced depolarization by (−)-isoprenaline (200 nM), group B by 5-hydroxytryptamine (10 μM) and group C by adenosine (20 μM). Calibrations: 5 min for all records; 500 μV for (A) and (C), 250 μV for (B).

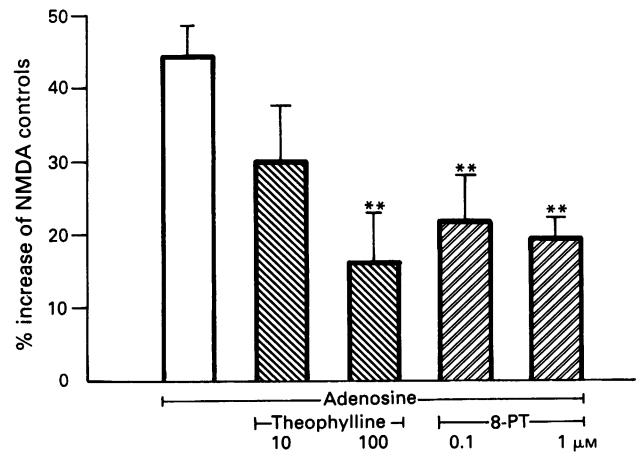

Figure 3 Histogram of the enhancement of N-methyl-D-aspartate (NMDA) depolarization induced by adenosine (20 μM) in the presence of theophylline 10 or 100 μM or 8-phenyltheophylline (8-PT) 0.1 or 1 μM. Columns show the mean with vertical bars indicating 1 s.e.mean ($n = 6$).
** $P < 0.01$ compared with the xanthine-free controls.

responses to NMDA alone. With both concentrations there was a reduction in the mean level of potentiation by adenosine though this antagonism was only significant at the higher concentration of xanthine. The more selective adenosine antagonist, 8-phenyltheophylline (8-PT), reduced adenosine responses significantly at a concentration of 100 nM and 1 μM (Figure 3).

In contrast, responses to 5-HT were not changed significantly by 100 nM or 1 μM 8-PT (Figure 4). The effect of 200 nM (−)-isoprenaline was reduced significantly by 100 μM theophylline; at 10 μM theophylline the response was reduced but not significantly. Since these effects of theophylline were not shared by 8-PT, it is unlikely they were related to any blockade of adenosine receptors (Figure 4).

Subacute and chronic treatment with theophylline

Adenosine In order to examine the persistence of any acute blockade of adenosine receptors by injected theophylline, one group of mice was killed 24 h after a single injection of theophylline 100 mg kg^{-1}, i.p. This procedure did not result in any diminution of adenosine response which now reached 140.8% ± 8.98 ($n = 5$) of control NMDA responses compared with 150.9 ± 15.9 ($n = 13$; NS) without xanthine administration.

Daily administration of theophylline for 14 days did result in a reduction of adenosine sensitivity. At a daily dose of 100 mg kg^{-1} the potentiation of NMDA by adenosine was reduced to only 101.7 ± 6.4 ($n = 6$) % of control (Figure 5).

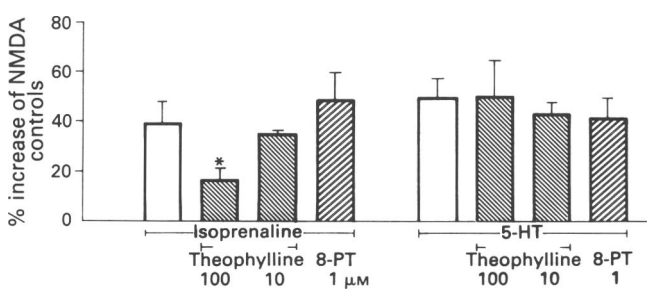

Figure 4 Histogram of the enhancement of N-methyl-D-aspartate (NMDA) depolarizations induced by (−)-isoprenaline (0.2 μM) and 5-hydroxytryptamine (5-HT, 10 μM) in the presence of theophylline 100 or 10 μM or 8-phenyltheophylline (8-PT) 1 μM. Columns show the mean with vertical bar indicating 1 s.e.mean ($n = 6$).
* $P < 0.05$ compared with the xanthine-free controls.

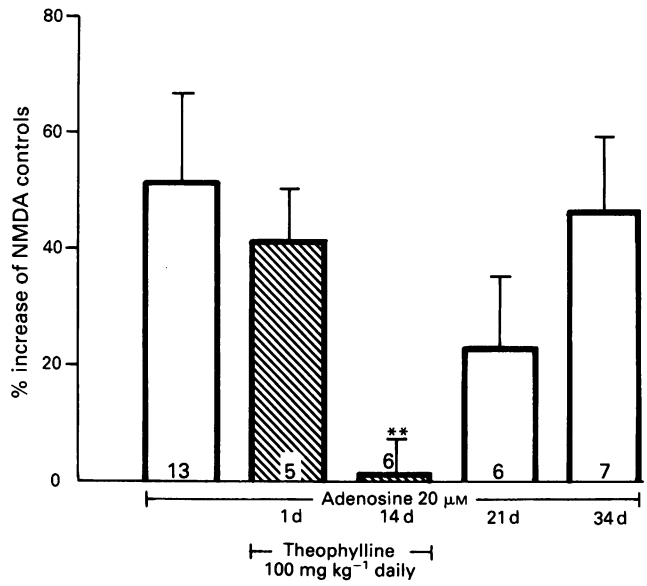

Figure 5 Histogram of the enhancement of N-methyl-D-aspartate (NMDA) depolarization induced by adenosine (20 μM) showing the time course of recovery of sensitivity 7 days and 20 days following treatment with theophylline 100 mg kg^{-1} daily. Columns show the mean with vertical bars indicating 1 s.e.mean with n shown within the columns.
** $P < 0.01$ compared with the xanthine-free controls.

The lower dose of 10 mg kg^{-1} daily for two weeks also abolished sensitivity to adenosine (Figure 6) with no change in the size of control responses to NMDA itself. In order to assess whether this loss of sensitivity represented a total absence of functional adenosine receptors or a shift in the dose-response curve, higher concentrations of adenosine were also examined in combination with this treatment schedule. As illustrated in Figure 6, responses could be obtained with 50 and 100 μM adenosine which represented significant potentiation of NMDA responses, though they also remained significantly below the amplitude of the respective control responses.

Two groups of mice were treated with theophylline at the high dose of 100 mg kg^{-1} daily for 14 days and then the xanthine treatment was stopped in order to follow events on

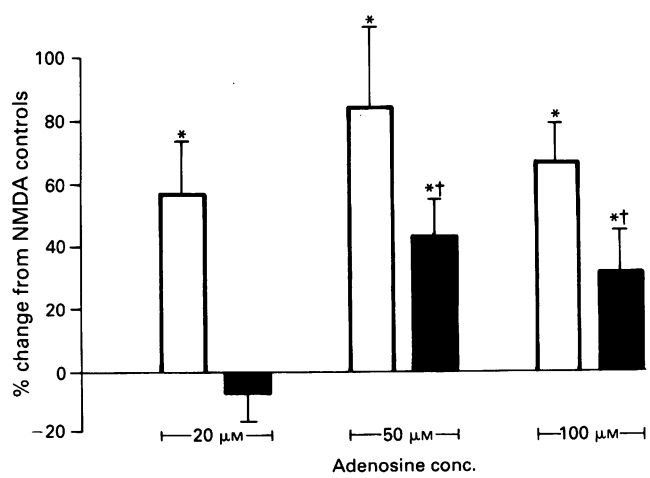

Figure 6 Histogram of the enhancement of N-methyl-D-aspartate (NMDA) depolarization induced by adenosine at 20, 50 and 100 μM in naive slices (open columns) or slices taken from animals pretreated with theophylline 10 mg kg^{-1} daily for 14 days (solid columns). Columns show the mean with vertical bars indicating 1 s.e.mean ($n = 7$).
* $P < 0.05$ compared with NMDA alone; † $P < 0.05$ compared with non-pretreated slices.

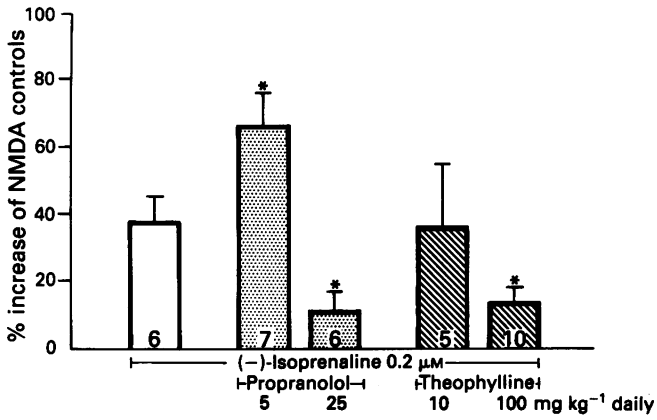

Figure 7 Histogram of the enhancement of N-methyl-D-aspartate (NMDA) depolarization induced by (−)-isoprenaline (0.2 μM) following pretreatment with propranolol 5 or 25 mg kg^{-1} daily for 14 days, or theophylline 10 or 100 mg kg^{-1} daily. Columns show the mean with 1 s.e.mean indicated by vertical bars; n is shown within each column.
* $P < 0.05$ compared with non-pretreated slices.

withdrawal. Seven days after the theophylline injections adenosine responses were again detectable, NMDA responses being potentiated significantly by 23.0% ± 12% ($n = 6$) (Figure 5). By 20 days after withdrawal adenosine responses had returned to control levels of enhancement of NMDA responses by 46.1% ± 12.4 ($n = 7$) compared with controls; $P < 0.05$ (Figure 5).

5-HT In contrast neither dose of theophylline had any effect on sensitivity to 5-HT (10 μM); at 100 mg kg^{-1} daily, 5-HT potentiated NMDA responses by 39% ± 13.2 ($n = 7$, $P < 0.001$) compared with 48.4% ± 8.8 ($n = 6$) in control slices. After chronic theophylline at 10 mg kg^{-1} daily, 5-HT still enhanced NMDA depolarization by 57.5% ± 6.5 ($n = 6$; NS).

Isoprenaline Treatment with the lower dose of theophylline (10 mg kg^{-1}, daily) did not result in any diminution of responses to (−)-isoprenaline, the potentiated NMDA response reaching 134.6% ± 18.5 ($n = 5$) of control compared with 136.4% ± 7.6 ($n = 6$) in slices from naive animals. The higher dose of 100 mg kg^{-1} daily did lead to a significant reduction of isoprenaline sensitivity (Figure 7) to control levels.

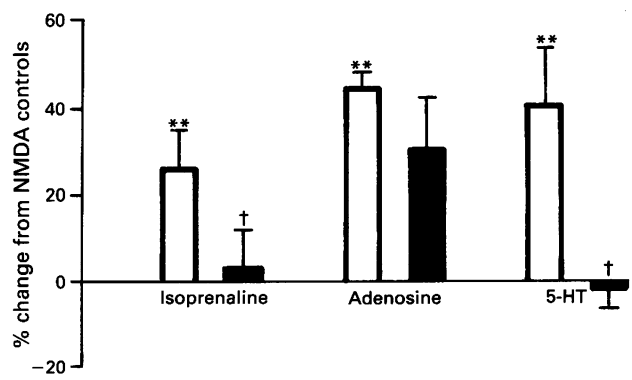

Figure 8 Histogram of the enhancement of N-methyl-D-aspartate (NMDA) depolarization induced by (−)-isoprenaline 0.2 μM, adenosine 20 μM or 5-hydroxytryptamine (5-HT) 10 μM in control slices (open columns) or in the presence of propranolol 40 μM (solid columns). Columns show the mean with 1 s.e.mean indicated by vertical bars ($n = 8$).
** $P < 0.05$ compared with NMDA alone. † $P < 0.05$ compared with control agonist responses.

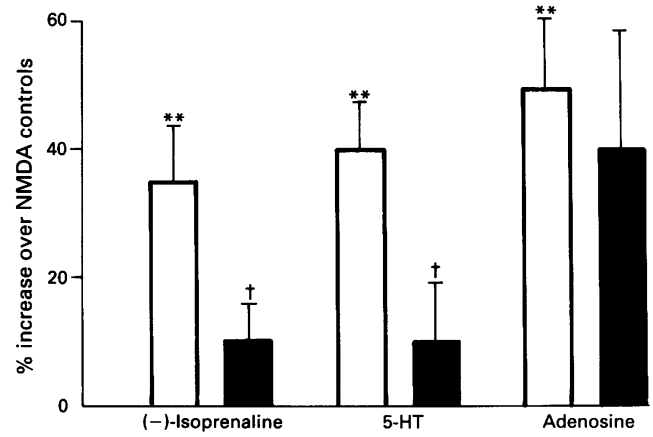

Figure 9 Histogram of the enhancement of N-methyl-D-aspartate (NMDA) depolarization induced by (−)-isoprenaline 0.2 μM, 5-hydroxytryptamine (5-HT) 10 μM or adenosine 20 μM in control slices (open columns) or following 14 days treatment with propranolol 25 mg kg^{-1} daily (solid columns). Columns show the mean with 1 s.e.mean shown by vertical bars ($n = 6$).
** $P < 0.05$ compared with NMDA alone. † $P < 0.05$ compared with agonist in propranolol naive slices.

Effect of propranolol superfusion

When slices were superfused with (±)-propranolol at a concentration of 40 μM, the potentiation of NMDA responses by (−)-isoprenaline (200 nM) or (+)-isoprenaline (10 μM) was abolished. Similarly, the potentiating effect of 5-HT (10 μM) was prevented by propranolol. Responses to adenosine were not changed significantly (Figure 8).

Subacute and chronic treatment with propranolol

Administration of propranolol at a dose of 25 mg kg^{-1} 24 h before death caused only small changes in the size of responses to (−)-isoprenaline (200 nM), (+)-isoprenaline (10 μM) or adenosine (20 μM), which were not significant. There was a somewhat larger reduction in the mean amplitude of responses to 5-HT (10 μM) but this did not reach statistical significance.

Chronic treatment with the same dose of propranolol, 25 mg kg^{-1} daily for 14 days, yielded a marked and significant loss of sensitivity to both (−)-isoprenaline and 5-HT (Figure 9). Responses to adenosine, however, were unaffected by chronic administration of propranolol.

A lower dose of propranolol (5 mg kg^{-1} daily given for 14 days) resulted in a small reduction of sensitivity to 5-HT which did not attain significance, but induced an elevation in the size of responses to (−)-isoprenaline (Figure 10). No statistically significant changes were seen in sensitivity to adenosine.

Discussion

The use of NMDA to provide a depolarization of neocortical neurones upon which the effects of other agents can be examined appears to be validated by the pharmacological confirmation that conventional receptors are involved in the effects of isoprenaline (stereospecific and blocked by propranolol) and adenosine (blocked by theophylline and 8-phenyltheophylline). The observation that adenosine enhanced the NMDA-induced depolarization rather than reducing it was unexpected. Adenosine is generally considered to be inhibitory on cortical neurones (Stone, 1989) increasing potassium conductance (Segal, 1981; Haas & Greene, 1984; Trussel & Jackson, 1985) and suppressing neuronal firing and transmitter release (Stone, 1981; 1982; Phillis, 1982). The enhancement of NMDA sensitivity may result from a disinhibitory action of adenosine within the cortex.

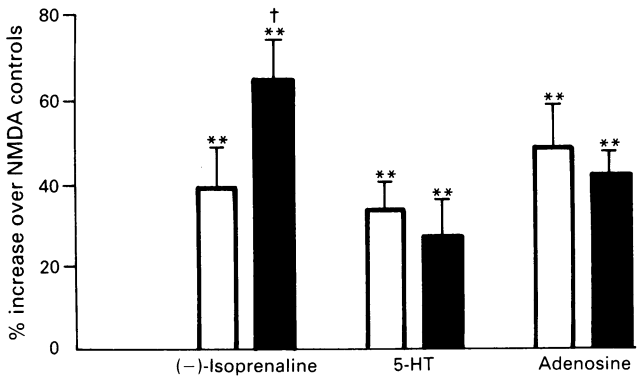

Figure 10 Histogram of the enhancement of N-methyl-D-aspartate (NMDA) depolarization induced by (−)-isoprenaline 0.2 μM, 5-hydroxytryptamine (5-HT) 10 μM or adenosine 20 μM in control slices (open columns) or following 14 days treatment with propranolol 5 mg kg^{-1} daily (solid columns). Columns show the mean with 1 s.e.mean shown by vertical bars (n = 6 or 7).
** $P < 0.05$ compared with NMDA alone. † $P < 0.05$ compared with agonist in propranolol-naive slices.

The potentiation of NMDA by isoprenaline is, however, consistent with reports that activation of β-adrenoceptors can have a net excitatory effect on central neurones. Thus noradrenaline can potentiate the excitatory effects of glutamate by an action at β-adrenoceptors (Madison & Nicoll, 1986; Wohlberg *et al.*, 1987; Marshall & Tsai, 1988; Mori-Okamoto & Tatsumo, 1989). This effect can occur without significant changes of spontaneous firing or against a background of reduced firing and hyperpolarization (Mori-Okamoto & Tatsumo, 1989), and probably results from a reduced calcium-activated potassium conductance (Haas & Rose, 1987; Madison & Nicoll, 1986). The nature of the 5-HT receptor involved in potentiating responses to NMDA has not been examined in this study in view of the complexity of 5-HT receptor pharmacology. These responses were, nevertheless, antagonized by propranolol which has become recognised as an antagonist at 5-HT$_1$ receptors in other neuronal systems (Weinstock & Schechter, 1975; Nahorski & Willcocks, 1983; Middlemiss, 1984; Richards, 1985). The failure of propranolol to affect responses to adenosine strongly suggests that it was not having any significant non-specific neuronal membrane stabilising action which might otherwise account for the antagonism of isoprenaline and 5-HT.

Chronic treatment with the lower dose of propranolol (5 mg kg^{-1} daily) for two weeks resulted in a significant increase in sensitivity to (−)-isoprenaline, consistent with previous work indicating an increase in the density of β-adrenoceptors in the CNS following propranolol treatment (Glaubiger & Lefkowitz, 1977; Wolfe *et al.*, 1978; 1982; Aarons & Molinoff, 1982). However, when animals were treated with propranolol at the higher dose of 25 mg kg^{-1} daily, sensitivity to (−)-isoprenaline was substantially diminished, a change noted also with responses to 5-HT. This may reflect a secondary down-regulation of receptor sensitivity or number resulting from a period of increased receptor sensitivity as obtained with the lower dose of propranolol. Again the absence of any change of adenosine responses excludes the involvement of membrane stabilisation or other non-specific membrane effects in the reduction of amine sensitivity.

Since the net effect of a high dose propranolol was qualitatively similar to the effect of acute superfusion it is important to note that, while little work has been done in mice, propranolol is rapidly metabolised by man, rat and guinea-pig with elimination half life estimates between 47 min and 3 h (Smits & Struyker-Boudier, 1979; Terao & Shen, 1983; 1984; Wooltrey *et al.*, 1989). It is thus unlikely that residual propranolol could accumulate sufficiently to account for the present observations 24 h after the last dose. The effects of low dose chronic propranolol are qualitatively different from acute superfusion so that residual propranolol cannot account for these effects.

Chronic administration of xanthines in contrast, has been shown clearly to increase the density of receptors for adenosine or its radiolabelled analogues. This result seems to follow administration of caffeine (Fredholm, 1982; Boulenger *et al.*, 1983; Marangos *et al.*, 1984; Daval *et al.*, 1989) or theophylline (Murray, 1982; Szot *et al.*, 1987; Zielke & Zielke, 1987; Sanders & Murray, 1988; Fastbom, 1988). In one of these studies a correlation was made between the increased number of adenosine receptors and the observed increase of seizure threshold (Szot *et al.*, 1987). In a different study Fredholm (1982) concluded that there was no relationship between the caffeine-induced increase of binding of phenylisopropyladenosine and the stimulation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels in the brain. This may merely reflect a preferential activity of the analogue at A$_1$ receptors (Stone, 1989), although both A$_1$ and A$_2$ receptors, examined separately do seem to up-regulate with caffeine treatment (Hawkins *et al.*, 1988; Daval *et al.*, 1989).

It is unlikely that any of these effects of theophylline can be attributed to the persistence of the xanthine in the brain since the tests performed with single injections of theophylline 24 h before death revealed no loss of adenosine sensitivity.

Furthermore kinetic studies have shown that little theophylline can be detected in brain 24 h after injection (Fredholm *et al.*, 1983) and since theophylline blockade of adenosine receptors is readily reversible *in vitro* the continuous flow of superfusion medium should rapidly relieve adenosine receptors from any residual xanthine. Indeed the half life of theophylline in different species has been measured at between 1 and 5 h (Walle & Walle, 1979; Madsen & Ribel, 1981; Chiou *et al.*, 1982). These various points together with the delay of at least 7 days before agonist responses returned to control levels after ceasing theophylline administration argues strongly against the possibility that theophylline could have accumulated in the brain sufficiently to account for the loss of adenosine sensitivity.

Previous biochemical work has led to reports that chronic xanthines can increase the inhibition of adenylate cyclase (Green & Stiles, 1986) and transmitter release by adenosine (Fredholm *et al.*, 1984) although it has also been emphasised that major regional differences exist in the ability of xanthines to alter adenosine sensitivity (Fastbom, 1988). The present study has attempted to examine a functional response to adenosine in an electropharmacological system. The results have demonstrated that, in contrast to the up-regulation of binding sites described previously, theophylline causes a dramatic loss of sensitivity to adenosine. The present work therefore emphasises the importance of confirming directly any changes of adenosine function postulated to occur on the basis of binding data alone. An additional finding was the reduced sensitivity to isoprenaline after theophylline treatment. This would be more consistent with binding studies which have revealed a down-regulation of β-adrenoceptor number after theophylline treatment (Goldberg *et al.*, 1982; Fredholm *et al.*, 1984). Since adenosine is known to suppress the release of noradrenaline from both peripheral and central neurones this phenomenon could be secondary to a reduction of adenosine sensitivity in the CNS as demonstrated here, with a consequent loss of adenosine inhibitory 'tone' and increased noradrenaline release.

In conclusion, the mouse neocortical slice may be a suitable preparation with which to investigate quantitatively changes of receptor function in the central nervous system following chronic treatment with drugs. The present data suggest that an explanation of some components of the xanthine withdrawal syndrome should be sought in terms of a paradoxical reduction of adenosine sensitivity rather than the increase predicted from binding data. Since alterations in sensitivity to isoprenaline have also been detected, however, β-adrenoceptors may also be involved.

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References

AARONS, R.D. & MOLINOFF, P.B. (1982). Changes in the density of β-adrenergic receptors in rat lymphocytes, heart and lung after chronic treatment with propranolol. *J. Pharmacol. Exp. Ther.*, **221**, 439–443.

ANTHONY, M. (1978). β-Blockers in migraine prophylaxis. *Drugs*, **15**, 249–250.

BOULENGER, J.-P., PATEL, J., POST, R.M., PARMA, A.M. & MARANGOS, P.J. (1983). Chronic caffeine consumption increases the number of brain adenosine receptors. *Life Sci.*, **32**, 1135–1142.

BRUMBACK, R.A., WILSON, H. & STANTON, R.D. (1984). Behavioural problems in children taking theophylline. Lancet, **i**, (8383) 958.

BURTON, N.R., SMITH, D.A.S. & STONE, T.W. (1987). The mouse neocortical slice: preparation and responses to excitatatory amino acids. *Comp. Biochem. Physiol.*, **88C**, 47–55.

BURTON, N.R., SMITH, D.A.S. & STONE, T.W. (1988). A quantitative pharmacological analysis of some excitatory amino acid receptors in the mouse neocortex *in vitro*. *Br. J. Pharmacol.*, **93**, 693–701.

CHIOU, W.L., LAM, G., CHEN, M.L. & LEE, N.G. (1982). Effect of arterio-venous plasma concentration differences on the determination of mean residence time of drugs in the body. *Res. Commun. Chem. Pathol. Pharmacol.*, **35**, 17–26.

CHOI, D.H., SHAMIM, M.T., PADGETT, W.L. & DALY, J.W. (1988). Caffeine and theophylline analogues: correlation of behavioural effects with activity as adenosine receptor antagonists and as PDE inhibitors. *Life Sci.*, **43**, 387–398.

DALY, J.W., BRUNS, R.F. & SNYDER, S.H. (1981). Adenosine receptors in the CNS: relationship to the central actions of methylxanthines. *Life Sci.*, **28**, 2083–2098.

DAVAL, J.-L., DECKERT, J., WEISS, S.R., POST, R.M. & MARANGOS, P.J. (1989). Upregulation of adenosine A1 receptors and forskolin binding sites following chronic treatment with caffeine or carbamazapine: a quantitative autoradiographic study. *Epilepsia*, **30**, 26–33.

DEFREITAS, B. & SCHWARTZ, G. (1979). Effects of caffeine in chronic psychiatric patients. *Am. J. Psychiat.*, **136**, 1337–1338.

FASTBOM, J. (1988). Adenosine receptors in the central nervous system. *Ph.D. Thesis, Karolinska Institute, Stockholm.*

FREDHOLM, B.B. (1982). Adenosine actions and adenosine receptors after 1 week treatment with caffeine. *Acta Physiol. Scand.*, **115**, 283–286.

FREDHOLM, B.B. & LINDGREN, E. (1983). Inhibition of soluble 5'nucleotidase from rat brain by different xanthine derivatives. *Biochem. Pharmacol.*, **32**, 2832–2834.

FREDHOLM, B.B., HEDQVIST, P. & VERNET, L. (1978). Effect of theophylline and other drugs on rabbit renal cyclic nucleotide phosphodiesterase, 5' nucleotidase and adenosine deaminase. *Biochem. Pharmacol.*, **27**, 2845–2850.

FREDHOLM, B.B., HERRERA-MARSCHITZ, M., JONZON, B., LINDSTROM, K. & UNGERSTEDT, U. (1983). On the mechanism by which methylxanthines enhance apomorphine-induced rotation behaviour in the rat. *Pharmacol. Biochem. Behav.*, **19**, 535–541.

FREDHOLM, B.B., JONZON, B. & LINDGREN, E. (1984). Changes in noradrenaline release and in Beta receptor number in rat hippocampus following long term treatment with theophylline or L-PIA. *Acta Physiol. Scand.*, **122**, 55–60.

FURUKAWA, C.T., SHAPIRO, G.G., DUHAMEL, T., WEIMER, L., PIERSON, W.E. & BIERMAN, C.W. (1984). Learning and behaviour problems associated with theophylline therapy. *Lancet* **i**, 621.

GLAUBIGER, G. & LEFKOWITZ, R.J. (1977). Elevated β-adrenergic receptor number after chronic propranolol treatment. *Biochem. Biophys. Res. Commun.*, **78**, 720–725.

GOLDBERG, M.R., CURATOLO, P.W., TUNG, CH. & ROBERTSON, D. (1982). Caffeine down-regulates β-adrenoceptors in rat forebrain. *Neurosci. Lett.*, **31**, 47–52.

GREEN, M.R. & STILES, G.L. (1986). Chronic caffeine ingestion sensitizes the A1 adenosine receptor-adenylate cyclase system in rat cerebral cortex. *J. Clin. Invest.*, **77**, 222–227.

GRIFFITHS, R.R., BIGELOW, G.E. & LIEBSON, I.A. (1986). Human coffee drinking – reinforcing and physical dependence producing effects of caffeine. *J. Pharmacol. Exp. Ther.*, **239**, 416–425.

HAAS, H.L. & GREENE, R.W. (1984). Adenosine enhances after-hyperpolarization and accommodation in hippocampal pyramidal cells. *Pflug. Arch.*, **402**, 244–247.

HAAS, H.L. & ROSE, G.M. (1987). Noradrenaline blocks potassium conductance in rat dentate granule cells *in vitro*. *Neurosci. Letts.*, **78**, 171–174.

HALLBERG, H. (1987). Blockade of central β-adrenoceptors attenuates tremor induced by 5-hydroxytryptamine (5HT)-receptor activation in rats. *Acta Physiol. Scand.*, **129**, 421–428.

HARRISON, N.L. & SIMMONDS, M. (1985). Quantitative studies on some antagonists of NMDA in slices of rat cerebral cortex. *Br. J. Pharmacol.*, **84**, 381–391.

HAWKINS, M., DUGICH, M.M., PORTER, N.M., URBANCIC, M. & RADULOVACKI, M. (1988). Effects of chronic administration of caffeine on adenosine A1 and A2 receptors in rat brain. *Brain Res. Bull.*, **21**, 479–482.

HEISER, J.F. & DEFRANCISCO, D. (1976). The treatment of pathological panic states with propranolol. *Am. J. Psychiat.*, **133**, 1389–1394.

KURODA, Y., KAKIGI, R. & SHIBASAKI, H. (1988). Treatment of essential tremor with arotinolol. *Neurology*, **38**, 650–652.

LINKEN, A. (1971). Propranolol for LSD-induced anxiety states. *Lancet*, **ii**, 1039–1040.

MADISON, D.V. & NICOLL, R.A. (1986). Actions of noradrenaline recorded intracellularly in rat hippocampal CA1 pyramidal neurones, *in vitro*. *J. Physiol.*, **372**, 221–244.

MADSEN, S.M. & RIBEL, U. (1981). Pharmacokinetics of theophylline and 3-methyl xanthine in guinea-pigs. Multiple dose administration. *Acta Pharmacol. Toxicol.*, **48**, 8–12.

MALLY, J. (1989). Aminophylline and essential tremor. *Lancet*, **ii**, 278–279.

MARANGOS, P.J., BOULENGER, J.-P. & PATEL, J. (1984). Effects of chronic caffeine on brain adenosine receptors: regional and ontogenetic studies. *Life Sci.*, **34**, 899–907.

MARSHALL, K.C. & TSAI, W.H. (1988). Noradrenaline induces short and long duration potentiation of glutamate excitations of cultured Purkinje neurons. *Can. J. Physiol. Pharmacol.*, **66**, 848–853.

MIDDLEMISS, D.N. (1984). Stereoselective blockade at ^{3}H 5HT binding sites and at the 5HT autoreceptor by propranolol. *Eur. J. Pharmacol.*, **101**, 289–293.

MORI-OKAMOTO, J. & TATSUNO, J. (1989). Participation of cAMP and β-adrenergic receptors in the facilitatory effect of noradrenaline on the response of cultured cerebellar neurons to glutamate. *Brain Res.*, **490**, 64–72.

MURRAY, T.F. (1982). Up-regulation of rat cortical adenosine receptors following chronic administration of theophylline. *Eur. J. Pharmacol.*, **82**, 113–114.

NAHORSKI, S.R. & WILLCOCKS, A.L. (1983). Interactions of β-adrenoceptor antagonists with 5HT receptor subtypes in rat cerebal cortex. *Br. J. Pharmacol.*, **78**, 107P.

PHILLIS, J.W. (1982). Evidence for an A2-like adenosine receptor on cerebral cortical neurones. *J. Pharm. Pharmacol.*, **34**, 453–454.

RALL, J.W. (1982). Evolution of the mechanism of action of methylxanthines: from calcium mobilizers to antagonists of adenosine receptors. *Pharmacologist*, **11**, 277–287.

RALL, J.W. (1985). Central nervous system stimulants: the xanthine. In *The Pharmacological Basis of Therapeutics, 7th Edn.* ed. Gilman, A.G., Goodman, L.S. & Gilman, A. pp. 589–602. New York: Macmillan.

RICHARDS, M.H. (1985). Efflux of ^{3}H 5HT from rat hypothalamic slices by continuous electrical stimulation: frequency dependent responses to serotonergic antagonists and 5HT. *Naunyn Schmiedebergs Arch. Pharmacol.*, **329**, 359–366.

SANDERS, R.C. & MURRAY, T.F. (1988). Chronic theophylline exposure increases agonist and antagonist binding to A1 adenosine receptors in rat brain. *Neuropharmacol.*, **27**, 757–760.

SATTIN, A. & RALL, J.W. (1970). The effect of adenosine and adenine nucleotides on the cyclic adenosine 3'-5'-phosphate content of guinea pig cerebral cortex slices. *Molec. Pharmacol.*, **6**, 13–23.

SEGAL, M. (1982). Intracellular analysis of a postsynaptic action of adenosine in the rat hippocampus. *Eur. J. Pharamcol.*, **79**, 193–199.

SMITS, J.M. & STRUYKER-BOUDIER, H.A. (1979). Propranolol in conscious spontaneously hypertensive rats. Disposition after subcutaneous and intracerebroventricular administration. *Naunyn Schmiedbergs Arch. Pharmacol.*, **308**, 19–24.

SNYDER, S.H., KATIMS, J.J., ANNAU, Z., BRUNS, R.F. & DALY, J.W. (1981). Adenosine receptors and behavioural actions of methylxanthines. *Proc. Natl. Acad. Sci., U.S.A.*, **78**, 3260–3264.

SPEALMAN, R.D. (1988). Psychomotor stimulant effects of methylxanthines in squirrel monkeys: relation to adenosine antagonism. *Psychopharmacol.*, **95**, 19–24.

STONE, T.W. (1981). Physiological roles for adenosine and adenosine 5'-triphosphate in the nervous system. *Neurosci.*, **6**, 523–555.

STONE, T.W. (1982). Purine receptors involved in the depression of neuronal firing in cerebral cortex. *Brain Res.*, **248**, 367–370.

STONE, T.W. (1989). Purine receptors and their pharmacological roles. In *Advances in Drug Research*, Vol. 18, pp. 291–429. New York,

London: Academic Press.

STONE, T.W. & BURTON, N.R. (1988). NMDA receptors and endogenous ligands. *Progr. Neurobiol.*, **30**, 333–368.

SZOT, P., SANDERS, R.C. & MURRAY, T.F. (1987). Theophylline induced up-regulation of A1 adenosine receptors associated with reduced sensitivity to convulsants. *Neuropharmacol.*, **26**, 1173–1180.

TAYLOR, E.A., JEFFERSON, D., CARROLL, J.D. & TURNER, P. (1981). Cerebrospinal fluid concentrations of propranolol, pindolol and atenolol in man: evidence for central actions of β-adrenoceptor antagonists. *Br. J. Clin. Pharmacol.*, **12**, 549–559.

TERAO, N. & SHEN, D.D. (1963). Effect of experimental renal failure on the disposition kinetics of 1-propranolol in rats. *J. Pharmacol. Exp. Ther.*, **227**, 295–301.

TERAO, N. & SHEN, D.D (1984). Pharmacokinetics of 1-propranolol during repetitive dosing in normal and uranyl nitrate induced renal failure in rats. *J. Pharmacokinet. Biopharmacol.*, **12**, 479–493.

TRUSSELL, L.O. & JACKSON, M.B. (1985). Adenosine activated potassium conductance in cultured striatrial neurons. *Proc. Natl. Acad. Sci., U.S.A.*, **82**, 4857–4861.

WALLE, T. & WALLE, K. (1979). Stereoselective oral bioavailability of $\pm$ propranolol in the dog. A GCMS study using a stable isotope technique. *Res. Commun. Chem. Pathol. Pharmacol.*, **23**, 453–464.

WATKINS, J.C. & EVANS, R.H. (1981). Excitatory amino acid transmitters. *Ann. Rev. Pharmacol.*, **21**, 165–204.

WEIBER, R.B. & REINMUTH, O.M. (1972). The treatment of migraine with propranolol. *Neurology*, **22**, 366–369.

WEINSTOCK, M. & SCHECHTER, Y. (1975). Antagonism by propranolol of the ganglion stimulant action of 5HT. *Eur. J. Pharmacol.*, **32**, 293–301.

WILLIAMS, M. (1987). Purine receptors in mammalian tissues: pharmacology and functional significance. *Ann. Rev. Pharmacol. Toxicol.*, **27**, 315–345.

WOHLBERG, C.J., HACKMAN, J.C. & DAVIDOFF, R.A. (1987). Epinephrine and norepinephrine modulate neuronal responses to excitatory amino acids and agonists in frog spinal cord. *Synapse*, **1**, 202–207.

WOLFE, B.B., HARDEN, T., SPORN, J.R. & MOLINOFF, P.B. (1978). Presynaptic modulation of β-adrenergic receptors in rat cerebral cortex after treatment. *J. Pharmacol. Exp. Ther.*, **207**, 446–457.

WOLFE, B.B., MINNEMAN, K.P. & MOLINOFF, P.B. (1982). Selective increases in the density of cerebellar β_1-adrenergic receptors. *Brain Res.*, **234**, 474–479.

WOOLTREY, S.G., PALIN, K.J. & DAVIS, S.S. (1989). The effect of Miglycol 812 oil on the oral absorption of propranolol in the rat. *J. Pharm. Pharmacol.*, **41**, 579–581.

YOUNG, R.R. (1986). Essential-familial tremor. In *Handbook of Clinical Neurology*. ed. Vinken, P.J., Bruyn, G.W. & Klawans, H.L. pp. 565–581. Amsterdam: Elsevier.

YOUNG, R.R., GROWDON, J.H., SHAHANI, B.T. & PHIL, D. (1975). β-adrenergic mechanisms in action tremor. *New Engl. J. Med.*, **293**, 950–953.

ZIELKE, C.L. & ZIELKE, H.R. (1987). Chronic exposure to subcutaneous implanted methylxanthines. Differential elevation of A1 adenosine receptors in mouse cerebellar and cerebral cortical membranes. *Biochem. Pharmacol.*, **36**, 2533–2538.

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Alkylation with β-funaltrexamine suggests differences between μ-opioid receptor systems in guinea-pig brain and myenteric-plexus

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1 The effects of pre-incubation with β-funaltrexamine (β-FNA) on the binding of [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin ([^{3}H]-DAMGO) to homogenates of guinea-pig brain and myenteric-plexus longitudinal muscle have been studied.

2 β-FNA pretreatment of brain homogenates in Tris-HCl buffer reduced the amount of [^{3}H]-DAMGO binding. This was principally due to a reduction in the maximal number of binding sites measurable. However, approximately 30% of sites labelled by 1 nM [^{3}H]-DAMGO were insensitive to 1 μM β-FNA. Similar findings were obtained when the alkylation was performed in brain homogenates prepared in Krebs solution buffered with HEPES.

3 β-FNA pretreatment of whole myenteric-plexus longitudinal muscle strips caused an increase in the IC_{50} values of μ-agonists, but not of κ-agonists. However, the binding of [^{3}H]-DAMGO to homogenates of myenteric-plexus longitudinal muscle was not altered by pre-incubation with β-FNA in Tris-HCl buffer. On the other hand when the pretreatment was carried out in whole tissue in Krebs solution, or in homogenates in the presence of NaCl and Gpp(NH)p, a marked reduction in [^{3}H]-DAMGO binding was observed.

4 These results suggest that a low affinity form of the μ-opioid receptor is the physiologically relevant site for β-FNA alkylation in the myenteric-plexus and that differences exist between μ-receptor systems in guinea-pig myenteric plexus and brain.

Introduction

β-Funaltrexamine (β-FNA) is an irreversible antagonist at μ-opioid receptors (Portoghese *et al.*, 1980). It is effective in preventing μ-receptor activation by opioids in both *in vitro* bioassay systems (Ward *et al.*, 1982a; 1986; Hayes *et al.*, 1985; Corbett *et al.*, 1985) and *in vivo* (Ward *et al.*, 1982b; Hayes *et al.*, 1986; Takemori & Portoghese, 1987). Ligand binding studies in brain homogenates suggest that this action is due to an irreversible reduction in the number of opioid binding sites (Rothman *et al.*, 1983; Ward *et al.*, 1985; Tam & Liu-Chen, 1986), possibly due to Michael addition with a thiol function close to the ligand-recognition site (Tam & Liu-Chen, 1986).

In homogenates of myenteric-plexus of the guinea-pig ileum, however, pre-incubation with β-FNA apparently does not lead to a reduction in the level of μ-opioid ligand binding. The loss of potency of μ-ligands in intact myenteric-plexus longitudinal muscle preparations following β-FNA treatment has therefore been attributed to an interference with the receptor-effector coupling system in this tissue, rather than a direct blockade of binding sites (Corbett *et al.*, 1985).

To understand this apparent anomaly, we have investigated the action of β-FNA at μ-opioid binding sites, in homogenates of guinea-pig brain and myenteric-plexus longitudinal muscle, as defined by the agonist [D-Ala^2,MePhe^{4}Gly-ol^5]enkephalin (DAMGO). We show that alkylation in the myenteric-plexus longitudinal muscle homogenates depends upon the incubation conditions, which may govern the presence of different affinity states of the μ-opioid receptor.

A preliminary account of this work has appeared (Franklin & Traynor, 1988).

Methods

Male Dunkin-Hartley guinea-pigs (400–500 g) (David Hall, Burton-on-Trent) were killed by cervical dislocation. Brains (without cerebellum) and segments of ileum were removed and placed in appropriate buffer solutions, either Tris-HCl (pH 7.4, 50 mM) or Krebs solution containing (mM): NaCl 118, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.6, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25 and glucose 11 without (Krebs) or with 25 mM HEPES (pH 7.4; Krebs-HEPES).

Binding assays

Homogenates of tissues, either strips of myenteric plexus longitudinal muscle from the ileum or whole brain (without cerebellum), were prepared in Tris buffer (pH 7.4, 50 mM), or Krebs-HEPES, essentially as described by Kosterlitz and colleagues (Gillan *et al.*, 1980; Corbett *et al.*, 1985). Homogenates (20 mg tissue ml^{-1}) were incubated with or without β-FNA (1 μM unless otherwise stated) for 60 min at 37°C in Tris-buffer or Tris-buffer containing NaCl (100 mM) and the guanosine triphosphate (GTP) analogue Gpp(NH)p (50 μM) or Krebs-HEPES. Both control and treated homogenates were washed four times in Tris-HCl buffer and finally resuspended to give a final tissue concentration of 20 mg ml^{-1}.

In a second set of experiments 2 cm strips of whole myenteric plexus longitudinal muscle preparation were treated with β-FNA (1 μM) for 60 min at 37°C in Krebs-HEPES or Tris-HCl buffer. The tissues were then washed for 60 min, homogenized, and resuspended in Tris-buffer at a tissue concentration of 20 mg ml^{-1}.

Binding assays on the above homogenates were performed; [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]-DAMGO) was used to label μ-sites, at 25°C for 40 min in Tris-HCl buffer (pH 7.4 50 mM) (Handa *et al.*, 1981). Specific binding was defined by use of 10 μM naloxone. To label μ-sites [^{3}H]-DAMGO (1 nM) was used in guinea-pig brain homogenates and [^{3}H]-DAMGO (4 nM) used in homogenates of myenteric plexus-longitudinal muscle. This higher concentration was employed to provide optimum specific binding and also because the affinity of [^{3}H]-DAMGO for μ-opioid binding sites in the myenteric plexus is lower than in brain tissue (Corbett *et al.*, 1985). Assuming affinities of [^{3}H]-DAMGO

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for the μ-site in myenteric plexus of 2.27 nM (Corbett *et al.*, 1985) and for the δ- and κ-sites of 407 nM (Cotton *et al.*, 1985) and 4960 (Kosterlitz *et al.*, 1981) respectively and a μ : δ : κ binding site ratio of 25% : 26% : 49% (Corbett *et al.*, 1985), then 97% of the [^{3}H]-DAMGO specifically bound can be calculated to be to μ-sites. A similar calculation for binding in guinea-pig brain homogenates, using an affinity of [^{3}H]-DAMGO for the μ-opioid site of 0.96 nM and a binding site ratio of μ (24): δ (32): κ (44) (Robson *et al.*, 1985), indicates that >99% of the specifically bound [^{3}H]-DAMGO is to μ-sites. IC_{50} values for the displacement of [^{3}H]-DAMGO were determined from Hill Plots of the data and converted to K_i values by the Cheng & Prussoff (1973) equation. Equilibrium dissociation constants (K_D) and binding capacities (B_{max}) were determined from Scatchard (1949) plots of binding isotherms using the programme EBDA (McPherson, 1985).

Bioassays

The myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum was set up in Krebs solution for field stimulation as previously described (Traynor *et al.*, 1987). Tissues were pre-incubated with β-FNA (100 nM) for 60 min and then washed with drug-free Krebs solution for 60 min. The potencies of agonists were obtained from cumulative dose-response curves and dose-ratios were calculated as IC_{50} after β-FNA/IC_{50} before β-FNA.

The equilibrium dissociation constant (K_e) of the κ-antagonist, norbinaltorphimine, was determined against morphine by the single-dose method (Kosterlitz & Watt, 1968). Norbinaltorphimine (0.3 nM) was allowed to equilibrate for 45 min.

Chemicals and drugs

[^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]-DAMGO) (60 Ci mmol^{-1}) was purchased from Amersham International plc and cold DAMGO from Sigma. The drugs used were gifts, as follows: fentanyl citrate (Janssen), methadone hydrochloride (Wellcome), β-funaltrexamine (β-FNA, Glaxo), morphine hydrochloride (Macfarlan Smith), naloxone hydrochloride (Endo Laboratories) and U-50,488H (*trans*-3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)cyclohexyl)-benzeneacetamide, Upjohn Company). 5'-Guanylylimidodiphosphate (Gpp(NH)p) was purchased from Sigma. All other reagents and chemicals were of analytical grade.

Results

Binding of [^{3}H]-DAMGO to homogenates of guinea-pig brain pretreated with β-funaltrexamine

Pre-incubation of homogenates of guinea-pig brain for 60 min, at 37°C in Tris-HCl buffer with different concentrations of β-FNA led to a reduction of specific [^{3}H]-DAMGO binding (Figure 1). Approximately half of the specifically bound [^{3}H]-DAMGO was sensitive to 100 nM β-FNA. The remaining binding sites for [^{3}H]-DAMGO were more resistant and 31.5 ± 4.9% (n = 3) of the specific binding of [^{3}H]-DAMGO was retained, even after pretreatment with 1 μM β-FNA. Use of Krebs-HEPES, rather than Tris-HCl, during the pre-incubation of homogenates with β-FNA did not significantly increase the degree of inactivation afforded (Figure 1).

Analysis of saturation binding isotherms of [^{3}H]-DAMGO binding to both untreated and β-FNA-treated homogenates revealed a single population of saturable high affinity sites (Table 1). The maximum capacity of [^{3}H]-DAMGO binding sites (B_{max}) in the homogenates after pre-incubation with β-FNA (1 μM) for 60 min at 37°C was one-third of that observed in control homogenates. The equilibrium dissociation constant (K_D) of [^{3}H]-DAMGO for these binding sites was not significantly changed in treated homogenates compared with control homogenates.

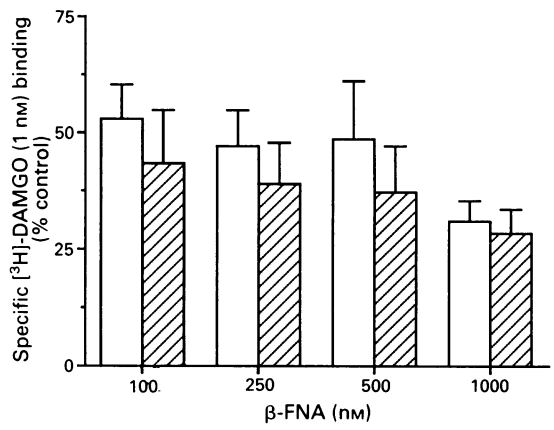

Figure 1 The effects of pre-incubation for 60 min at 37°C with different concentrations of β-funaltrexamine (β-FNA) in Tris-HCl (open columns) or Krebs-HEPES (hatched columns) on the specific binding of [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]-DAMGO, 1 nM) in homogenates of guinea-pig brain. Each column is the mean of three experiments. Vertical bars represent s.e.mean. All values are significantly different from controls treated in the absence of β-FNA ($P < 0.01$, Student's t test).

The competitive displacement of specific [^{3}H]-DAMGO (1 nM) binding by various μ-opioid ligands was similar in both control homogenates and homogenates pretreated with β-FNA (1 μM) for 60 min at 37°C (Table 2), even though specific binding in the latter homogenates was only 40% of that seen in control homogenates.

Table 1 The effects of pre-incubation with β-funaltrexamine (β-FNA) 1 μM for 60 min on the binding characteristics of [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin in homogenates of guinea-pig brain

Pre-incubation	*Binding capacity* (fmol mg^{-1} protein)	*Dissociation constant* (K_D, nM)
Control (Tris-HCl)	96.8 ± 26.8	4.5 ± 0.3
β-FNA	31.5 ± 6.4*	6.8 ± 0.7

The values are means ± s.e.mean from three experiments. Homogenates were incubated for 60 min in the absence or presence of 1 μM β-FNA then washed as described in the methods.
* Significantly different from control $P < 0.01$ (Student's t test).

Table 2 The effects of pre-incubation with β-funaltrexamine (β-FNA) 1 μM for 60 min on the inhibitory effects of opioids on the binding of [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]-DAMGO, 1 nM) in homogenates of guinea-pig brain

	K_i (nM)	
Competing ligand	*Control*	*After β-FNA*
DAMGO	2.1 ± 0.2	3.3 ± 0.5
Fentanyl	2.3 ± 0.4	2.5 ± 0.4
Morphine	6.3 ± 0.8	11.8 ± 2.5
Naloxone	2.7 ± 0.8	3.2 ± 1.3

The values are means ± s.e.mean from three experiments. Homogenates were incubated in the absence or presence of 1 μM β-FNA for 60 min, then washed as described in the methods.

Table 3 The effects of pre-incubation with β-funaltrexamine (β-FNA) 100 nM on the agonist potencies of opioids in the myenteric plexus-longitudinal muscle preparation of the guinea-pig

	Agonist potency (IC_{50}, nM)		
	Before β-FNA	*After β-FNA*	*Mean dose-ratio*
DAMGO	7.5 ± 0.7	359 ± 86.0*	39.7 ± 15.0
Methadone	23.6 ± 1.7	616 ± 58.7*	26.7 ± 3.4
Morphine	108 ± 16.4	577 ± 24.9*†	7.5 ± 0.7
U-50488H	1.1 ± 0.2	1.7 ± 0.4	1.6 ± 0.1

The values are means ± s.e.mean from three observations. Tissues were pre-incubated with 100 nM β-FNA for 60 min, then washed for 60 min with drug-free Krebs solution. Dose-ratio is the ratio of IC_{50} after incubation with β-FNA to the IC_{50} before treatment. † Based on a maximum inhibition of 50%.
* $P < 0.01$ compared to control values (Student's t test).

Agonist action of opioids following treatment of the myenteric plexus-longitudinal muscle with β-funaltrexamine

Following pre-incubation of strips of myenteric plexus-longitudinal muscle with β-FNA for 60 min the potency of the μ-agonists DAMGO, methadone and morphine were markedly attenuated with the shift for DAMGO and methadone being much greater than for morphine. In addition, morphine acted as a partial agonist following β-FNA treatment of the tissue and maximally only 50% inhibition of the electrically-induced contractions was observed. The potency of the κ-agonist U-50488H was unaffected (Table 3).

The agonist action of morphine before β-FNA treatment of the myenteric plexus was antagonized by the κ-antagonist norbinaltorphimine affording a K_e value of 2.04 ± 0.48 nM ($n = 3$). Following β-FNA treatment this shifted to a value of 0.10 ± 0.01 nM ($n = 3$) similar to the K_e for norbinaltorphimine of 0.07 ± 0.01 nM ($n = 3$) determined against the selective κ-agonist U-50488H.

Binding of [^{3}H]-DAMGO to homogenates of myenteric-plexus longitudinal muscle of the guinea-pig ileum following pre-incubation with β-funaltrexamine

In contrast to brain tissue, pre-incubation of homogenates of myenteric-plexus longitudinal muscle, even with an increased concentration (1 μM) of β-FNA for 60 min at 37°C in Tris-HCl buffer, caused no reduction in the level of specifically bound [^{3}H]-DAMGO (4 nM). Values of 7.1 ± 0.7 and 8.8 ± 1.4 fmol mg^{-1} protein before and after treatment respectively were obtained. On the other hand the level of specifically bound [^{3}H]-DAMGO (4 nM) in homogenates treated with 1 μM β-FNA in Tris-buffer containing NaCl (100 mM) and Gpp(NH)p (50 μM) was 3.9 ± 0.9 fmol mg^{-1} protein. This represents a reduction of 56% when compared with the control value of 7.9 ± 1.9 fmol mg^{-1} protein (Figure 2).

A similar loss in the level of specific [^{3}H]-DAMGO (4 nM) binding from 9.7 ± 2.2 to 4.4 ± 1.5 fmol mg^{-1} was seen when whole strips of myenteric plexus-longitudinal muscle, in Krebs-HEPES, were incubated with β-FNA (1 μM) prior to homogenization. (Figure 2). The same effect was seen when the pre-incubation was carried out in Tris-HCl buffer, although the amount of specifically bound tritiated ligand was reduced affording values of 4.5 ± 0.5 and 2.5 ± 0.4 fmol mg^{-1} protein ($n = 3$) at 4 nM [^{3}H]-DAMGO in control and β-FNA treated homogenates respectively, presumably due to damage to the tissue as a result of incubation in the very non-physiological medium.

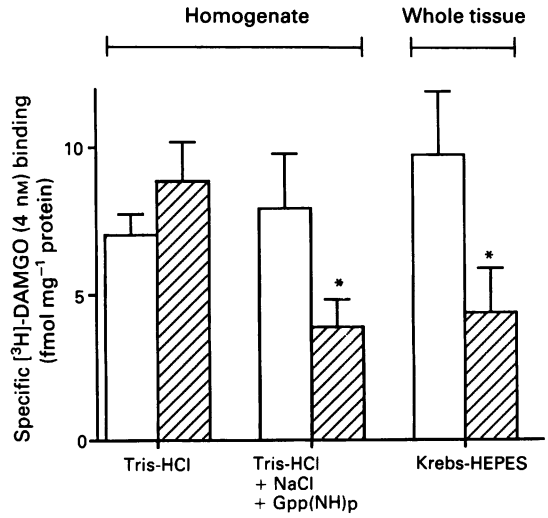

Figure 2 The effects of pre-incubation of guinea-pig myenteric plexus longitudinal muscle without (open columns) or with (hatched columns) 1 μM β-funaltrexamine (β-FNA) for 60 min, on the specific binding of [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]-DAMGO, 4 nM). Homogenates of myenteric plexus longitudinal muscle were treated with β-FNA in Tris-HCl (pH 7.4, 50 mM) or in Tris-HCl in the presence of NaCl (100 mM) and Gpp(NH)p (50 μM), then washed as described in the methods. For whole tissue studies 2 cm strips of myenteric plexus-longitudinal muscle were treated with β-FNA as above but in Krebs-HEPES, washed and homogenates prepared as described in the methods. Each column is the mean of three experiments. Vertical bars represent s.e.mean. * $P < 0.01$ vs controls in the absence of β-FNA (Student's t test).

Discussion

The results demonstrate that the binding of [^{3}H]-DAMGO to the μ-opioid binding site in homogenates of myenteric plexus can be irreversibly blocked by pretreatment with β-FNA. The loss of μ-agonist potency of DAMGO, methadone and morphine in the myenteric plexus, following β-FNA treatment is therefore a result of a reduction in the number of functional μ-receptors. The myenteric plexus contains both μ- and κ-receptors (Chavkin & Goldstein, 1981). The results with the κ-antagonist norbinaltorphimine suggest that the lesser shift seen with the alkaloid morphine is due to an action of this compound at κ-receptors, following β-FNA treatment, as a result of its lesser selectivity (Magnan *et al.*, 1982) and efficacy (Smith & Rance, 1983) compared with DAMGO. This finding is in agreement with the results of Ward *et al.* (1986) who obtained a significant increase in the K_e value for naloxone, measured against morphine, but not when measured against the μ-selective peptide RX 78-3030, following β-FNA treatment. Also the reduced response obtained with morphine suggests that at the κ-receptor in the myenteric plexus this ligand acts as a partial agonist.

However, the alkylation by β-FNA of μ-receptors in the plexus differs from that observed in brain homogenates. In the latter homogenates [^{3}H]-DAMGO binding is reduced if pretreatment with β-FNA is carried out in Tris buffer. This reduction in binding is principally due to a reduction in the maximal binding capacity, as observed by others (Rothman *et al.*, 1983; Ward *et al.*, 1985; Tam & Liu-Chen, 1986). Indeed, the results obtained agree with the findings of Tam & Liu-Chen (1986) and those of Ward *et al.* (1985) that irreversible binding of β-FNA occurs in Tris-buffer. However, these groups do report enhancement of alkylation in the presence of Na^+ ions or Krebs-Ringer, whilst in the present study the difference between alkylation in Tris-buffer and Krebs-HEPES did not reach significance. The reasons for this discrepancy are unknown, but could for example relate to the composition of Krebs solution used, since ions are known to have marked effects on binding properties at opioid receptors (Paterson *et al.*, 1986; Kosterlitz *et al.*, 1987). In contrast to results with

brain tissue, [^{3}H]-DAMGO binding in homogenates of myenteric-plexus longitudinal muscle is sensitive to β-FNA only when Na^+ ions and Gpp(NH)p are present, or the alkylation is performed in whole tissue.

In the myenteric plexus therefore the findings suggest that homogenization in Tris buffer alters the μ-opioid binding site to a form different from that found in intact tissue. The μ-opioid receptor is a G-protein coupled receptor (for reviews see Gilman, 1987; Milligan, 1988). Homogenization in Tris buffers generates a tightly coupled high agonist affinity opioid receptor (Wong *et al.*, 1989). Addition of Na^+ ions and Gpp(NH)p restores the receptor to a form susceptible to alkylation by β-FNA and therefore presumably similar to the form present in intact tissue. Several different agonist affinity states of opioid receptors are believed to exist in equilibrium (Werling *et al.*, 1988) and Na^+ ions and guanine nucleotides are known to shift the opioid receptor to a conformational state which is recognised by agonists with low affinity. However antagonist affinity is not reduced (Pert & Snyder, 1974; Simon *et al.*, 1975; Blume, 1978). The results suggest that a low agonist affinity conformation of the μ-opioid receptor represents a predominant state present in intact myenteric plexus. This would explain why opioid antagonists are better able to protect against β-FNA alkylation in whole tissue than opioid agonists and also perhaps why the agonist equivalent of β-FNA, namely β-fluoxymorphamine, does not act irreversibly (Portoghese & Takemori, 1983). Furthermore the results support the suggestion (Carroll *et al.*, 1988) that the low agonist affinity form represents the physiologically relevant state of the μ-opioid receptor.

The different alkylation observed in brain and myenteric plexus must mean that the μ-opioid receptors in the two tissues differ. Certainly it would appear that the high affinity μ-opioid site in guinea-pig brain, that is the binding of [^{3}H]-DAMGO observed in Tris-buffer, is susceptible to alkylation by β-FNA, or that during incubation in the presence of β-FNA the binding site converts to a lower affinity form susceptible to alkylation, whilst high affinity μ-binding in myenteric plexus under these conditions is not susceptible to alkylation. Additionally, the affinity of [^{3}H]-DAMGO measured in brain and myenteric plexus differs slightly (Corbett *et al.*, 1985) and Takemori and colleagues (1974) have suggested a difference between central and peripheral μ-receptors may exist based on responses with an anileridine affinity label. The observed different interactions of β-FNA in brain and myenteric plexus homogenates may reside in the binding site, the efficiency of coupling between receptor and G-protein, the involvement of different G-proteins or the presence of alternative coupling systems.

Any discussion of β-FNA alkylation must consider those receptors, representing some 30% of the μ-binding site population labelled under the experimental conditions, which are resistant to β-FNA. Such β-FNA-insensitive sites have been observed previously (Rothman *et al.*, 1983; 1987). In the competition experiments described in this paper, μ-receptor ligands do not differentiate β-FNA sensitive and insensitive μ-sites. The insensitive sites would thus appear to be a form of the μ-receptor, although it must be realised that the binding studies were performed in Tris buffer which generates a high agonist affinity state. It is interesting to note that 30% of μ-receptors solubilised from 7315c cells are still sensitive to GTP after 18 h treatment of the cells with pertussis toxin (Frey *et al.*, 1989), which may indicate that part of the G-protein associated with the μ-receptor is in some way protected from ADP-ribosylation by the toxin. It is possible then, as discussed by Corbett and colleagues (1985), that it is the coupling system that interacts irreversibly with β-FNA rather than ligand binding domain and that in myenteric plexus, but not brain, this system is only sensitive in the presence of Na^+ ions and guanine nucleotide. Alkylation of G-protein by agents such as N-ethylmaleimide and pertussis toxin generates a low agonist affinity state of the receptor (Smith & Harden, 1984; Spain & Coscia, 1987; Werling *et al.*, 1988). If this is the case with β-FNA alkylation, then the observed reduction in B_{max} could be due to a large reduction in affinity of the [^{3}H]-DAMGO for the μ-opioid receptor, such that binding cannot be measured in a conventional ligand-binding assay, rather than a direct loss of sites due to alkylation of the ligand binding domain by β-FNA.

In conclusion the data presented show that the observed reduction in μ-ligand potency following β-FNA treatment of myenteric-plexus is due to alkylation of a functional μ-receptor. The susceptible form of the receptor is probably a low agonist affinity state, which predominates under physiological conditions. Furthermore β-FNA does uncover differences between μ-opioid receptor sites in brain and myenteric plexus which may suggest differential coupling systems in the two tissues.

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References

BLUME, A.J. (1978). Interaction of ligands with the opiate receptor of brain membranes: regulation by ions and nucleotides. *Proc. Natl. Acad. Sci., U.S.A.*, **75**, 1713–1717.

CARROLL, J.A., SHAW, J.S. & WICKENDEN, A.D. (1988). The physiological relevance of low agonist affinity binding at opioid μ-receptors. *Br. J. Pharmacol.*, **94**, 625–631.

CHAVKIN, C. & GOLDSTEIN, A. (1981). Specific receptor for the opioid peptide dynorphin: structure activity relationships. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 6543–6547.

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymic reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.

CORBETT, A.D., KOSTERLITZ, H.W., McKNIGHT, A.T., PATERSON, S.J. & ROBSON, L.E. (1985). Pre-incubation of guinea-pig myenteric plexus with β-funaltrexamine: discrepancy between binding assays and bioassays. *Br. J. Pharmacol.*, **85**, 665–673.

COTTON, R., KOSTERLITZ, M.W., PATERSON, S.J., RANCE, M.J. & TRAYNOR, J.R. (1985). The use of [^{3}H]-[D-Pen^2, D-Pen^5] enkephalin as a highly selective ligand for the δ-binding site. *Br. J. Pharmacol.*, **84**, 927–932.

FRANKLIN, T.G. & TRAYNOR, J.R. (1988). Action of β-funaltrexamine at μ-opioid sites in guinea-pig ileum. *Br. J. Pharmacol.*, **95**, 861P.

FREY, E.A., GOSSE, M.E. & COTE, T.E. (1989). Reconstitution of the solubilised μ-opioid receptor coupled to a GTP-binding protein. *Eur. J. Pharmacol.*, **172**, 347–356.

GILLAN, M.C.G., KOSTERLITZ, H.W. & PATERSON, S.J. (1980). Comparison of the binding characteristics of tritiated opiates and opioid peptides. *Br. J. Pharmacol.*, **70**, 481–490.

GILMAN, A.G. (1987). G. Proteins: Transducers of receptor-generated signals. *Annu. Rev. Biochem.*, **56**, 615–649.

HANDA, B.K., LANE, A.C., LORD, J.R., MORGAN, B.A., RANCE, M.J. & SMITH, C.F.C. (1981). Analogues of β-LPH possessing selective agonist activity at μ-opiate receptors. *Eur. J. Pharmacol.*, **70**, 531–540.

HAYES, A.G., SHEEHAN, M.J. & TYERS, M.B. (1985). Determination of the receptor selectivity of opioid agonists in the guinea-pig ileum and mouse vas deferens by use of β-funaltrexamine. *Br. J. Pharmacol.*, **86**, 899–904.

HAYES, A.G., SKINGLE, M. & TYERS, M.B. (1986). Reversal by β-funaltrexamine of the antinoiceptive effect of opioid agonists in the rat. *Br. J. Pharmacol.*, **88**, 867–872.

KOSTERLITZ, H.W., PATERSON, S.J. & ROBSON, L.E. (1981). Characterisation of the κ-subtype of the opiate receptor in the guinea-pig brain. *Br. J. Pharmacol.*, **73**, 939–949.

KOSTERLITZ, H.W., PATERSON, S.J., ROBSON, L.E. & TRAYNOR, J.R. (1987). Effects of cations on binding, in membrane suspensions, of various opioids at μ-sites of rabbit cerebellum and κ-sites of guinea-pig cerebellum. *Br. J. Pharmacol.*, **91**, 431–437.

KOSTERLITZ, H.W. & WATT, A.J. (1968). Kinetic parameters of narcotic agonists and antagonists with particular reference to N-allylnoroxymorphone (naloxone). *Br. J. Pharmacol.*, **33**, 266–270.

MAGNAN, J., PATERSON, S.J., TAVANI, A. & KOSTERLITZ, H.W. (1982). The binding of narcotic analgesic drugs with different agonist and antagonist properties. *Naunyn-Schmeidebergs Arch. Pharmacol.*, **319**, 197–205.

McPHERSON, G.A. (1985). Analysis of radioligand binding experiments: a collection of computer programs for the IBM PC. *J. Pharmacol. Methods*, **14**, 213–218.

MILLIGAN, G. (1988). Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. *Biochem. J.*, **225**, 1–13.

PATERSON, S.J., ROBSON, L.E. & KOSTERLITZ, H.W. (1986). Control by cations of opioid binding in guinea-pig brain membranes. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 6216–6220.

PERT, C.B. & SNYDER, S.H. (1974). Opiate receptor binding of agonists and antagonists affected differentially by sodium. *Mol. Pharmacol.*, **10**, 868–879.

PORTOGHESE, P.S., LARSEN, D.L., SAYER, L.M., FRIES, D.S. & TAKEMORI, A.E. (1980). A novel opioid receptor site directed alkylating agent with irreversible narcotic antagonistic and reversible agonistic activities. *J. Med. Chem.*, **23**, 233–234.

PORTOGHESE, P.S. & TAKEMORI, A.W. (1983). Different receptor sites mediate opioid agonism and antagonism. *J. Med. Chem.*, **26**, 1341–1343.

ROBSON, L.E., GILLAN, M.G.C. & KOSTERLITZ, H.W. (1985). Species differences in the concentration and distributions of opioid binding sites. *Eur. J. Pharmacol.*, **112**, 65–71.

ROTHMAN, R.B., BOWMAN, W.D., SCHUMACHER, U.K. & PERT, C.B. (1983). The effect of β-FNA on opiate receptor binding: preliminary evidence for two types of μ-receptors. *Eur. J. Pharmacol.*, **95**, 147–148.

ROTHMAN, R.B., JACOBSEN, A.E., RICE, K. & HERKENHAM, M. (1987). Autoradiographic evidence for two classes of mu-opioid binding sites in rat brain using ^{125}I FK33824. *Peptides*, **8**, 1015–1021.

SCATCHARD, G. (1949). The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.*, **51**, 660–672.

SIMON, E.J., MILLER, J.M., GROTH, J. & EDELMAN, I. (1975). Further properties of stereospecific opiate binding sites in rat brain: on the nature of the sodium effect. *J. Pharmacol. Exp. Ther.*, **192**, 531–537.

SMITH, C.F.C. & RANCE, M.J. (1983). Opiate receptors in rat vas deferens. *Life Sci.*, **33**, (Suppl. 1), 327–330.

SMITH, M.M. & HARDEN, T.K. (1984). Modification of receptor-mediated inhibition of adenylate cyclase in NG108-15 neuroblastoma × glioma cells by N-ethylmaleimide. *J. Pharmacol. Exp. Ther.*, **228**, 425–433.

SPAIN, J.W. & COSCIA, C.J. (1987). Multiple interconvertible affinity states for the δ-opioid agonist-receptor complex, *J. Biol. Chem.*, **262**, 8948–8951.

TAKEMORI, A.E. & PORTOGHESE, P.S. (1987). Evidence for the interaction of morphine with kappa and delta receptors to induce analgesia in β-funaltrexamine-treated mice. *J. Pharmacol. Exp. Ther.*, **243**, 91–94.

TAKEMORI, A.E., WARD, A., PORTOGHESE, P.S. & TELANG, V.G. (1974). Potential non-equilibrium analgetic receptor inactivators. Further pharmacologic studies of N-acylanileridines. *J. Med. Chem.*, **17**, 1051–1054.

TAM, S.W. & LIU-CHEN, L.Y. (1986). Reversible and irreversible binding of β-funaltrexamine to mu, delta and kappa opioid receptors in guinea-pig brain membranes. *J. Pharmacol. Exp. Ther.*, **239**, 351–357.

TRAYNOR, J.R., CORBETT, A.D. & KOSTERLITZ, H.W. (1987). Diprenorphine has agonist activity at opioid κ-receptors in the myenteric plexus of the guinea-pig ileum. *Eur. J. Pharmacol.*, **137**, 85–89.

WARD, S.J., FRIES, D.S., LARSEN, D.L., PORTOGHESE, P.S. & TAKEMORI, A.E. (1985). Opioid receptor binding characteristics of the non equilibrium μ-antagonist, β-funaltrexamine (β-FNA). *Eur. J. Pharmacol.*, **107**, 323–330.

WARD, S.J., LOPRESTI, D. & JAMES, D.W. (1986). Activity of mu- and delta-selective opioid agonists in the guinea-pig ileum preparation: differentiation into peptide and nonpeptide classes with β-funaltrexamine. *J. Pharmacol. Exp. Ther.*, **238**, 625–631.

WARD, S.J., PORTOGHESE, P.S. & TAKEMORI, A.E. (1982a). Pharmacological profiles of β-funaltrexamine (β-FNA) and β-chlornaltrexamine (β-CNA) on the mouse vas deferens preparation. *Eur. J. Pharmacol.*, **80**, 377–384.

WARD, S.J., PORTOGHESE, P.S. & TAKEMORI, A.E. (1982b). Pharmacological characterisation *in vivo* of the novel opiate β-funaltrexamine. *J. Pharmacol. Exp. Ther.*, **220**, 494–498.

WERLING, L.L., PUTTFARCKEN, P.S. & COX, B.M. (1988). Multiple agonist-affinity states of opioid receptors: regulation of binding by guanyl nucleotides in guinea-pig cortical, NG108-15, and 7315c cell membranes. *Mol. Pharmacol.*, **33**, 423–431.

WONG, Y.H., DEMOLIOU-MASON, C.D. & BARNARD, E.A. (1989). Opioid receptors in magnesium-digitonin-solubilised rat brain membranes are tightly coupled to a pertussin toxin-sensitive guanine nucleotide binding protein. *J. Neurochem.*, **52**, 999–1009.

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Dissociation of a ferric maltol complex and its subsequent metabolism during absorption across the small intestine of the rat

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1 The fate and disposition of [^{59}Fe]-ferric [^{3}H]-maltol after intravenous administration were investigated in anaesthetized rats. Immediate dissociation of ferric iron from maltol took place in the circulation even with high doses of ferric maltol (containing 1 mg elemental iron). In plasma samples withdrawn within 1 min of injection and subjected to gel filtration, ^{59}Fe eluted with the high molecular weight proteins whilst the tritium was associated with low molecular weight material.

2 The rates of elimination of ^{59}Fe and of tritium from the plasma and their ultimate fate were very different. The half life for ^{59}Fe in the plasma was around 70 min and ^{59}Fe appeared mainly in the bone marrow and liver. There was an initial rapid exit of tritium from the plasma with a half life of around 12 min. This was followed either by a plateau or by a rise in tritium levels, involving entry of maltol metabolites into the circulation. These metabolites could be recovered in the urine.

3 Entry of ^{59}Fe and of tritium into the blood plasma after intraduodenal administration of [^{59}Fe]-ferric [^{3}H]-maltol was also very different. At low doses of ferric maltol (containing 100 μg elemental iron), the tritium appeared in the plasma in highest amounts within seconds and then decreased whilst there was a slow rise in ^{59}Fe levels. At higher doses of ferric maltol (containing 7 mg elemental iron), levels of ^{59}Fe in the plasma were highest at 5 min and then fell whereas tritium levels rose steadily. Mucosal processing of ^{59}Fe prevented further entry of iron at high dose into the circulation.

4 Initial rates of uptake of [^{3}H]-maltol into isolated intestinal fragments were measured over a range of concentrations and revealed that maltol alone could diffuse freely into the tissues whereas maltol complexed to iron showed saturable uptake kinetics similar to those seen with the iron itself.

5 After intestinal uptake, ^{59}Fe and tritium were associated with different subcellular fractions, maltol itself being metabolized to the glucuronide conjugate within the intestinal mucosa.

6 It is concluded that dissociation of metal and ligand takes place before entry into the intestinal mucosa. Iron is then taken up on the endogenous carrier and processed in the normal way whilst maltol enters by diffusion, its rate of entry being limited by the degree of dissociation. It is subsequently metabolized by conjugation and eliminated rapidly from the body in the urine.

Introduction

It is generally supposed that ferrous preparations are more effective in the oral treatment of iron deficiency than ferric compounds partly at least because of the low bioavailability of ferric iron (Dietzfelbinger, 1987). Ferrous preparations may cause irritation and damage to the mucosal lining, particularly in overdose (Nayfield *et al.*, 1976). Thus compounds that can hold ferric iron in absorbable form might be a therapeutic advantage. The hydroxypyrone, maltol, has a very high affinity for ferric iron (K_{aff} value of ferric iron for maltol of log $\beta 3 = 28$) and appears able to hold the metal in soluble form within the pH range which would be encountered in the duodenal lumen i.e., up to 8 (Hungerford & Linder, 1983; Hider *et al.*, 1984). Yet the ferric maltol complex is kinetically labile and will readily donate its iron to transferrin (half life of transfer to apotransferrin of 2 min (M.E. Bakaj, personal communication). It has already been shown in rats that substantial absorption of iron can occur after oral treatment with ferric maltol (Barrand *et al.*, 1987).

Since the intestinal mucosa provides the only means of controlling body iron status, it is important to ensure that the mechanism by which iron from ferric maltol crosses the intestinal wall does not involve by-passing the normal regulatory system, thereby leading to iron overload. It was originally supposed that the uncharged ferric maltol complex (structure given in Barrand *et al.*, 1987) on account of its relatively high oil/water partition coefficient (0.5; Bakaj, 1984), could diffuse across the intestinal mucosa and donate the ferric ions directly to transferrin in the blood. Recent studies have indicated however, that the iron taken up from ferric maltol probably passes to the normal control pathways within the intestinal wall (Barrand & Callingham, 1991). Furthermore, it appears that reduction of ferric iron to the ferrous form can occur within the intestinal lumen and may well precede any uptake (Barrand *et al.*, 1990). If this is so, then metal and ligand must dissociate before iron absorption, since maltol has a very low affinity for the ferrous form of iron.

The present work extends these previous studies by following concomitantly the passage of metal and ligand across the intestinal wall and by investigating the fate and metabolism of the ligand itself. Further evidence that dissociation takes place before entry is provided indicating that the ferric maltol complex itself never exists beyond the lumen of the gut. Preliminary results of this work have been reported to the British Pharmacological Society (Gee *et al.*, 1987).

Methods

Absorption and pharmacokinetic studies in whole animals

Male Wistar rats, 150–400 g body weight, were fasted for 18 h before use. For pharmacokinetic studies, rats were anaesthetized with sodium pentobarbitone (Sagatal, Rhône Poulenc, Dagenham, Essex; 60 mg kg^{-1}, i.p.) and cannulae

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inserted into the jugular vein and carotid artery. Heparin (500 i.u.), further anaesthetic as required and the iron solutions were introduced via the jugular venous cannula. Blood was sampled from the carotid arterial cannula at suitable time intervals, care being taken to ensure that total blood loss was kept to a minimum, i.e., no more than 10% of the blood volume. The volume of blood removed in each sample was replaced by an equivalent volume of physiological saline. In studies where ferric maltol solutions were given into the duodenum the jugular vein was not cannulated and heparin and further anaesthetic were administered i.p. The animals remained under anaesthesia throughout the experiments and were killed by overdose at the end. Samples of liver together with the kidneys, femurs and urine were taken for analysis of their ^{59}Fe content in a Packard Auto Gamma 500 counter. The blood samples taken during the experiments were centrifuged at 6000 g for 5 min to remove the cells and the plasma analysed for ^{59}Fe and tritium content by liquid scintillation β-particle spectrometry with automatic external standardization to correct for quenching and to allow conversion to d.p.m. for each radioisotope. The efficiency of counting for tritium was around 30% whilst that for ^{59}Fe almost 100%.

Gel filtration

Plasma samples, up to 1 ml in volume, were passed down 10 ml columns (PD-10) of Sephadex G-25 (Pharmacia, Uppsala, Sweden) previously equilibrated with 20 mM HEPES buffer at pH 7.4 and eluted in 1 ml fractions. The supernatants obtained after centrifugation of intestinal homogenates (see below) were subjected to gel filtration in a similar manner. Recovery of tritium from the columns was around 96% to 98% of that applied whilst that of ^{59}Fe was 80% to 90%. Some dissociation of iron from maltol occurred on mixing the ferric maltol complex *in vitro* with either plasma or intestinal homogenates due to the presence of transferrin in these samples. Ferric iron has a higher affinity for transferrin than for maltol and rapidly transfers from the ligand to the protein (Barrand *et al.*, 1987).

Thin layer chromatography

Samples of the gel fractions were concentrated by freeze-drying and spotted in 100 μl volumes on to cellulose thin layer plates together with 2 μl samples of unlabelled maltol (10 mg ml^{-1}) and run to a distance of 12 cm (1 to 2 h duration) at room temperature in the following solvent system: methyl ethyl ketone:acetone:water:formic acid (80:6:12:2 by volume). The unlabelled maltol spots could be visualized under u.v. light at 254 nm. The plates were divided into 1 cm lengths and the tritium content of the cellulose analysed after dissolution in scintillation fluid. Recovery of tritium was generally over 95% of that applied to the plates.

Uptake into isolated intestinal fragments

Uptake of ^{59}Fe and of tritium into isolated fragments of small intestine was carried out as described previously (Levey *et al.*, 1988). Intestinal fragments, 30 to 50 mg in weight, were removed immediately from rats, which had been killed by cervical dislocation, and immersed in buffer containing (mM): HEPES 16, NaCl 125, KCl 3.5, $CaCl_2$ 1, $MgSO_4$ 10 and D-glucose 10 at pH 7.4. After incubation for 10 min at 37°C with the appropriate ^{59}Fe and tritium solutions (see above), fragments were removed, digested in Soluene-350 (Packard, Reading) and analysed for their ^{59}Fe and/or tritium content. Where uptake of tritium alone was being monitored, [^{14}C]-mannitol (0.001 μCi ml^{-1}) was included in the incubation mixture as an extracellular fluid marker. Calculations of ^{59}Fe, ^{3}H or their simultaneous accumulation were corrected for wet weight of each tissue fragment and for extracellular fluid volume. Over the course of 10 min, both ^{59}Fe and tritium accumulation increased in a linear fashion so it was possible to calculate initial rates of uptake from the data.

Maltol metabolite studies

Isolated intestinal fragments, 2 to 3 cm in length, were incubated for 10 to 20 min with appropriate ^{59}Fe and tritium solutions, the mucosa then scraped off with a glass slide and homogenized in 20 mM HEPES buffer at pH 7.4. After centrifugation for 5 min at 600 g to remove unbroken cells, the homogenate was centrifuged at 100,000 g for 1 h. Samples of pellet and supernatant were analysed for ^{59}Fe and tritium content by liquid scintillation counting. Supernatant samples were also subjected to gel filtration (see above). In studies to investigate the nature of metabolites formed, mucosal scrapings from isolated intestinal fragments, previously incubated for 30 min in 0.1 mM [^{3}H]-maltol, were homogenized in 80% ethanol. The precipitated proteins were centrifuged into a pellet and excess ethanol was removed from the supernatant under a jet of N_2. Supernatant samples were subjected to gel filtration and the tritium-rich fractions concentrated by freeze-drying before incubation for 1 h with 10–20 units of β-glucuronidase in Na acetate buffer at pH 5. It was found from preliminary experiments in which the incubation time was varied that incubation for at least 1 h was necessary for conversion of 75%–100% of the tritiated metabolite. After incubation, the pH was readjusted to 7 and 100 μl samples spotted on to thin layer plates to undergo separation as described above. In studies to determine the effect of maltol concentration on conjugation, uncentrifuged homogenates were adjusted to pH 6 by addition of 0.05 M HCl and were washed 3 times with equal volumes of chloroform to remove unchanged [^{3}H]-maltol before being analysed for tritiated metabolite content. Initial recovery experiments showed that, with this extraction technique, 96–100% maltol could be removed leaving 95–100% of the metabolite for radiochemical assay. Conjugation rates can be much modified by isolation conditions and it is probable that tissue systems more closely mimic the *in vivo* situation than subcellular fractions (Koster & Noordhoek, 1983; Koster, 1985).

Preparation of [^{3}H]-maltol

Preparation of [^{3}H]-methylfurfurylalcohol Methyl iodide (3.63 g) was dissolved in 75 ml sodium-dried ether, chilled in ice and mixed with [^{3}H]-methyl iodide (Amersham, 25 mCi; 101 mCi mg^{-1}). The specific radioactivity of the methyl iodide used in the first stage of the synthesis was therefore 1 Ci mol^{-1}. The ethereal methyl iodide solution was poured into an addition funnel attached to a 250 ml 3-necked round-bottomed flask containing 0.77 g Mg turnings. After running approximately half of the methyl iodide solution on to the magnesium, a crystal of iodine was placed in the flask, to initiate formation of the Grignard reagent. Over a period of a few min the yellow iodine colour disappeared, being replaced by the white 'cloudiness' of the Grignard reagent. At this stage, stirring was begun and the mixture heated until the ether refluxed. Stirring was continued until the reaction subsided. The remaining methyl iodide solution was added dropwise over a 5 min period with continued stirring. The mixture was refluxed for a further 15 min in order to ensure maximum Grignard preparation. On cooling a cloudy white solution resulted with little magnesium being visible.

Furfural (2.49 g) was dissolved in 25 ml sodium-dried ether, poured into a second addition funnel and added dropwise to the Grignard reagent over a period of 10 min with constant stirring. On completion of this addition the mixture was refluxed for 15 min to ensure completion of the reaction.

After cooling, 75 ml of ice/water was added to the reaction flask and the mixture shaken until all the resulting solid dissolved. The ethereal/aqueous mixture was decanted into a separating funnel and thoroughly mixed with 100 ml saturated NaCl together with 100 ml 1 M sodium metabisulphite solu-

tion. The aqueous layer was washed with dichloromethane (2 × 200 ml), and the organic fractions were combined for final washing with 1 M sodium metabisulphite (2 × 200 ml), dried over anhydrous sodium sulphate, and evaporated *in vacuo* to give [^{3}H]-methylfurfuryl alcohol as an oil, 1.9 g, 66% yield.

Preparation of [^{3}H]-maltol A mixture of methanol (4 ml) and water (6 ml) contained in a 250 ml 3-necked round-bottomed flask was chilled to −10°C by external application of dry ice/acetone. Two additional funnels attached to the flask contained [^{3}H]-methylfurfuryl alcohol (1.9 g) dissolved in methanol (8 ml)/water (4 ml) and bromine (5.7 g) respectively. The alcohol and bromine were added simultaneously in a rapid dropwise fashion, maintaining the temperature of about −10°C, such that all the alcohol was added when 1/3 of the bromine remained. Stirring was maintained throughout the additions, the final portion of bromine being added slowly at −10°C.

After replacing one of the additional funnels with a sidearm and condenser, the flask was heated to 80°C and an 8 ml portion of methanol was distilled from the reaction mixture. The sidearm was then rapidly replaced by a double condenser and the mixture was refluxed at 90–95°C for 3.5 h.

The brown mixture was cooled, decanted into a beaker and adjusted to pH 8 by the addition of 2 M NaOH. After reducing the volume of the resulting solution to approximately 5 ml by rotary evaporation, it was extracted with dichloromethane (4 × 30 ml). The combined organic layers were dried over anhydrous Na_2SO_4 filtered and evaporated *in vacuo* to give [^{3}H]-maltol, 0.75 g, 35% yield. The crude product was recrystallized from absolute ethanol. The purity of each batch of [^{3}H]-maltol was tested alongside unlabelled maltol on thin layer silica gel plates with methanol:chloroform (10:1 by volume) as the solvent system.

Materials

$^{59}FeCl_3$ and [^{14}C]-mannitol were purchased from Amersham International (Bucks). Unlabelled maltol (Veltol) was obtained from Pfizer Ltd (Sandwich, Kent). All other chemicals either were bought from Sigma Chemical Co Ltd (Poole, Dorset) or were standard laboratory reagents of analytical grade. For the *in vitro* uptake experiments, non-radioactive stock solutions of ferric maltol were prepared by mixing $FeCl_3$ with maltol in HEPES buffer to a metal:ligand ratio of 1:4. It is known from *in vitro* studies that on mixing ferric iron with maltol at neutral pH, there is immediate formation of an uncharged 1:3 iron-maltol complex (Stefanovic *et al.*, 1968). Radioactive stocks were prepared in a similar way, $^{59}FeCl_3$ and [^{3}H]-maltol being added so as to achieve final concentrations in the incubation media of 0.05–0.25 μCi ml^{-1} for ^{59}Fe and 0.1–0.5 μCi ml^{-1} for tritium. Where appropriate, [^{14}C]-mannitol was used at a final concentration of 0.01 μCi ml^{-1}. For the whole animal uptake studies, ferric maltol solutions were prepared just before use by mixing solid $FeCl_3$, $^{59}FeCl_3$[^{3}H]-maltol and maltol powder at a molar ratio of 1:4 in sufficient saline to provide 100 μg, 1 mg or 7 mg elemental iron, 5, 15 or 25 μCi of tritium and 1, 3 or 5 μCi of ^{59}Fe in each 500 μl dose.

Results

Pharmacokinetic studies after intravenous injection of [^{59}Fe]-ferric [^{3}H]-maltol

The elimination of ^{59}Fe and of tritium from the blood plasma after intravenous injection of [^{59}Fe]-ferric [^{3}H]-maltol at doses of 100 μg and of 1 mg elemental iron was followed by analysing samples of blood plasma taken at 10 to 20 min intervals for ^{59}Fe and tritium content. Elimination of ^{59}Fe appeared to obey single compartment first order kinetics with a half life of around 70 min (Figure 1); 60 min after injection of 100 μg of [^{59}Fe]-ferric maltol, the tissues with the highest

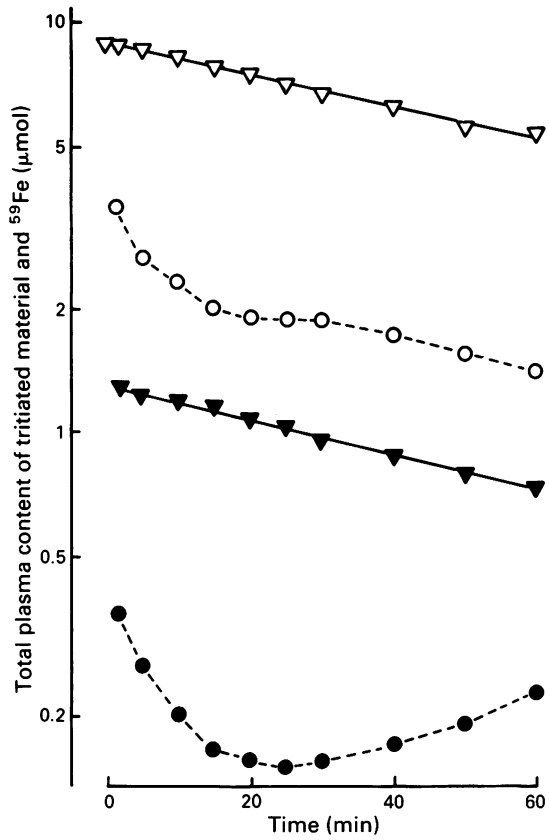

Figure 1 Changes in total plasma content of ^{59}Fe (▼, ▽) and tritium (●, ○) during 1 h following intravenous injections of [^{59}Fe]-ferric [^{3}H]-maltol at doses containing 100 μg (closed symbols) and 1 mg (open symbols) of elemental iron. Each value is the mean of data from 3 animals and is given as μmol of radioactive material in the total plasma.

content of ^{59}Fe were the bone marrow (11 ± 2% of administered dose, n = 4 rats) and liver (18 ± 1%). A small amount of ^{59}Fe was detected in the urine (2.6 ± 1%). The shapes of the elimination curves for tritium were much more complex. There seemed to be an initial phase with a half life of about 12 min which was followed in some cases by a pleateau and in some cases by a rise in tritium content. Tissue content of tritium was not measured but a substantial proportion of the dose injected was found in the urine after 60 min (30–40% at 100 μg, 5–10% at 1 mg).

Disposition of ^{59}Fe and tritium in the plasma after intravenous injection

Gel filtration of plasma samples taken 2 and 60 min after injection of [^{59}Fe]-ferric [^{3}H]-maltol at a dose containing 1 mg elemental iron showed that even within 2 min, metal and ligand no longer were present as the complex (Figure 2). ^{59}Fe eluted from the gel at the void volume which corresponded to high molecular weight proteins whereas tritium was retained within the gel. Partial dissociation of ferric maltol can also occur in plasma samples *in vitro*, presumably due to the ready donation of ferric iron to apotransferrin. In the plasma samples taken after 2 min, the tritium eluted with material corresponding to a molecular weight of maltol itself but in the 60 min sample, the tritium peak was shifted slightly to a position corresponding to material slightly larger than maltol. The tritium in the urine showed a similar elution profile. Identical results were obtained after injection of ferric maltol at the 100 μg dose.

The tritium containing fractions were analysed further by a thin layer chromatography system that separated the relatively lipophilic maltol from more hydrophilic compounds.

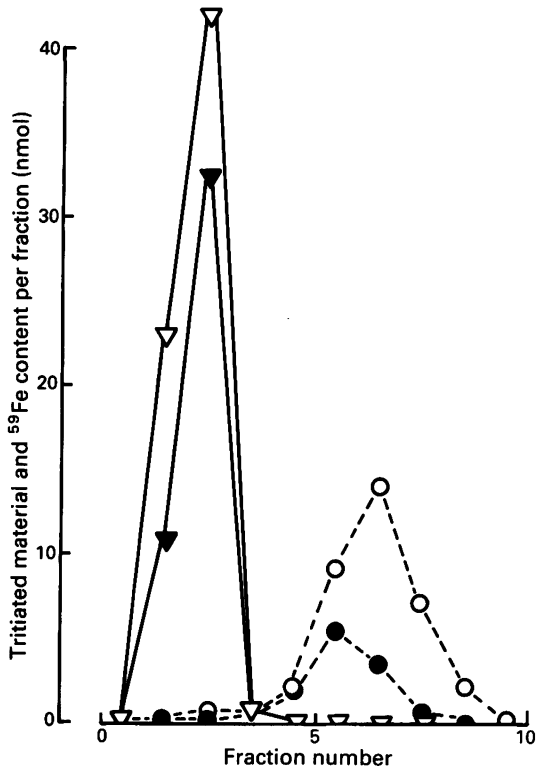

Figure 2 Gel filtration profiles of ^{59}Fe (▼, ▽) and tritium (●, ○) in plasma samples taken 2 min (open symbols) and 60 min (closed symbols) after intravenous injection of [^{59}Fe]-ferric [^{3}H]-maltol at a dose of 1 mg elemental iron; 100 μl volumes were applied to 10 ml columns of Sephadex G-25 and 1 ml fractions eluted. Values are shown as nmol of radioactive material in each fraction.

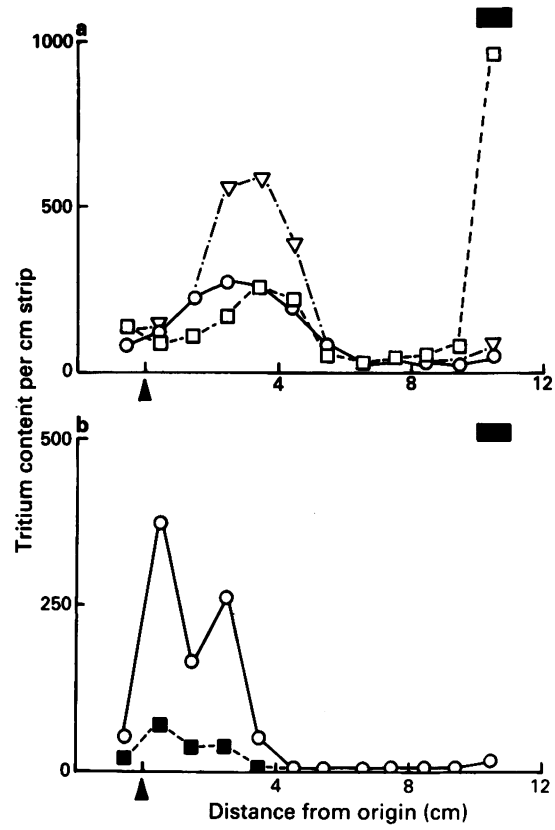

Figure 3 Thin layer chromatographic separation of tritiated material in plasma samples taken 2 min (□), 5 min (■), 20 min (▽) and 60 min (○) following intravenous (a) or intraduodenal (b) administration of [^{59}Fe]-ferric [^{3}H]-maltol at a dose of (a) 1 mg or (b) 7 mg elemental iron. Radioactive fractions obtained from plasma samples after gel filtration were concentrated, spotted on to thin layer plates and developed 1–2 h in methyl ethyl ketone:acetone:water:formic acid (80:6:12:2 by volume). Values are given as d.p.m. of tritiated material eluted from each 1 cm strip. The position of non-radioactive maltol is indicated by the black band and the origin is shown by the arrow head.

Most of the tritium in the gel fractions obtained from the 2 min plasma samples ran with unlabelled maltol just behind the solvent front (Figure 3a). But the tritium contained in the gel fractions from the 60 min plasma sample appeared in a peak near the origin, corresponding to a substance of more hydrophilic nature, most probably a conjugate. Although the nature of this conjugate was not analysed, the identity of the maltol conjugate formed by intestinal tissue was investigated in more detail as described below.

Pharmacokinetics after intraduodenal administration of [^{59}Fe]-ferric [^{3}H]-maltol

The entry of ^{59}Fe and of tritium into the plasma after intraduodenal administration of [^{59}Fe]-ferric [^{3}H]-maltol was followed by measuring the ^{59}Fe and tritium content of plasma samples taken at 5 to 10 min intervals. After the lower dose of ferric maltol (100 μg elemental iron), tritium entered the circulation rapidly, peak values being attained within 10 min whilst ^{59}Fe levels rose slowly reaching a plateau after about 60 min (Figure 4a). After the higher doses of ferric maltol (7 mg elemental iron), tritium levels rose gradually over the course of 60 min whilst the ^{59}Fe content was highest initially and then declined (Figure 4b). At both doses therefore ^{59}Fe and tritium must cross the intestinal wall at different rates. Thin layer analysis of plasma samples taken 5 and 60 min after intraduodenal administration (Figure 3b) indicated that maltol must enter the circulation entirely in the conjugated form, no unchanged maltol being detected. The presence of two radioactive peaks near the origin suggests that two separate conjugates may have been formed.

[^{3}H]-maltol uptake into isolated intestinal fragments

The way in which maltol itself can cross the brush border membranes and enter the intestinal cells was investigated by studying tritium accumulation in isolated fragments of rat small intestine. In the absence of iron, [^{3}H]-maltol appeared to diffuse rapidly into the fragments (Figure 5a) but when presented to the tissues as the ferric maltol complex, [^{3}H]-maltol entry was very much slower and showed saturable kinetics (Figure 5b) similar to those seen previously for iron entry from ferric maltol (Levey *et al.*, 1988). Indeed when entry of both ^{59}Fe and tritium were followed simultaneously, the uptake curves obtained for metal and for ligand were superimposable.

Disposition of ^{59}Fe and tritium in intestinal tissue

The disposition of ^{59}Fe and of tritium within the intestinal fragments after uptake was investigated. This was done by analysing the distribution of radioactivity in pellet and supernatant following centrifugation of homogenates of mucosal scrapings at 100,000 *g* for 1 h. After incubation of isolated fragments for 20 min with 0.05 mM [^{59}Fe]-ferric [^{3}H]-maltol, 99% of the absorbed tritium was localized in the supernatant whilst 60–70% of the ^{59}Fe appeared bound to the membrane pellet.

Metabolism of maltol

The nature of the tritiated material recovered from the supernatant was identified following gel filtration and thin layer chromatography. After incubation of intestinal fragments with 0.1 mM [^{3}H]-maltol, tritium was found in the gel fractions containing material slightly larger than maltol itself. After thin

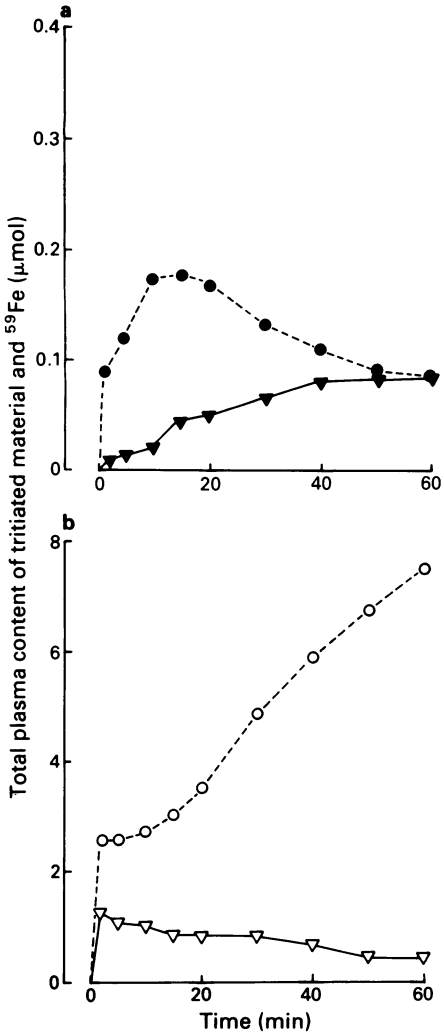

Figure 4 Changes in total plasma content of ^{59}Fe (▼, ▽) and tritium (●, ○) during 1 h following intraduodenal administration of [^{59}Fe]-ferric [^{3}H]-maltol at doses containing (a) 100 μg (closed symbols) and (b) 7 mg (open symbols) of elemental iron. Each value is the mean of data from 3 animals and is shown as μmol of radioactive material in the total plasma.

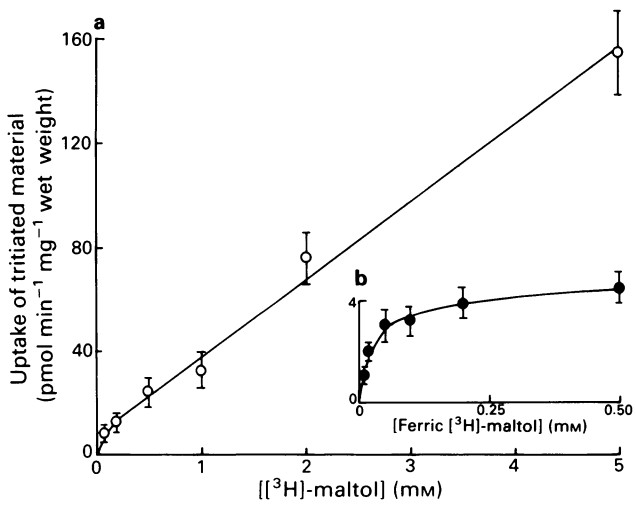

Figure 5 Effect of concentration on initial rates of uptake of tritium into isolated intestinal fragments after incubation with (a) [^{3}H]-maltol (○) or (b) ferric [^{3}H]-maltol (●). Each value represents the mean of data from 3 separate experiments, quadruplicate determinations being obtained from each experiment; s.e.mean shown by vertical bars. Values are given as pmol of tritiated material min^{-1} mg^{-1} wet weight of tissue.

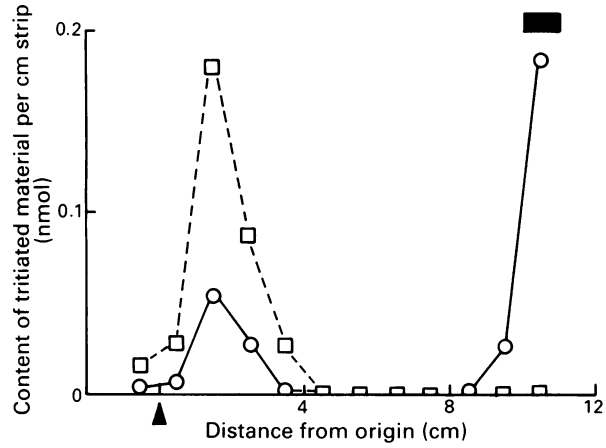

Figure 6 Thin layer chromatographic separation of tritiated material obtained from intestinal mucosa after incubation for 30 min with 0.1 mM [^{3}H]-maltol. Following homogenization and gel filtration, tritiated fractions were concentrated and incubated with (○) or without (□) β-glucuronidase before thin layer separation. Values are shown as nmol of tritiated material eluted from each 1 cm strip. The position of non-radioactive maltol is indicated by the black band and the origin is shown by the arrowhead.

layer separation it was detectable as a single peak of material near the origin, thus more hydrophilic than maltol (Figure 6). After incubation of the gel fractions with β-glucuronidase, the tritiated material then ran on the thin layer plates just behind the solvent front in a position corresponding to the unchanged maltol (Figure 6). The amount of tritiated metabolite converted to maltol varied from experiment to experiment, ranging from 75% to 100%. It is possible that in some cases the incubation time or amount of enzyme available was insufficient for complete conversion.

The proportion of maltol transformed to the conjugate varied with maltol concentration. After incubation of intestinal fragments with 1 mM [^{3}H]-maltol, two tritium peaks could be discerned on the thin layer plates, one corresponding to the hydrophilic metabolite and the other to the unchanged maltol. Uptake and metabolism of maltol over a range of maltol con-

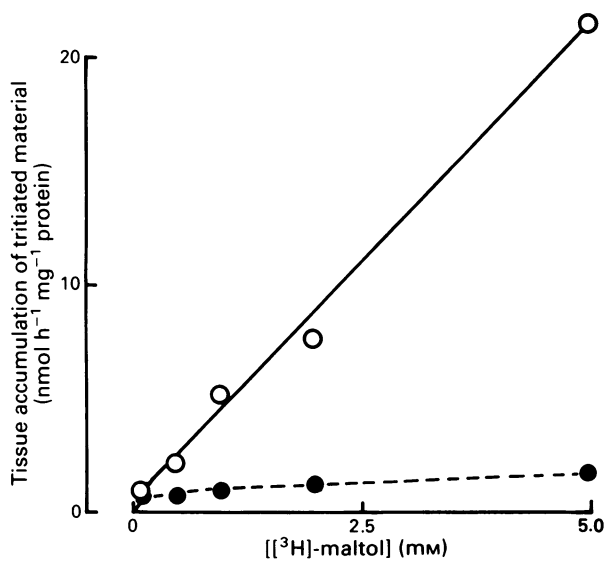

Figure 7 Effect of concentration on the proportion of maltol metabolized during uptake into isolated intestinal fragments. Following incubation at 37°C for 30 min with [^{3}H]-maltol, mucosal scrapings from the fragments were homogenized and the content of total tritium (○) and of tritiated metabolite (●) remaining after chloroform extraction of unchanged maltol analysed. Each value is the mean of triplicate determinations and is given as nmol of tritiated material h^{-1} mg^{-1} protein in the homogenate.

centrations were investigated together in the isolated intestinal fragment, unchanged maltol being separated from its metabolite by chloroform extraction. Conjugation was clearly saturable (Figure 7) so that at the higher maltol concentrations, most of the tritium accumulated by the isolated fragments was still in the form of unchanged maltol. Attempts to study maltol metabolism in intestinal homogenates proved unsuccessful. Although the homogenates retained the ability to conjugate other phenols, metabolism of maltol was not detectable despite the addition of the requisite cofactors (Bock et al., 1983) in the absence of normal subcellular compartmentation.

Discussion

It is apparent from these results that ferric maltol dissociates rapidly within the circulation. The complex is known to be kinetically labile and able to donate the iron rapidly to apotransferrin (Barrand et al., 1987). This dissociation also occurred with doses of iron that must have far exceeded the iron carrying capacity of transferrin in the plasma. It is possible that under these conditions iron then binds to albumin. Generally in the iron overload situation, non-transferrin-bound iron in the plasma is complexed to low molecular weight compounds such as amino acids or to citrate (Grootveld et al., 1989) but to a lesser extent attachment to albumin may also occur (Brissot et al., 1985; Wright et al., 1988). Gel filtration revealed the ^{59}Fe still to be associated with high molecular weight proteins although the identity of these proteins was not resolved. Separation of albumin and transferrin cannot be achieved on the basis of molecular size but requires exploitation of differences in charge and such methodology might well alter the capacity of these two proteins to bind the iron. Although non-transferrin bound iron may enter body tissues by routes different from those involved in transferrin-bound iron uptake (Brissot et al., 1985; Wright et al., 1988), no obvious effects on elimination of ^{59}Fe from the plasma were seen after the intravenous ferric maltol dose containing 1 mg elemental iron.

After dissociation, [^{3}H]-maltol itself seems to remain in the plasma as low molecular weight material, unbound by proteins. Its complicated elimination pattern together with the slight change in gel filtration profiles for tritium seen between 2 min and 60 min after injection into the circulation strongly suggest the presence of more than one species of tritiated material. This was confirmed by thin layer chromatography, which revealed the presence of a metabolite, larger and more hydrophilic in nature than the original maltol and therefore probably a conjugate. Conjugation is a common pathway for metabolism and elimination of phenols (Smith & Williams, 1966). Certainly the tritiated material collected in the urine after 1 h appeared to be entirely in the form of the conjugate. The liver is probably the main site for conjugation of maltol injected directly into the circulation but conjugation can also take place within the small intestines. This was demonstrated by incubating isolated intestinal fragments with [^{3}H]-maltol. Both glucuronide and sulphate conjugation can take place in the rat small intestine, the relative proportions of each depending on the nature of the substrate (Koster, 1985). The conjugate formed from maltol in the intestines appeared to be a glucuronide since enzymic digestion with β-glucuronidase led to recovery of the original [^{3}H]-maltol. Previous studies in dogs identified both glucuronide and sulphate conjugates in the urine after oral dosing with ethyl maltol (Rennhard, 1971). Preliminary data (Hider, unpublished observations) suggests that both forms of conjugate may be produced from maltol and eliminated in the urine in human subjects.

During absorption of ferric maltol from the duodenal lumen, ^{59}Fe and tritium enter the circulation at different rates, the pattern of entry depending on the dose of ferric maltol given. This strongly suggests that dissociation of metal and ligand takes place before reaching the blood. Subcellular fractionation studies indicated that once within the intestinal tissues, iron and maltol become localized to different compartments. The initial uptake of maltol across the intestinal brush border in the presence of iron is a saturable process and similar to the uptake of the iron itself. However, in the absence of iron, maltol can diffuse rapidly into the tissues. This would seem to suggest that iron and maltol pass into the intestinal cells as the complex before dissociation. However, this seems unlikely since previous studies have shown that reduction of the ferric iron to the ferrous state occurs in the intestinal lumen and appears to be a vital step in the uptake of iron from ferric maltol (Barrand et al., 1990). Dissociation of iron and maltol takes place on reduction of the iron in the gut lumen. Thus iron and ligand will be able to enter the tissues by separate pathways, the ferrous iron binding to the endogenous carrier at the cell surface and maltol simply diffusing across the cell membrane. The entry of maltol will still appear to be saturable since the rate of its diffusional entry will be governed by the rate of release of maltol from its complex with iron.

The different rates of entry of ^{59}Fe and of tritium into the circulation from the duodenum with the different doses of ferric maltol may be explained in the following way. At low dose, rapid and extensive dissociation may occur in the gut lumen. Free maltol would then be able to diffuse rapidly into the blood whilst the iron would be passed into the intestinal cells for normal mucosal processing. It has already been established that at low doses of ferric maltol much of the iron absorbed into the intestinal mucosa of iron replete animals becomes associated with ferritin (Callingham & Barrand, 1987; Levey et al., 1988; Barrand & Callingham, 1991). Transfer of iron from the serosal surface to transferrin in the circulation will then be regulated by the demands for iron in the peripheral tissues (Peters et al., 1988) and may well take place as a slow release. At high doses, dissociation of metal and ligand may be very slow, hence the slow entry of maltol into the circulation. Indeed after oral administration with high doses of ferric maltol i.e. those containing 1 or more mg elemental iron, it has been possible to visualize the deep red coloured undissociated ferric maltol complex not only in the stomach but also throughout the length of the small intestine (Barrand & Callingham, 1991). Certainly the rate of ferrous iron formation from ferric maltol decreases at the higher concentrations (Barrand et al., 1990). Iron passing initially into the blood will quickly saturate all binding sites being in much higher absolute amounts, so no further transfer of iron from serosal surface to blood will take place even though dissociation of iron and maltol continue. Presumably the released iron is either precipitated in the gut lumen or sequestered by ferritin in the mucosal cells and eventually shed back into the gut lumen. Either way, it does not gain access to the circulation and so lead to iron overload in peripheral tissues.

By following the fates of metal and ligand independently during absorption of ferric maltol, it has been possible to demonstrate that dissociation of iron and maltol probably due to reductive mechanisms occurs in the gut lumen before uptake of either component. The ligand, maltol, is then free to diffuse across the intestinal wall. Conjugation both within the intestines and in other peripheral tissues renders the hydroxypyrone more hydrophilic and it is rapidly eliminated from the body in the urine. The reduced iron, no longer complexed to maltol, passes into the intestinal mucosa on the endogenous carrier and is processed via normal regulatory mechanisms. Ferric maltol thus provides a way of administering ferric iron to the gut lumen in bioavailable form. Since maltol limits the lipid peroxidation brought about by ferrous iron generation (Singh & Barrand, 1990) ferric maltol acts as a reservoir of soluble iron in a form less likely to produce tissue damage (Slivka et al., 1989). Once reduced, however, the iron is readily available for uptake at the intestinal cell surface.

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References

BAKAJ, M.E. (1984). Facilitation of iron absorption. *MSc. Thesis, University of Essex.*

BARRAND, M.A., CALLINGHAM, B.A. & HIDER, R.C. (1987). Effects of the pyrones, maltol and ethyl maltol, on iron absorption from the rat small intestine. *J. Pharm. Pharmacol.*, **39**, 203–211.

BARRAND, M.A. & CALLINGHAM, B.A. (1991). Evidence for regulatory control of iron uptake from ferric maltol across the small intestine of the rat. *Br. J. Pharmacol.*, (in press).

BARRAND, M.A., HIDER, R.C. & CALLINGHAM, B.A. (1990). The importance of reductive mechanisms for intestinal uptake of iron from ferric maltol and ferric nitrilotriacetic acid. *J. Pharm. Pharmacol.*, **42**, 279–282.

BOCK, K.W., BURCHELL, B., DUTTON, G.J., HANNINEN, O., MULDER, G.J., OWENS, I.S., SIEST, G. & TEPHLY, T.R. (1983). UDP-glucuronosyltransferase activities. Guidelines for consistent interim terminology and assay conditions. *Biochem. Pharmacol.*, **32**, 953–955.

BRISSOT, P., WRIGHT, T.L., MA, W-L. & WEISIGER, R.A. (1985). Efficient clearance of non-transferrin-bound iron by rat liver. *J. Clin. Invest.*, **76**, 1463–1470.

CALLINGHAM, B.A. & BARRAND, M.A. (1987). Iron uptake in the presence of maltol into normal and iron deficient rat duodenal fragments. *IUPHAR 10th Internat. Congr. Pharmacol.*, Abstract P1388.

DIETZELFBINGER, H. (1987). Bioavailability of bi- and trivalent oral iron preparations. *Arzneim. Forsch.*, **37**, 105–107.

GEE, A.S., BARRAND, M.A. & CALLINGHAM, B.A. (1987). Metabolism of the pyrone, maltol, during intestinal absorption of an iron-maltol complex in the rat. *Br. J. Pharmacol.*, **91**, 477P.

GROOTVELD, M., BELL, J.D., HALLIWELL, B., ARUOMA, O.I., BOMFORD, A. & SADLER, P. (1989). Non-transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. *J. Biol. Chem.*, **264**, 4417–4422.

HIDER, R.C., KONTOGHIORGHES, G. & SILVER, J. (1984). *UK patent GB 2136807A.*

HUNGERFORD, D.M. & LINDER, M.C. (1983). Interactions of pH and ascorbate in intestinal iron absorption. *J. Nutr.*, **113**, 2615–2622.

KOSTER, A.S. (1985). Intestinal glucuronidation. *In vivo* and *in vitro* model systems. In *Advances in Glucuronide Conjugation.* ed. Matern, S., Bock, K.W. & Gerock, W. pp. 177–195. Lancaster: HTP Press.

KOSTER, A.S. & NOORDHOEK, J. (1983). Glucuronidation in the rat intestinal wall. Comparison of isolated mucosal cells, latent microsomes and activated microsomes. *Biochem. Pharmacol.*, **32**, 895–900.

LEVEY, J.A., BARRAND, M.A., CALLINGHAM, B.A. & HIDER, R.C. (1988). Characteristics of iron (III) uptake by isolated fragments of rat small intestine in the presence of the hydroxypyrones, maltol and ethyl maltol. *Biochem. Pharmacol.*, **37**, 2051–2057.

NAYFIELD, S.G., KENT, T.H. & RODMAN, N.F. (1976). Gastrointestinal effects of acute ferrous sulphate poisoning in rats. *Arch. Pathol. Lab. Med.*, **100**, 325–328.

PETERS, T.J., RAJA, K.B., SIMPSON, R.J. & SNAPE, S. (1988). Mechanisms and regulation of intestinal iron absorption. *Ann. N.Y. Acad. Sci.*, **526**, 141–147.

RENNHARD, H.H. (1971). The metabolism of ethyl maltol and maltol in the dog. *J. Agr. Food Chem.*, **19**, 152–154.

SINGH, R.K. & BARRAND, M.A. (1990). Lipid peroxidation effects of a novel iron compound, ferric maltol. A comparison with ferrous sulphate. *J. Pharm. Pharmacol.*, **42**, 276–279.

SLIVKA, A., KANG, J. & COHEN, G. (1986). Hydroxyl radicals and the toxicity of oral iron. *Biochem. Pharmacol.*, **35**, 553–556.

SMITH, R.L. & WILLIAMS, R.T. (1966). Implications of the conjugation of drugs and other exogenous compounds. In *Glucuronic Acid, Free and Combined, Chemistry, Biochemistry, Pharmacology and Medicine.*, ed. Dutton, G.J. pp. 457–490. New York: Academic Press.

STEFANOVIC, A., HAVEL, J. & SOMMER, L. (1968). On the reaction of iron (III) with maltol. *Coll. Czech. Chem. Commun.*, **33**, 4198–4214.

WRIGHT, T.L., FITZ, J.G. & WEISIGER, R.A. (1988). Non-transferrin-bound iron uptake by rat liver. *J. Biol. Chem.*, **263**, 1842–1847.

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Peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) activates capsaicin-sensitive primary afferent nerves in guinea-pig atria and urinary bladder

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1 We have investigated the ability of the N-formyl-methionyl-leucyl-phenylalanine (FMLP) a synthetic analogue of a chemotactic peptide derived from a variety of bacteria, to activate capsaicin-sensitive primary afferents in the guinea-pig atria and urinary bladder.

2 In the isolated, electrically-driven left atria from reserpine-pretreated guinea-pigs (atropine in the bath), FMLP (3 nM–1 μM) produced a biphasic positive inotropic response. The late component of this response was selectively abolished by *in vitro* capsaicin pretreatment while both the early and late responses were abolished by indomethacin.

3 The inotropic response to FMLP in the guinea-pig atria was unaffected by ruthenium red. The late but not the early response was strongly inhibited or abolished by tetrodotoxin (TTX), ω-conotoxin (CTX) or by the C-terminal fragment (8–37) of human α-calcitonin gene-related peptide (hCGRP). hCGRP-(8-37) acts as competitive antagonist at CGRP receptors.

4 In the guinea-pig isolated bladder, FMLP (10 nM–10 μM) produced a concentration-dependent contraction which was unchanged by previous *in vitro* capsaicin, TTX or CTX pretreatment. The response to low concentrations of FMLP was suppressed by indomethacin, irrespective of the capsaicin pretreatment.

5 FMLP (10 μM) produced a significant increase in the outflow of CGRP-like immunoreactivity (CGRP-LI) from superfused guinea-pig atria or urinary bladder. CGRP-LI outflow induced by FMLP was blocked by indomethacin or *in vitro* capsaicin pretreatment.

6 These findings indicate that FMLP activates the 'efferent' function of capsaicin-sensitive primary afferents via prostanoid generation. This action could provide a neurogenic contribution to the overall inflammatory response produced by bacteria-derived peptides in inflamed tissues. In addition the present data indicate that endogenous prostanoids generated during exposure to FMLP produce peptide secretion from sensory nerves via a TTX- and CTX-sensitive but ruthenium red-resistant mechanism.

Introduction

N-formyl-methionyl-leucyl-phenylalanine (FMLP) is a powerful synthetic analogue of a chemotactic peptide derived from a variety of bacteria (Schiffmann *et al.*, 1975; Showell *et al.*, 1976). FMLP is known to produce a variety of biological responses including airway smooth muscle contraction (Hamel *et al.*, 1984; Smith *et al.*, 1985), vascular contraction and relaxation (Crowell *et al.*, 1989; Laplante *et al.*, 1989), leucocyte and macrophage chemotaxis and granule enzyme secretion (Smith & Iden, 1980). The action of FMLP on granulocytes is mediated by specific receptors (Aswanikumar *et al.*, 1977) and some of its biological actions are indirectly mediated by production of prostanoids (Laplante *et al.*, 1989; Crowell *et al.*, 1989).

In recent years much attention has been directed to investigation of the mechanisms leading to activation of the 'efferent' function played by capsaicin-sensitive afferents (Szolcsányi 1984; Maggi & Meli, 1988). Several chemical stimuli including bradykinin (Geppetti *et al.*, 1988; Saria *et al.*, 1988) or arachidonic acid metabolites (Manzini *et al.*, 1990) have been shown to induce secretion of neuropeptides from peripheral endings of capsaicin-sensitive afferents. This process is thought to be of relevance for the pathophysiology of various organs and systems and particularly to play a pivotal role in the genesis of neurogenic inflammation.

In this study we present functional and neurochemical evidence indicating that FMLP activates, via prostanoid generation, capsaicin-sensitive afferents in the guinea-pig atria and urinary bladder. Additionally, we investigated the effects of various pharmacological tools, such as ω-conotoxin and ruthenium red on the response to FMLP in the guinea-pig atria. These pharmacological tools have been recently used (Maggi *et al.*, 1988; 1989a) to distinguish between different modes of peptide secretion from peripheral endings of capsaicin-sensitive afferents.

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Methods

General

Male albino guinea-pigs weighing 250–300 g were stunned and bled. The urinary bladder and left atria were excised and placed in oxygenated (96% O_2 and 4% CO_2, pH 7.4 at 37°C) Krebs solution (urinary bladder) or Tyrode solution (atria), as described previously (Maggi *et al.*, 1988; 1989a; Giuliani *et al.*, 1989).

Functional experiments

The left atria or longitudinal strips of the urinary bladder (mucosa-free, 1 cm long 3 mm wide) were placed in 5 ml baths for isolated organs, maintained at 37°C. Tension was recorded isometrically (atria, load 5 mN) or isotonically (urinary bladder 10 mN) by means of Basile 7050 Unirecord. The atria were obtained from reserpine-pretreated (5 mg kg^{-1}, i.p., 72–96 h beforehand) animals and placed in Tyrode solution containing atropine (1 μM), and driven at a frequency of 3 Hz (0.5 ms pulse width, maximal voltage) by means of a Grass S88 stimulator, as described previously (Maggi *et al.*, 1988; 1989a; Giuliani *et al.*, 1989).

All experiments started after a 90 min equilibration period. In the atria, the positive inotropic response produced by trains of electrical field stimulation (10 Hz for 2.5 s, 1 ms pulse width, 60 V) was determined at 15–20 min intervals by means of the second output channel of the stimulator until reproducible inotropic responses, shown previously to depend on antidromic activation of sensory nerves (Maggi *et al.*, 1988; 1989a).

Concentration-response curves to FMLP were obtained for the atria or urinary bladder with 30 min intervals between doses. In both cases the effect of FMLP was allowed to develop for 10 min before washing. No significant desensitization was found with this protocol for the response to 10 μM FMLP checked at the end of the equilibration period or at the end of the concentration-response curve.

In some experiments the effect of FMLP was studied in preparations which were desensitized to capsaicin *in vitro*. This was done by applying 10 μM capsaicin for 15 min at the end of the equilibration period followed by washing.

The effects of indomethacin (10 μM) on responses to FMLP was investigated by adding the drug to the Krebs or Tyrode solution from the beginning of the experiment.

In the atria the response to 1 μM FMLP was also investigated in the presence of 0.1 μM ω-conotoxin fraction GVIA (CTX, 20 min before), tetrodotoxin (TTX, 0.3 μM, 10 min before), ruthenium red (10 μM, 10 min before) or the CGRP antagonist, hCGRP-(8-37) (1 μM, 10 min before). The concentrations of CTX, TTX and ruthenium red were selected from previous studies in which these drugs have been shown to inhibit responses produced by activation of sensory nerves without exerting postjunctional effects (Maggi *et al.*, 1988; 1989a). The C-terminal fragment of hCGRP, hCGRP-(8-37) is a competitive antagonist at CGRP receptors, as shown by Chiba *et al.* (1989). The concentration of the CGRP antagonist used here was selected from a previous study in which it was shown to antagonize competitively the response to hCGRP in guinea-pig left atria without affecting the inotropic response to isoprenaline (Maggi *et al.*, 1991).

In the atria the response to FMLP was expressed as % variation of resting inotropism. In the urinary bladder it was expressed as % of the response to KCl (20 mM, added to the bath for 60 s) chosen as an appropriate internal standard. The response to KCl was not modified by previous exposure to capsaicin or indomethacin.

Release experiments

Release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) was determined by radioimmunoassay in superfusates from guinea-pig atria and mucosa-free urinary bladder, using the same technique as described previously (Maggi *et al.*, 1989a,b). Tissue slices (100–150 mg) were placed in a 2 ml bath maintained at 37°C and superfused at a rate of 0.3 ml min^{-1} with oxygenated (96% O_2 and 4% CO_2) Krebs solution containing 1 μM thiorphan. Five-min (1.5 ml) fractions were collected before (2 fractions), during (4 fractions) and after (1 fraction) the stimulus. After a 90 min equilibration period the tissues were exposed for 20 min to the stimulus. Samples were added with acetic acid to give 2 N final concentration and freeze-dried. At the end of the experiments the tissues were blotted two–three times with filter paper and weighed.

Statistical analysis

Each value in the text and figures is mean ± standard error of the mean (s.e.mean). Statistical analysis was performed by means of the Student's *t* test for paired or unpaired data or by means of analysis of variance, as indicated in the legends. A P value <0.05 was considered statistically significant.

Drugs

Drugs used were: N-formyl-Met-Leu-Phe (FMLP), human calcitonin gene-related peptide (hCGRP), hCGRP-(8-37), ω-conotoxin GVIA (CTX) (Peninsula), thiorphan, indomethacin, tetrodotoxin (TTX) (Sigma), ruthenium red (Aldrich), capsaicin, reserpine (Serva).

Results

Functional experiments: guinea-pig atria

FMLP (3 nM–1 μM) produced, after a latency of 5–10 s, a concentration-dependent slowly developing positive inotropic effect (Figures 1 and 2) that was fully reproducible at 30 min interval. At 0.1–1 μM the response to FMLP was clearly biphasic, as shown in a typical tracing in Figures 1 and 2. During

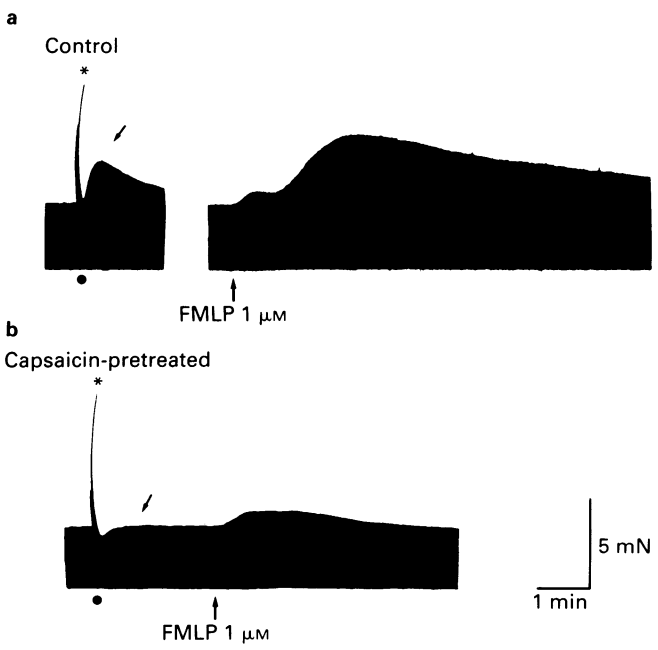

Figure 1 Typical tracings showing the positive inotropic response to electrical field stimulation (EFS 10 Hz, 1 ms, 60 V for 2.5 s) and to N-formyl-Met-Leu-Phe (FMLP, 1 μM) administration in control (a) and in capsaicin-pretreated (10 μM for 15 min) electrically-driven guinea-pig left atria (b). EFS, applied at the dots, produced a post stimulus potentiation (indicated by asterisks) followed by a delayed neurogenic positive inotropic response (indicated by arrows) which is due to antidromic activation of capsaicin-sensitive sensory nerves. FMLP produced a biphasic positive inotropic effect. *In vitro* capsaicin desensitization abolished both the delayed positive inotropic response to EFS and the late inotropic response to FMLP.

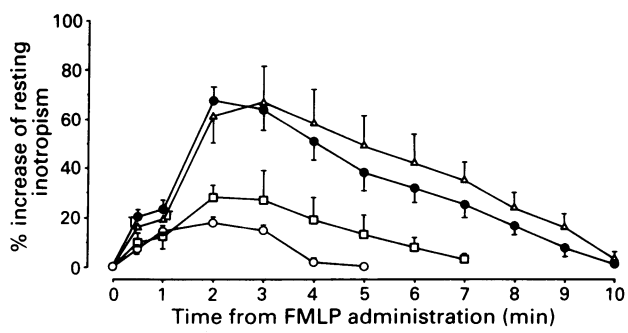

Figure 2 Time-course of the inotropic effect of N-formyl-Met-Leu-Phe (FMLP) on guinea-pig isolated left atria at various concentrations (○ 3 nM; □ 10 nM; △ 0.1 μM; ● 1 μM). Maximal effect was reached at 0.1–1 μM concentration with a clearly biphasic response. Each value is the mean of 5–6 experiments with s.e. shown by vertical bars.

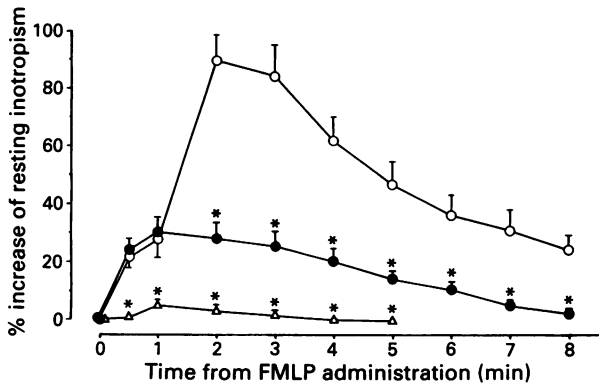

Figure 3 Time-course of the inotropic response to N-formyl-Met-Leu-Phe (FMLP) at 1 μM concentration (○ control response) on isolated electrically-driven left atria of guinea-pig. Pre-exposure to capsaicin (● 10 μM for 15 min) significantly reduced the late inotropic response to 1 μM FMLP leaving the early one unchanged. In the presence of indomethacin (△ 10 μM) the inotropic response to FMLP was abolished.
* $P < 0.05$ significantly different from control response (Student's t test for paired data). Each value is the mean of 5–7 experiments with s.e.mean shown by vertical bars.

the first min after FMLP application inotropism slowly increased and reached a plateau at about 20–30% over resting tension. At this stage a further, marked increase in atrial inotropism was observed which peaked at about 2–3 min after FMLP administration. Data in Figures 3 and 4 show that pre-exposure of the preparations to a desensitizing concentration of capsaicin (10 μM for 15 min) failed to affect the early inotropic response to FMLP while the late component was significantly reduced and the overall response to FMLP appeared as a monophasic inotropic response with a peak at 2 min after FMLP administration. In the presence of indomethacin (10 μM) the inotropic response to FMLP was abolished (Figures 3 and 4).

In subsequent experiments we investigated the action of various substances on the biphasic inotropic response to 1 μM FMLP on the assumption that the biphasic behaviour observed in control conditions reflected activation of two distinct mechanisms, the latter involving peptide release from capsaicin-sensitive afferents. Tension developed in the presence of FMLP was therefore calculated at 1 and 3 min after FMLP administration and the effect of test substances evaluated at these two times after FMLP administration.

Data in Figure 4 show that the early response to FMLP was unaffected by either TTX (0.3 μM), CTX (0.1 μM), ruthenium red (10 μM) or the CGRP antagonist, h-CGRP(8-37) (1 μM). Ruthenium red was also without effect on the delayed response to FMLP which was, by contrast, significantly and markedly reduced by TTX, CTX or the CGRP antagonist (Figure 4). Data in Figure 4 also show, for comparison, the effect of various pretreatments on the inotropic response produced in the same preparation by electrical field stimulation which, in the present experimental conditions, has been proposed to depend upon CGRP release from capsaicin-sensitive afferents (Saito *et al.*, 1987; Maggi *et al.*, 1988; 1989a): the response to electrical field stimulation was abolished or markedly inhibited by *in vitro* capsaicin desensitization, TTX, CGRP antagonist, CTX while it was unaffected by indomethacin or ruthenium red (Figure 4).

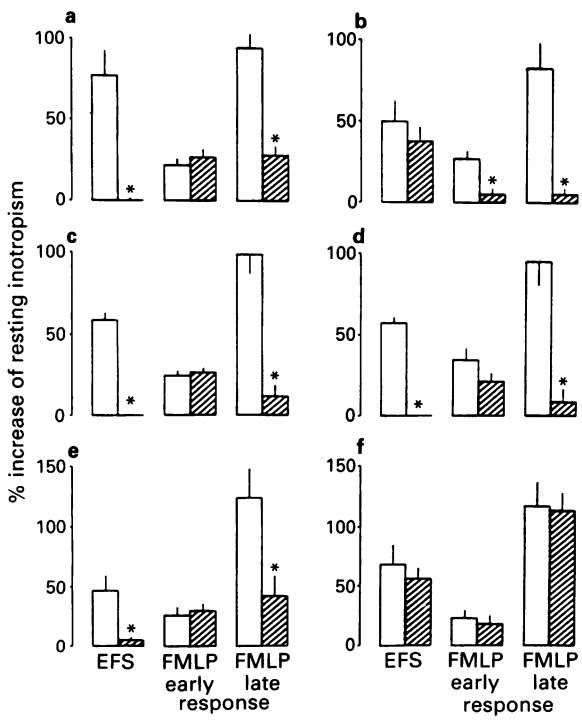

Figure 4 Effect of capsaicin, indomethacin, tetrodotoxin (TTX), human calcitonin gene-related peptide-(8-37) (hCGRP-(8-37)), conotoxin (CTX) and ruthenium red (RR) on the inotropic effect (early and late responses calculated at 1 and 3 min respectively) produced by N-formyl-Met-Leu-Phe (FMLP, 1 μM) and for comparison by electrical field stimulation (EFS 10 Hz, 1 ms, 60 V for 2.5 s). In all panels (a–f), open columns are controls; hatched columns: in (a) capsaicin 10 μM; (b) indomethacin 10 μM; (c) TTX 0.3 μM; (d) hCGRP-(8-37) 1 μM; (e) CTX 0.1 μM; (f) RR 10 μM.
* $P < 0.05$ significantly different from the respective control (Student's t test for paired data). Each column is the mean of 4–6 experiments with s.e.mean shown by vertical lines.

Functional experiments: guinea-pig urinary bladder

FMLP (10 nM–10 μM) produced a concentration-dependent contraction of mucosa-free strips from the guinea-pig urinary bladder. At maximal concentrations (1–10 μM) the response approached 40–50% of that to KCl (20 mM) and was not significantly affected by indomethacin (10 μM), capsaicin-pretreatment (10 μM for 15 min followed by repeated washings) or a combination of these two treatments (Figure 5, $n = 5$ for each group). At low FMLP concentrations (10–100 nM) no response was observed in the presence of indomethacin (Figure 5). The response to FMLP in capsaicin-pretreated strips was not different from controls (Figure 5). Likewise, the response to FMLP was not significantly modified by pretreatment with either TTX (0.3 μM) or CTX (0.1 μM) ($n = 4$ each, Figure 5).

Release experiments

Data in Figure 6, show that 10 μM FMLP evoked a significant increase in CGRP-LI outflow from both guinea-pig atria and urinary bladder. The FMLP-evoked peptide release was abolished, in both tissues, in the presence of indomethacin (10 μM) or in tissues pre-exposed to capsaicin (10 μM for 15 min) in order to inactivate capsaicin-sensitive primary afferents (Figure 6).

Discussion

The present findings provide functional and neurochemical evidence indicating that the bacterial chemotactic peptide FMLP activates indirectly, via prostanoid generation, the 'efferent' function of capsaicin-sensitive primary afferents and determines the release of CGRP-LI. In both the guinea-pig atria and urinary bladder, CGRP-LI is entirely stored in peripheral endings of capsaicin-sensitive primary afferents (Su *et al.*, 1986; Franco-Cereceda *et al.*, 1987) from which it has been shown to be released in response to a variety of stimuli including capsaicin, high potassium depolarizing media or bradykinin (Maggi & Meli, 1988 for review).

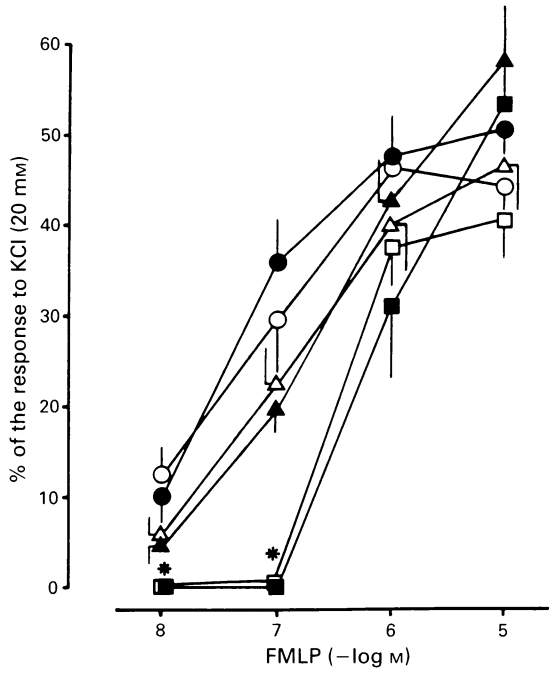

Figure 5 Concentration-response curves showing the contractile effect of N-formyl-Met-Leu-Phe (FMLP, 0.01–10 μM) on guinea-pig isolated urinary bladder (○ control response) alone and in the presence of indomethacin (□ 10 μM), following *in vitro* capsaicin desensitization (● 10 μM for 15 min), a combination of these two treatments (■), tetrodotoxin (△) or conotoxin (▲).
* $P < 0.05$ significantly different from control (Student's t test for unpaired data). Each value is the mean of 4–5 experiments; vertical lines show s.e.mean.

In the atria, the functional response produced by FMLP was clearly biphasic with a late component ascribable to sensory nerve activation. The early response, which persisted after *in vitro* capsaicin desensitization, is most likely ascribable to the inotropic effect of prostanoids generated by FMLP being abolished by indomethacin. Release experiments indicate that FMLP activates the efferent function of capsaicin-sensitive afferents even in the guinea-pig urinary bladder. As in the case of the atria, this effect of FMLP can be ascribed to prostanoid generation, CGRP-LI release being abolished by indomethacin. However, in the case of the guinea-pig bladder, peptide release from sensory nerves by FMLP does not play a relevant role in the local motor response, which was unchanged by capsaicin, TTX or CTX pretreatment. In spite of this, the contraction of the bladder induced by FMLP is partially indomethacin-sensitive. It appears therefore that the motor effect produced by sensory nerve activation in the guinea-pig bladder is masked by the larger contraction produced by FMLP through capsaicin-insensitive mechanisms. However, we cannot exclude the possibility that the local release of sensory neuropeptides by FMLP in the bladder might have a prominent action on other smooth muscle cells, e.g. in the regulation of blood flow and vascular permeability, thus contributing to the overall symptoms of cystitis.

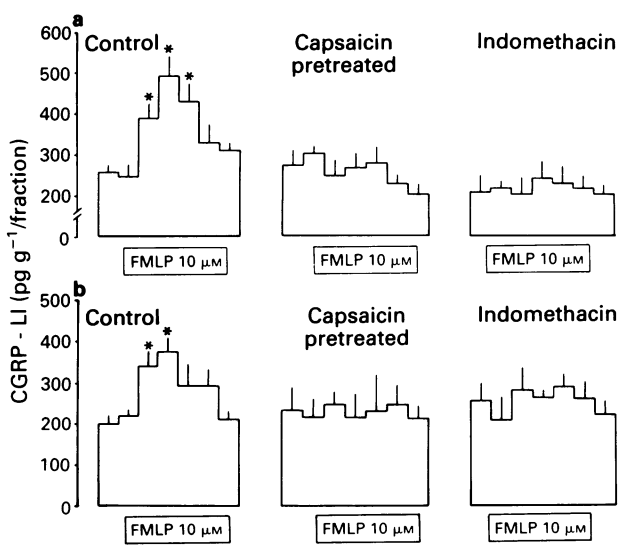

Figure 6 Effect of superfusion with N-formyl-Met-Leu-Phe (FMLP, 10 μM) on the calcitonin-gene related peptide like (CGRP-LI) outflow from slices of guinea-pig urinary bladder (a) or atria (b) in control preparations (left panels), preparations pre-exposed to capsaicin (10 μM for 15 min, middle panels) or in presence of 10 μM indomethacin (right panels).
* $P < 0.05$ significantly different from the basal values (by Analysis of Variance). Each bar indicates the mean of at least 5 experiments; vertical lines show s.e.mean.

The electrically driven left atria from reserpine-pretreated guinea-pigs (atropine in the bath) have been repeatedly shown to provide an excellent bioassay for functional responses produced by activation of capsaicin-sensitive primary afferents (Saito *et al.*, 1988; Maggi *et al.*, 1988; 1989a). In these particular experimental conditions, sensory nerve activation determines a positive inotropic response which is thought to be mediated by endogenous CGRP (Franco-Cereceda *et al.*, 1987; Saito *et al.*, 1988). This is further supported by the observation that hCGRP-(8-37), which acts as a competitive antagonist at certain CGRP receptors (Chiba *et al.*, 1989), selectively blocked the inotropic response to hCGRP (pA_2 6.8) without affecting that to isoprenaline (Maggi *et al.*, 1991). The CGRP antagonist blocked the positive inotropic response produced by electrical field stimulation and the late response to FMLP without affecting the inotropic response to isoprenaline (Maggi *et al.*, 1991) or the early response to FMLP. This result provides pharmacological evidence for an involvement of CGRP in the late component of the functional response to FMLP.

Recently, evidence has been presented indicating that two distinct mechanisms for activation of the 'efferent' function of capsaicin-sensitive primary afferents exist (Maggi *et al.*, 1988; 1989a). The first mechanism, activated by capsaicin itself, involves a TTX-resistant direct stimulation of sensory nerve endings that is selectively blocked by ruthenium red and does not involve the voltage-sensitive calcium channels. The second mechanism, activated by electrical field stimulation, involves the generation of a propagated action potential (TTX-sensitive) which invades antidromically sensory nerve endings and determines peptide secretion via CTX-sensitive calcium channels. This second mode of sensory nerve activation is unaffected by ruthenium red at concentrations which block the effect of capsaicin (Maggi *et al.*, 1989a).

Present data indicate that the indirect action of FMLP on sensory nerves, via prostanoid generation, fits this second mechanism of activation e.g. mimics an axon reflex arrangement.

As discussed in the Introduction, FMLP is a synthetic chemoattractant peptide derived from bacteria. It has been proposed that FMLP-like peptides generated during bacterial infections play a pathogenic role in inflammatory diseases by virtue of their powerful chemotactic activity. The present results indicate an additional mechanism through which bacteria-derived peptides could exert a local pro-inflammatory action i.e. sensory neuropeptide release from peripheral endings of capsaicin-sensitive primary afferents. This might be important in the case of the urinary bladder bacterial cystitis and, in the heart, for pericarditis/myocarditis. The action of FMLP on sensory nerves at this level could participate in the genesis of symptoms by stimulating reflexes (pain, cardiovascular reflexes) and, through peptide release from sensory nerves, contribute to the overall inflammatory process.

References

ASWANIKUMAR, S., CORCORAN, B., SCHIFFMANN, E., DAY, A.R., FREER, R.J., SHOWELL, H.J., BECKER, E.L. & PERT, C.B. (1977). Demonstration of a receptor on rabbit neutrophils for chemotactic peptides. *Biochem. Biophys. Res. Commun.*, **74**, 810–817.

CHIBA, T., YAMAGUCHI, A., YAMATANI, T., NAKAMURA, A., MORISHITA, T., INUI, T., FUKASE, M., NODA, T. & FUJITA, T. (1989). Calcitonin gene-related peptide receptor antagonist human CGRP (8-37). *Am. J. Physiol.*, **256**, E331–E335.

CROWELL, R.E., VAN EPPS, D.E. & REED, W.P. (1989). Responses of isolated pulmonary arteries to synthetic peptide F-Met-Leu-Phe. *Am. J. Physiol.*, **257**, H107–H112.

FRANCO-CERECEDA, A., HENKE, H., LUNDBERG, J.M., PETERMANN, J.B., HÖKFELT, T. & FISHER, J.A. (1987). Calcitonin gene-related peptide (CGRP) in capsaicin-sensitive substance P-immunoreactive sensory neurons in animals and man: distribution and release by capsaicin. *Peptides*, **8**, 399–410.

GEPPETTI, P., MAGGI, C.A., PERRETTI, F., FRILLI, S. & MANZINI, S. (1988). Simultaneous release by bradykinin of substance P and calcitonin gene-related peptide immunoreactivities from capsaicin-sensitive structures in guinea-pig heart. *Br. J. Pharmacol.*, **94**, 288–290.

GIULIANI, S., MAGGI, C.A. & MELI, A. (1989). Prejunctional modulatory action of neuropeptide Y on peripheral terminals of capsaicin-sensitive sensory nerves. *Br. J. Pharmacol.*, **98**, 407–412.

HAMEL, R., FORD-HUTCHINSON, A.W., LORD, A. & CIRINO, M. (1984). Bronchoconstriction induced by N-formyl-methionyl-leucyl-phenylalanine in the guinea pig; involvement of arachidonic acid metabolites. *Prostaglandins*, **28**, 43–56.

LAPLANTE, C., TREMBLAY, B. & MARCEAU, F. (1989). Relaxant effect of N-Formyl-Methionyl-Leucyl-Phenylalanine on rabbit vascular strips. *J. Pharmacol. Exp. Ther.*, **248**, 774–780.

MAGGI, C.A. & MELI, A. (1988). The sensory-efferent function of capsaicin-sensitive sensory neurons. *Gen. Pharmacol.*, **19**, 1–43.

MAGGI, C.A., PATACCHINI, R., GIULIANI, S., SANTICIOLI, P. & MELI, A. (1988). Evidence for two independent modes of activation of the efferent function of capsaicin-sensitive nerves. *Eur. J. Pharmacol.*, **156**, 367–373.

MAGGI, C.A., PATACCHINI, R., SANTICIOLI, P., GIULIANI, S., DEL BIANCO, E., GEPPETTI, P. & MELI, A. (1989a). The 'efferent' function of capsaicin-sensitive nerves: ruthenium red discriminates between mechanisms of activation. *Eur. J. Pharmacol.*, **170**, 167–177.

MAGGI, C.A., PATACCHINI, R., SANTICIOLI, P., GEPPETTI, P., CECCONI, R., GIULIANI, S. & MELI, A. (1989b). Multiple mechanisms in the motor responses of the guinea-pig isolated urinary bladder to bradykinin. *Br. J. Pharmacol.*, **98**, 619–629.

MAGGI, C.A., CHIBA, T. & GIULIANI, S. (1991). Human CGRP-(8-37) as an antagonist of exogenous and endogenous calcitonin gene-related peptide. *Eur. J. Pharmacol.*, (in press).

MANZINI, S., BALLATI, L., GEPPETTI, P., RUBINI, I., MEINI, S. & PERRETTI, F. (1990). Arachidonic acid-induced bronchomotor responses are partially mediated by release of sensory neuropeptides from capsaicin-sensitive structures. *Br. J. Pharmacol.*, **98**, 1077–1079.

SAITO, A., ISHIKAWA, T., KIMURA, S. & GOTO, K. (1987). Role of calcitonin gene-related peptide as cardiotonic neurotransmitter in guinea-pig left atria. *J. Pharmacol. Exp. Ther.*, **243**, 731–740.

SARIA, A., MARTLING, C.R., YAN, Z., THEODORSSON-NORHEIM, E., GAMSE, R. & LUNDBERG, J.M. (1988). Release of multiple tachykinins from capsaicin-sensitive sensory nerves in the lung by bradykinin, histamine, dimethylphenylpiperazinium and vagal nerve stimulation. *Am. Rev. Resp. Dis.*, **137**, 1330–1335.

SCHIFFMANN, E., CORCORAN, B. & WAHL, S.M. (1975). N-formylmethionyl peptides as chemoattractants for leucocytes. *Proc. Natl. Acad. Sci., U.S.A.*, **72**, 1059–1062.

SHOWELL, H.J., FREER, R.J., ZIGMOND, S.H., SCHIFFMANN, E., ASWANIKUMAR, S., CORCORAN, B. & BECKER, E. (1976). The structure-activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal enzyme secretion for neutrophils. *J. Exp. Med.*, **143**, 1154–1169.

SMITH, J.K., McCORMICK, J.R. & ELGUINDI, A.S. (1985). Contraction of human and guinea pig airway by a chemotactic peptide. *Chest*, **88**, 25S.

SMITH, R.J. & IDEN, S.S. (1980). Pharmacological modulation of chemotactic factor-elicited release of granule-associated enzymes from human neutrophils. Effects of prostaglandins, nonsteroid anti-inflammatory agents and corticosteroids. *Biochem. Pharmacol.*, **79**, 2389–2395.

SU, H.C., WHARTON, J., POLAK, J.M., MULDERRY, P.K., GHATEI, M.A., GIBSON, S.J., TERENGHI, G., MORRISON, J.F.B., BALLESTA, J. & BLOOM, S.R. (1986). Calcitonin gene-related peptide immunoreactivity in afferent neurons supplying the urinary tract: combined retrograde tracing and immunohistochemistry. *Neuroscience*, **18**, 727–747.

SZOLCSÁNYI, J. (1984). Capsaicin-sensitive chemoceptive neural system with dual sensory-efferent function. In *Antidromic Vasodilation and Neurogenic Inflammation* ed. Chahl, L.A., Szolcsányi, J. & Lembeck, F. pp. 26–52. Budapest: Akademiai Kiado.

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Selectivity of Ca^{2+} channel blockers in inhibiting muscular and nerve activities in isolated colon

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1 Potency and efficacy of nifedipine, verapamil and diltiazem and of Bay K 8644 in modifying propulsion and nerve or smooth muscle activities have been compared in the guinea-pig isolated distal colon. Both the neuronal and muscular effects of Ca^{2+} channel blockers seem to develop at concentrations that are devoid of any significant effect apart from that on Ca^{2+} channels.

2 Nifedipine, verapamil and diltiazem were all able to impair propulsion, resting and stimulated acetylcholine (ACh) release and smooth muscle contractility in a concentration-dependent way. However, some degree of selectivity for neuronal and muscular effects could be observed. Nifedipine was more than 500 fold more potent than verapamil in relaxing musculature but less than twice as potent in reducing ACh release. On the other hand, verapamil was the most efficacious Ca^{2+} channel blocker tested in inhibiting ACh release, its effects being inversely correlated to the external Ca^{2+} concentration, and completely abolished by Bay K 8644.

3 By comparing the potencies exhibited by each drug against peristaltic reflex, smooth muscle contractility and ACh release, verapamil proved to be almost as potent in slowing the peristaltic reflex as in reducing ACh release, while nifedipine was about 100 fold more potent against the peristaltic reflex than against ACh release, but nearly equal against the peristaltic reflex and smooth muscle tone. Therefore, interference with cholinergic neurotransmission is likely to play a major role in the antipropulsive effect of verapamil, while peristaltic reflex impairment by nifedipine is likely to be dependent on inhibition of smooth muscle.

4 A facilitatory effect of Bay K 8644 on both the efficiency of the peristaltic reflex and the non-adrenergic, non-cholinergic (NANC) nerve-mediated relaxation could be observed at concentrations at least 10 fold lower than those required to affect ACh release or smooth muscle.

5 It is concluded that the effects of Ca^{2+} channel blockers on neurotransmitter release may be relevant to their effects on the gastrointestinal motor function.

Introduction

There is growing evidence that the high affinity binding sites for organic Ca^{2+} channel blockers, which are known to be present in high concentrations in gastrointestinal neuromuscular tissues, are associated with functional calcium channels. Indeed, a good correlation between functional effects and competition for nitrendipine binding sites has been shown for a series of dihydropyridine analogues (Bolger *et al.*, 1983; Godfraind & Wibo, 1985). However, although Ca^{2+} channel blockers are reported to be at least as potent in inhibiting intestinal smooth muscle contractility as they are in relaxing small blood vessels (Rosenberg *et al.*, 1979; Barone *et al.*, 1986; Godfraind *et al.*, 1986; Yousif & Triggle, 1986), at present there seems to be little scope for the currently available drugs of this class to be employed in motor disorders of the gastrointestinal tract (Traube & McCallum, 1984; Castell, 1985; Prior *et al.*, 1987). Nevertheless, constipation is not infrequently associated with long term treatment with verapamil (Hedner, 1986).

It is well known that in the gastrointestinal tract most myogenic and nerve-sustained motor activity show a high degree of Ca^{2+} dependence (Wood, 1987). Synaptic events not dependent on external Ca^{2+} seem not to play a significant role in sustaining neurotransmission in the enteric plexuses, as suggested by the strict dependence of both neurotransmitter release and neuronal excitation on Ca^{2+} entry (Alberts & Stjarne, 1982; Yokoyama *et al.*, 1986; Wood, 1987). However, only recently has the functional role of drug-sensitive Ca^{2+} channels at the level of enteric neuronal synapses and neuromuscular junctions received attention (Bornstein *et al.*, 1985; Miller *et al.*, 1988; Katsuragi *et al.*, 1990).

In the present study the effects of drugs, representative of the major classes of L-type Ca^{2+} channel blockers, were investigated for their ability to modify some neuromuscular functions in the distal portion of the guinea-pig large intestine.

Methods

Experiments were carried out in the guinea-pig isolated distal colon. The capability of Ca^{2+} channel blockers and of the Ca^{2+} channel activator Bay K 8644 to interfere with responses related to enteric neurone activation was studied by measuring peristaltic reflex, non-adrenergic, non-cholinergic (NANC) nerve-mediated relaxation of the longitudinal muscle in response to transmural stimulation (TS) and resting and stimulated acetylcholine (ACh) release. Moreover, the ability of drugs to modify circular muscle resting tone and contractility was also assessed.

Isolated specimens of terminal colon 5–7 cm long were taken from male guinea-pigs weighing 300–400 g, mounted in organ baths perfused with Tyrode solution under a tension of 0.5–1.5 g through an isotonic force transducer.

The peristaltic reflex was elicited by a radial distension of the lumen applied at the proximal end of the specimens by means of an intraluminal balloon. Longitudinal muscle movements and displacement of the balloon towards the distal end of the colon were recorded. Since the velocity of propulsion is dependent on the degree of distension (Frigo & Lecchini, 1970), supramaximal stimulation was employed and the maximal velocity of propulsion taken as a measure of the efficiency of the peristaltic reflex. Changes in peristaltic action are indicated as the percentage variation of the propulsion velocity with respect to the control value and were estimated as described by Frigo *et al.* (1984).

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Resting and stimulated ACh release was measured by incubating the specimens in a 3 ml organ bath with physostigmine sulphate (1.5×10^{-5} M) during 20 min collection periods. Samples were obtained during resting and stimulation periods in a sequence similar to that described by Lecchini *et al.* (1969). Stimulation-induced release was obtained by applying rectangular pulses of supramaximal strength, 1 ms duration and 1 Hz frequency delivered by coaxial silver wire electrodes for the first 10 min in a way similar to that described by Frigo *et al.* (1984). The 1 Hz frequency of stimulation was chosen because of its relative selectivity in stimulating the enteric cholinergic neurones (Cowie *et al.*, 1978). ACh was assayed on the guinea-pig isolated ileum incubated with morphine sulphate (6.6×10^{-6} M) and physostigmine sulphate (7.7×10^{-9} M) by the method of Paton & Vizi (1969). To allow for any effects that modifying drugs might have on the ACh assay, the concentrations of such drugs in the test samples were duplicated in the standard ACh solutions during the assay. The effects of compounds on ACh release were expressed as percentage variations with respect to the control level.

Transmural stimulation was used to excite NANC intrinsic nerves by delivering trains of rectangular pulses of supramaximal strength, 0.5 ms duration and frequencies ranging from 0.2 to 2 Hz for 20 s through coaxial silver wire electrodes to specimens pretreated with hyoscine sulphate (3×10^{-6} M) and guanethidine sulphate (3×10^{-6} M). Suppression by tetrodotoxin (TTX, 5×10^{-7} M) was used to check that NANC relaxation was entirely neurogenic. Electrical stimulations were applied at 10 min intervals and inhibitory responses of the longitudinal muscle indicated as percentage of the maximal responses. Maximal response was the greatest response obtained independent of the frequency employed. Frequency-response relationships for TS-induced NANC nerve-mediated relaxations were obtained over the range of 0.2–1 Hz. The effect of Ca^{2+} channel blockers used at the highest concentrations that were ineffective on longitudinal muscle resting tone was tested on frequency-response relationships.

Circular muscle activity was measured in isolated strips of circular muscle deprived of the mucosal layer and connected to an isotonic force transducer under a tension of 0.5–1 g. The basal tension chosen during the experiments was that which allowed rhythmic activity to develop after setting up the preparation. The tone of the circular muscle was fairly constant during the experiment. In any case tension variations such as those produced mechanically by changing the load applied to the preparation did not significantly change the amplitude of carbachol- or $BaCl_2$-induced contractions. In order to exclude any neurogenic component in the drug-induced variation of resting tone, the experiments were carried out in the presence of TTX (5×10^{-7} M). Maximal contraction to both $BaCl_2$ and carbachol were used to test the effect of both Bay K 8644 and Ca^{2+} channel blockers on muscular contractility. For this purpose, the concentrations of $BaCl_2$ and carbachol were in the range of $5–20 \times 10^{-7}$ M and $1–5 \times 10^{-4}$ M, respectively. The Ca^{2+} channel blocker-induced reduction and Bay K 8644-induced increase of circular muscle resting tone were measured as a fraction of the maximal relaxation induced by papaverine and as a fraction of carbachol-induced maximal contraction, respectively. The effects of compounds on carbachol- or $BaCl_2$-induced contractions were expressed as percentage variation with respect to the control value.

The effects of both Ca^{2+} channel blockers and of the Ca^{2+} channel activator Bay K 8644 were evaluated after a preincubation period of at least 20 min for each concentration of the compounds.

The Tyrode solution was of the following composition (mM): NaCl 136.9, KCl 2.7, $MgCl_2$ 1.04, $NaHCO_3$ 11.9, NaH_2PO_4 0.4 and glucose 5.5. Unless otherwise indicated, the experiments were carried out in solutions containing Ca^{2+} 1.8 mM.

The dependence of the efficiency of the peristaltic reflex, resting ACh release and NANC nerve-mediated relaxation on external Ca^{2+} was studied by changing the Ca^{2+} concentration in the Tyrode solution from 0 to 8 mM. The effect of each Ca^{2+} concentration was evaluated after 20 min incubation. Ca^{2+}-free solution was made by omitting $CaCl_2$. No osmolar compensation was made for Ca^{2+} modified solutions.

The drugs used were: acetylcholine chloride (ACh, Sigma); carbamylcholine chloride (carbachol, Sigma); diltiazem hydrochloride (Sigma); hyoscine sulphate (Sigma); guanethidine sulphate (Ciba); morphine hydrochloride (SIFAC); papaverine hydrochloride (Sigma); physostigmine sulphate (Sigma); tetrodotoxin (TTX, Sankyo); yohimbine hydrochloride (Sigma). Nifedipine and Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) were kindly supplied by Dr Thomas (Bayer, Milano) and (±)-verapamil hydrochloride by Knoll (Italia). Dihydropyridines were dissolved in Tween 80 prior to dilution in distilled water. All solutions were protected from light and experiments performed under yellow light.

Concentration-effect relationships were obtained in order to calculate the concentrations able to produce half maximum effects (EC_{50}) and the 30% or 50% inhibitory concentrations (IC_{30} or IC_{50}). Linear regression analysis was performed and potency ratios calculated according to Finney (1978).

For each Ca^{2+} channel blocker, the ratio of the potencies exhibited in inhibiting circular muscle tone versus resting ACh release, as derived from the analysis of log concentration-response relationships, was calculated and indicated as a muscular versus neuronal selectivity index.

Antagonism by Bay K 8644 of nifedipine, verapamil and diltiazem effects was studied according to the method of Arunlakshana & Schild (1959).

Statistical significance of differences between groups was analyzed by applying Student's *t* test for unpaired data.

Results

Ca^{2+}-dependence of acetylcholine release and nerve-mediated motor activities

The peristaltic reflex, resting ACh release and NANC nerve-mediated relaxation of longitudinal muscle were dependent on the external Ca^{2+} concentration. In effect, although a small fraction of the ACh release and NANC relaxation still persisted in Ca^{2+}-free solution, a Ca^{2+} concentration-related increase of the velocity of propulsion, the resting ACh release and the amplitude of NANC relaxation was observed in the concentration range of 0–1.8 mM (Figure 1). All the parameters considered reached a maximum in the concentration range of 1.5–2 mM Ca^{2+}, while the peristaltic reflex and ACh release rapidly declined above 4 mM Ca^{2+}.

Effects of the Ca^{2+} channel activator Bay K 8644

Opposite effects of Bay K 8644 on both the peristaltic reflex and the amplitude of TS-induced NANC relaxation could be observed depending on the concentration employed. A slight but significant increase in the peristaltic reflex and in the amplitude of NANC relaxation, reaching a maximum of 20 and 33% of the controls, respectively, could be observed in the concentration-range of $0.8–8 \times 10^{-9}$ M (Figures 2 and 3). Propulsion was reduced by a concentration of 3×10^{-8} M and was prevented by concentrations of 3×10^{-6} M. Concentrations of Bay K 8644 able to facilitate both peristaltic reflex and NANC relaxation were at least 10 fold lower than the concentrations required to affect both circular and longitudinal smooth muscle.

In fact, Bay K 8644 ($3 \times 10^{-8}–3 \times 10^{-6}$ M) increased in a concentration-dependent manner both the circular muscle resting tone and the contractions in response to $BaCl_2$ and carbachol. The average maximum increase of the maximal $BaCl_2$- and carbachol-induced contractions were 61.2 ± 8.6 and 18.4 ± 1.9 (mean ± s.e.mean, $n = 5$), respectively.

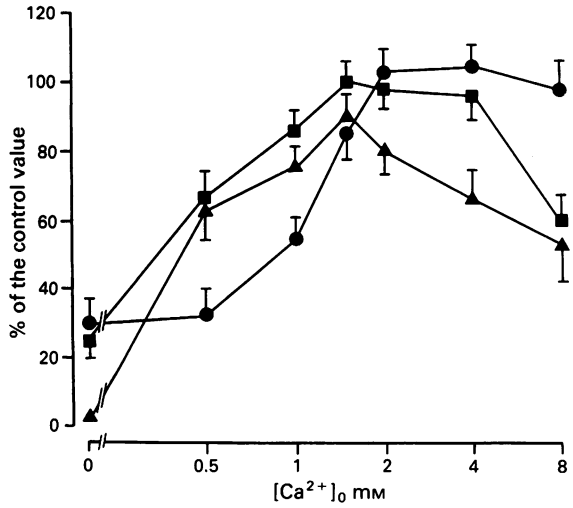

Figure 1 Ca^{2+}-dependence of some nerve-mediated functions in guinea-pig isolated colon. Effect of varying external Ca^{2+} concentration on propulsion velocity (▲); resting acetylcholine release (■); amplitude of TS-induced NANC relaxation of longitudinal muscle (0.5 Hz, supramaximal strength) (●). Values are expressed as percentages of the controls and are plotted against log Ca^{2+} concentration. Controls are considered the values obtained in standard Tyrode solution containing 1.8 mM Ca^{2+}. Each value represents the mean of 5 experiments. Vertical bars indicate s.e.mean.

Bay K 8644 produced a concentration-dependent increase of resting ACh release in the concentration range of 8×10^{-8}–3×10^{-6} M. The calculated EC_{50} value (with 95% confidence limits) was 2.01 (1.20–3.35) $\times 10^{-7}$ M. The Bay K 8644-induced increase of ACh release could be antagonized by verapamil and nifedipine. The reduction of the Bay K 8644-induced increase of ACh release was linearly related to concentration only for nifedipine (Figure 4).

Effects of Ca^{2+} channel blockers

Peristaltic reflex Ca^{2+} channel blockers were able to impair propulsive activity in a concentration-dependent manner. Log concentration-response relationships of nifedipine, verapamil and diltiazem in reducing the peristaltic reflex and the effect of

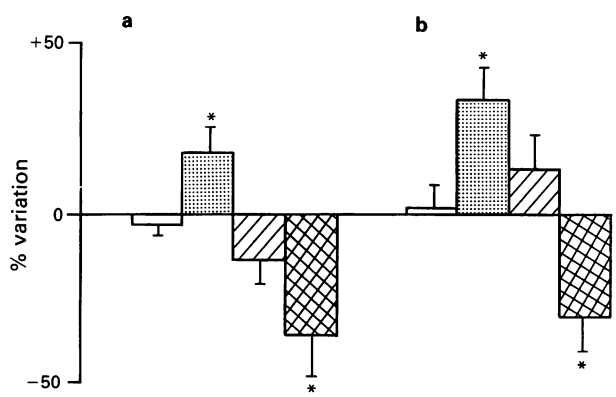

Figure 2 Bay K 8644-induced changes of the NANC nerve-mediated inhibition of longitudinal muscle and of the peristaltic reflex in guinea-pig isolated colon. Columns represent the percentage variation of the velocity of propulsion (a) and of the TS-induced NANC relaxation (2 Hz, supramaximal strength) (b) with respect to the control values in the presence of Bay K 8644: 3×10^{-10} M (open columns); 3×10^{-9} M (stippled columns); 3×10^{-8} M (hatched columns); 3×10^{-7} M (cross-hatched columns). Each column represents the mean of 5 experiments. Vertical bars indicate s.e.mean. * Significantly different from the control value ($P < 0.05$).

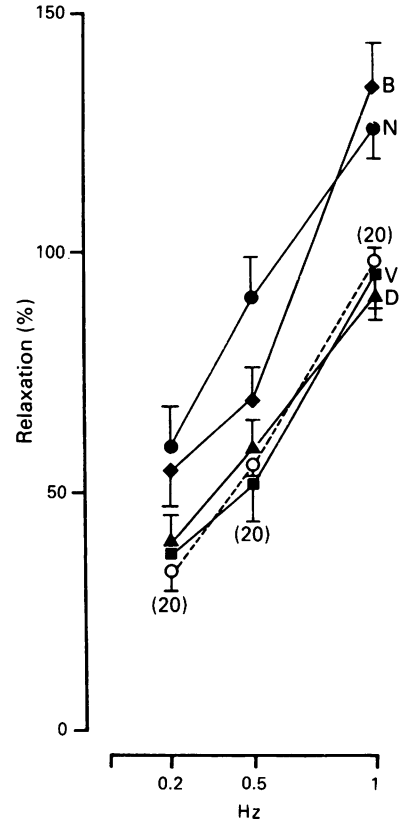

Figure 3 Effect of Ca^{2+} channel blockers and Bay K 8644 on NANC nerve-mediated relaxation of longitudinal muscle in guinea-pig isolated colon. Log frequency-response relationships for TS-induced NANC relaxation in the absence (○) and in the presence of nifedipine (N) 3×10^{-8} M (●); verapamil (V) 7×10^{-7} M (■); diltiazem (D) 7×10^{-7} M (▲) or Bay K 8644 (B) 3×10^{-9} M (◆). Values on the ordinate scale are expressed as percentage of the maximal response. Unless otherwise indicated, each point represents the mean of 5 experiments. Vertical bars indicate s.e.mean. For each Ca^{2+} channel blocker, the concentration chosen was devoid of any effect on muscle tone.

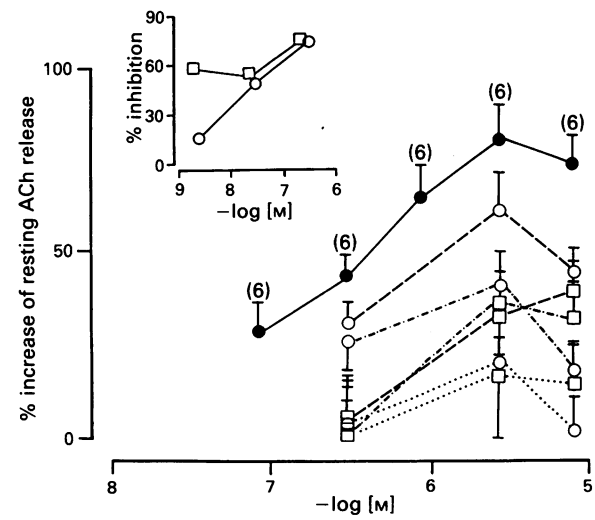

Figure 4 Bay K 8644-induced increase of resting acetylcholine (ACh) release in guinea-pig isolated colon and its modification by organic Ca^{2+} channel blockers. Log concentration-response relationships for Bay K 8644 (●——●) and for Bay K 8644 plus nifedipine (○) or verapamil (□) at the concentrations of 3×10^{-9} (---), 3×10^{-8} (-·-·) and 3×10^{-7} (·····) M in increasing resting acetylcholine release. Log concentrations are plotted against percentage increase of acetylcholine release. Unless otherwise indicated, each point represents the mean of 4 experiments. Vertical bars indicate s.e.mean. The concentration dependence of the nifedipine but not of the verapamil effect is shown by plotting concentration against percentage reduction of Bay K 8644 maximally stimulated acetylcholine release (inset); (○) $r = 0.99$; (□) r = p.75 (NS).

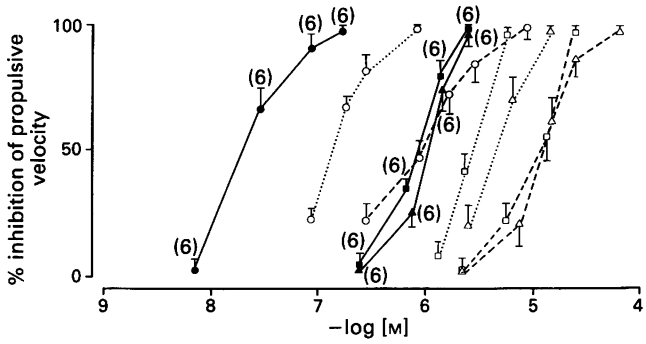

Figure 5 Bay K 8644 antagonism towards Ca^{2+} channel blocker-induced inhibition of peristaltic reflex in guinea-pig isolated colon. Log concentrations of Ca^{2+} channel blockers are plotted against percentage inhibition of velocity of propulsion. Log concentration-response relationships were obtained for nifedipine (●), verapamil (■) and diltiazem (▲) and for nifedipine (○), verapamil (□) and diltiazem (△) plus Bay K 8644 at the concentrations of 3×10^{-8} (·····) and 3×10^{-7} (– – –) M. Unless otherwise indicated, each point represents the mean of 4 experiments. Vertical bars indicate s.e.mean.

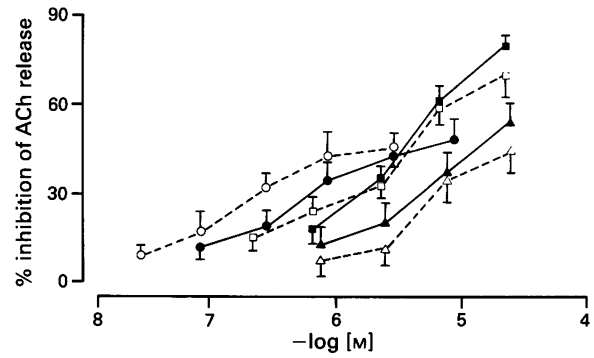

Figure 6 Log concentration response relationships of Ca^{2+} channel blockers in reducing acetylcholine (ACh) release from guinea-pig isolated colon. Log concentrations of nifedipine (●, ○), verapamil (■, □) and diltiazem (▲, △) are plotted against percentage inhibition of acetylcholine release during rest (——) and during TS stimulation (1 Hz, supramaximal strength) (– – –). Each point represents the mean of 6 experiments. Vertical bars indicate s.e.mean.

Bay K 8644 are shown in Figure 5. The IC_{50} values with 95% confidence limits were 2.70 (1.10–6.64) $\times 10^{-8}$ M for nifedipine, 7.04 (4.76–10.42) $\times 10^{-7}$ M for verapamil and 8.50 (4.95–14.95) $\times 10^{-7}$ M for diltiazem, the corresponding potency ratios being: nifedipine (1) > verapamil (0.038) ⩾ diltiazem (0.031). Higher concentrations were required to prevent the onset of the peristaltic reflex. The average blocking concentrations (± s.e.mean) were 8.48 (± 0.24) $\times 10^{-8}$ M for nifedipine, 1.53 (± 0.26) $\times 10^{-6}$ M for verapamil and 2.08 (± 0.21) $\times 10^{-6}$ M for diltiazem. In the presence of blocking concentrations of Ca^{2+} channel blockers the peristaltic reflex could be completely restored by increasing the Ca^{2+} concentration to 6 mM. The effect of nifedipine was antagonized in an apparently competitive way by Bay K 8644, the apparent pA_2 value being 8.22. Bay K 8644 did not fit the requirements for competitive antagonism against verapamil and diltiazem, the slopes of Schild plots being 0.44 and 0.63, respectively.

Acetylcholine release Ca^{2+} channel blockers were found to reduce both resting and TS-induced ACh release in a concentration-dependent manner. The corresponding log concentration-response relationships are depicted in Figure 6. The calculated IC_{30} values (with 95% confidence limits) were 6.51 (3.50–12.13) $\times 10^{-7}$ M for nifedipine, 1.40 (0.97–2.02) $\times 10^{-6}$ M for verapamil and 3.82 (2.05–7.24) $\times 10^{-6}$ M for diltiazem in inhibiting resting ACh release and 2.53 (1.42–4.51) $\times 10^{-7}$ M for nifedipine, 1.53 (0.82–2.84) $\times 10^{-6}$ M for verapamil and 5.13 (2.29–11.50) $\times 10^{-6}$ M for diltiazem in inhibiting TS-induced ACh release. The corresponding potency ratios were: nifedipine (1) ⩾ verapamil (0.47) > diltiazem (0.17); and nifedipine (1) > verapamil (0.16) ⩾ diltiazem (0.049), respectively. Nifedipine was significantly more potent against TS-induced than resting ACh release, the corresponding IC_{30} potency ratio of resting against stimulated ACh release being 2.57. Nifedipine although more potent, was much less efficacious than verapamil. The potency of verapamil was strongly dependent on changes in the external Ca^{2+} concentration. Ca^{2+}-related changes of log concentration-response relationships of verapamil in reducing resting ACh release are shown in Figure 7. Verapamil (6×10^{-6} M)-induced inhibition of resting ACh release was abolished by pretreatment with Bay K 8644 (3×10^{-6} M), but unaffected by pretreatment with yohimbine (1×10^{-6} M).

NANC nerve-mediated relaxation The frequency-response relationships for NANC inhibitory responses in the presence of different Ca^{2+} channel blockers at concentrations insufficient to modify smooth muscle tone but effective in inhibiting

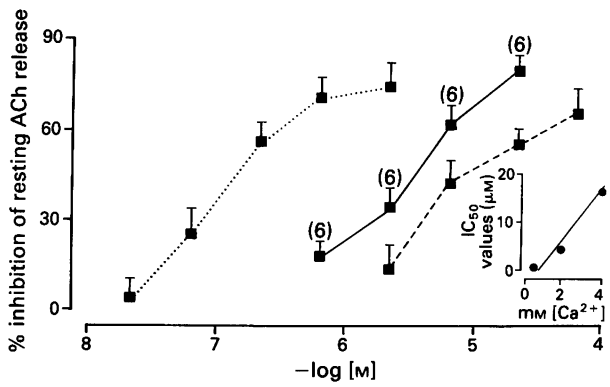

Figure 7 Ca^{2+}-dependence of verapamil-induced inhibition of acetylcholine (ACh) release from guinea-pig isolated colon. Log concentration-response relationships were obtained in organs perfused with Tyrode solution containing 0.5 mM Ca^{2+} (·····); 1.8 mM Ca^{2+} (——); 4 mM Ca^{2+} (– – –). Log concentrations of verapamil are plotted against percentage inhibition of resting acetylcholine release. Unless otherwise indicated, each point represents the mean of 4 experiments. Vertical bars indicate s.e.mean. A significant correlation can be observed between external Ca^{2+} concentration and IC_{50} values of verapamil (inset).

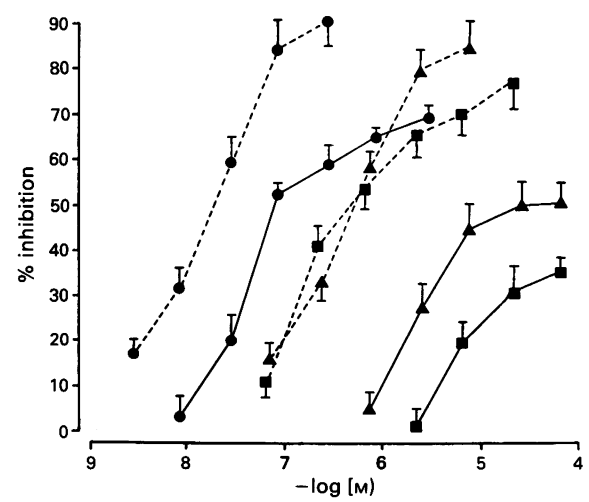

Figure 8 Log concentration-response relationships of Ca^{2+} channel blockers in inhibiting circular muscle contractility in guinea-pig isolated colon. Log concentrations of nifedipine (●); verapamil (■); diltiazem (▲) are plotted against percentage inhibition of contraction elicited by carbachol at supramaximal concentration (– – –) and the fractional tone inhibition (——) in respect to the maximal relaxation produced by papaverine. Each point represents the mean of 5 experiments. Vertical bars indicate s.e.mean.

Table 1 Ratios of the potencies calculated for each Ca^{2+} channel blocker in inhibiting propulsion, acetylcholine (ACh) release and smooth muscle contractility in guinea-pig isolated colon, and muscular versus neuronal selectivity indices, calculated from the analysis of log concentration-response relationships

	Propulsion versus resting ACh release	*Propulsion versus circular muscle tone*	*Carbachol-induced contraction versus resting ACh release*	*Selectivity index**
Nifedipine	107.82 (82.24–141.35)†	3.59 (2.55–5.05)	229.24 (167.22–314.26)	22.69 (14.99–34.35)
Verapamil	5.96 (4.97–7.16)	71.41 (57.51–88.67)	4.51 (3.23–6.30)	0.039 (0.025–0.061)
Diltiazem	9.94 (7.77–12.72)	9.45 (7.30–12.23)	28.83 (20.93–39.71)	1.09 (0.69–1.71)

* Selectivity index for each Ca^{2+} channel blocker was calculated from the log concentration-response relationships in inhibiting circular muscle tone versus resting ACh release.
† (95% confidence limits)

peristaltic reflex are represented in Figure 3. The dihydropyridines nifedipine and Bay K 8644 were both able to increase NANC relaxation, the effect being independent of the frequency employed, while verapamil and diltiazem had no effect.

Circular muscle resting tone and contractility Log concentration-response relationships of nifedipine, verapamil and diltiazem in inhibiting the resting tone and carbachol-induced contraction of circular muscle are shown in Figure 8. All drugs exhibited higher potency and efficacy against contracted than against resting musculature. This feature was more evident for verapamil than for nifedipine. Potency ratios for the inhibition of resting tone and carbachol-induced contraction as derived from IC_{50} or IC_{30} values were: nifedipine (1) > diltiazem (0.012) > verapamil (0.001); and nifedipine (1) > diltiazem (0.034) ≥ verapamil (0.022), respectively.

Discussion

It is well documented that several types of Ca^{2+} channels with some tissue specificity are present in excitable cells, which are believed to contribute to the differences in sensitivity to Ca^{2+} channel blockers exhibited by nervous and muscular tissues (Lee & Tsien, 1983; Hurwitz, 1986; Callewaert *et al.*, 1989). The physiological relevance of dihydropyridine binding sites in neuronal cells has long been questioned (Miller & Freedman, 1984). However, data have recently accumulated suggesting a functional role for drug-sensitive Ca^{2+} channels both in the central and in the peripheral nervous systems (Middlemiss & Spedding, 1985; Turner & Goldin, 1985; Thayer *et al.*, 1986; Miller, 1987; Hirning *et al.*, 1988; Tsien *et al.*, 1988; Miller *et al.*, 1988). Under certain conditions sensitivity of cholinergic as well as adrenergic and glutamatergic neurones to Ca^{2+} channel blockers of different classes has been reported (Starke *et al.*, 1984; Nordstrom *et al.*, 1986; Bartfai & Vizi, 1986). As far as the enteric nervous system is concerned, most neurophysiological studies indicate a low sensitivity of intramural nerves to Ca^{2+} channel blockers (Wood, 1987), although cholinergic and NANC inhibitory neurones have recently been reported to be modulated by dihydropyridines (Bornstein *et al.*, 1985; Katsuragi *et al.*, 1990). On the other hand, although spontaneous myoelectrical and mechanical activities can hardly be affected even by high concentrations of organic Ca^{2+} channel blockers, inhibition of intestinal smooth muscle has been reported to occur at subnanomolar concentrations during K^+ or receptor-mediated excitation (Rosenberg *et al.*, 1979; Golenhofen, 1981; Barone *et al.*, 1986; Terada *et al.*, 1987). However, to what extent smooth muscle inhibition or impairment of neurotransmission in the enteric plexuses could contribute to the end effect of Ca^{2+} channel blockers on intestinal complex motor function (e.g. propulsive activity) remains to be determined. Peristaltic reflex in the isolated intestine is a sensitive and reliable method to assess the viability of enteric neuronal synapses during activation by a physiological stimulus, and ACh release and NANC responses reflect the activity of functionally relevant enteric neurone subpopulations.

In order to interpret the mechanism of Ca^{2+} channel blocker motor inhibitory effects, it is worth noting that in our experiments substances supposed to act by opening or blocking Ca^{2+} channels were able to affect in opposite directions release of ACh from enteric neurones in a concentration-dependent manner. However, a unique profile for each Ca^{2+} channel blocker emerged when the potencies exhibited by each substance against peristaltic reflex, muscle contractility and ACh release were compared (Table 1). Indeed, the muscular versus neuronal selectivity index was about 23 for nifedipine and 0.04 for verapamil. While verapamil proved to be nearly as potent in slowing the peristaltic reflex as it was in reducing ACh release, the potency of nifedipine was about 100 fold higher against the peristaltic reflex than against ACh release, but nearly equal against peristaltic reflex and smooth muscle tone. This is highly suggestive of a substantial contribution of cholinergic neuronal inhibition to the impairment of the peristaltic reflex in the case of verapamil, while it contributes little to the end effect of nifedipine. Whatever the neural or muscular mechanisms responsible for the Ca^{2+} channel blocker-induced inhibition of peristaltic reflex, their effects, which can be easily suppressed both by increasing Ca^{2+} concentration or by Bay K 8644, are likely to be entirely dependent on interference with Ca^{2+} channels. However, the lack of competitive antagonism by Bay K 8644 of the inhibitory effects of verapamil and diltiazem on the peristaltic reflex seems to exclude competition for the same binding site and is compatible with a widely accepted model assuming an interaction with different binding sites on the same channel (Glossmann *et al.*, 1983).

Although Ca^{2+} channel blockers showed similar orders of potency to inhibitors of muscular and of nerve-mediated effects, the highest potency being exhibited in both cases by nifedipine, the ratios of the potencies were markedly different. In fact nifedipine, which was more than 500 fold more potent than verapamil in relaxing musculature, was less than twice as potent as verapamil in reducing ACh release.

Circular muscle sensitivity to verapamil was strongly dependent on the degree of excitation, a feature which occurred only to a minor extent with nifedipine. Indeed, the ratio of potencies in inhibiting carbachol-induced contraction and in relaxing resting circular muscle was about 260 for verapamil and only 7.19 for nifedipine.

The question can be raised as to whether the effect of verapamil on enteric cholinergic neurones is entirely dependent on Ca^{2+} channel blockade. In fact, verapamil is well known to bind to several neurotransmitter receptors, such as α_1- and α_2-adrenoceptors, muscarinic, and opioid receptors, and to block Na^+ channels besides Ca^{2+} channels (see Janis & Scriabine, 1983). All these properties could be responsible for the effects of verapamil on cholinergic neurotransmission. However, the low concentrations able to affect ACh release (see the concentration-response curve at 0.5 mM external Ca^{2+}) and the antagonism both by external Ca^{2+} and Bay K 8644 are all in favour of a selective action of verapamil on neuronal Ca^{2+} channels in our preparation.

The muscular and neuronal effects of Bay K 8644 could also help in interpreting the role and location of Ca^{2+} channels on the intestinal structures. Our experiments are suggestive of a correlation between the improvement of peristaltic reflex efficiency and the facilitatory effect on NANC neurotransmission, which both appear at concentrations far below those required to enhance either contractility or ACh release. A similar facilitatory effect of nifedipine on NANC inhibition, possibly due to a partial agonist activity (Glossmann *et al.*, 1985), has been reported previously in the ileum (Bornstein *et al.*, 1985).

In conclusion, by assuming all the effects of Ca^{2+} channel blockers observed in our preparations result from an interference with Ca^{2+} channels, the following hypotheses can be advanced: (a) Drug-sensitive Ca^{2+} channels are likely to be present on neurones of the enteric plexuses and to be involved in the modulation of both ACh release and NANC inhibition; (b) the Ca^{2+} channels modulating resting ACh release seem to be more efficiently blocked by verapamil-like drugs. However, dihydropyridine sensitive Ca^{2+} channels appear to be involved for a significant fraction (about 50%) of ACh released at rest unless this release is secondary to stretch caused by spontaneous muscle contractions. This fraction increased during Bay K 8644 induced excitation (up to 80%). (c) Ca^{2+} channels located on NANC neurones seem to exhibit the highest sensitivity for dihydropyridine moieties.

Impairment of peristaltic activity could result from blockade of either neuronal or muscular Ca^{2+} channels. While all Ca^{2+} channel blockers were more potent against propulsion than in inhibiting ACh release or the smooth muscle directly, they showed different nervous-muscular activity ratios. The spectrum of activities ranges from the high muscular-low neuronal profile of nifedipine to the low muscular-high neuronal profile of verapamil, diltiazem standing in an intermediate position.

Due to the high sensitivity of enteric neurones to Ca^{2+} channel blockers, in our opinion the neurotropic intestinal effects deserve to be investigated in the search for moieties with higher selectivity for the gastrointestinal tract.

References

ALBERTS, P. & STJARNE, L. (1982). Role of calcium in muscarinic autoinhibition of ^{3}H-acetylcholine secretion in guinea-pig ileum myenteric plexus. *Acta Physiol. Scand.*, **115**, 487–491.

ARUNLAKSHANA, O. & SCHILD, H.P. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BARONE, F.C., WHITE, R.F., ORMSBEE, H.S. & WASSERMAN, M.A. (1986). Effects of calcium channel entry blockers, nifedipine and nivaldipine, on colonic motor activity. *J. Pharmacol. Exp. Ther.*, **237**, 99–106.

BARTFAI, T. & VIZI, E.S. (1986). Prevention by nimodipine, a calcium entry blocker, of the effect of α-adrenoceptor blocking agents on noradrenaline release: differential effects of nimodipine on (^{3}H)noradrenaline and (^{14}C)acetylcholine release measured concomitantly from the guinea-pig ileum. *Arch. Int. Pharmacodyn.*, **284**, 212–224.

BOLGER, G.T., GENDO, P., KLOCKOWSKI, R., SIEGEL, H., JANIS, R.A., TRIGGLE, A.M. & TRIGGLE, D.J. (1983). Characterization of binding of the Ca^{++} channel antagonist, ^{3}H nitrendipine, to guinea-pig ileal smooth muscle. *J. Pharmacol. Exp. Ther.*, **225**, 291–309.

BORNSTEIN, J.C., LEES, G.M. & McBAIN, C.J. (1985). Comparative effects of calcium antagonists on inhibitory junction potentials of circular muscle of guinea-pig ileum. *Br. J. Pharmacol.*, **86**, 724P.

CALLEWAERT, G., HANBAUER, I. & MORAD, M. (1989). Modulation of calcium channels in cardiac and neuronal cells by an endogenous peptide. *Science*, **243**, 663–666.

CASTELL, D.O. (1985). Calcium-channel blocking agents for gastrointestinal disorders. *Am. J. Cardiol.*, **55**, 210B–313B.

COWIE, A.L., KOSTERLITZ, H.W. & WATERFIELD, A.A. (1978). Factors influencing the release of acetylcholine from the myenteric plexus of the ileum of the guinea-pig and rabbit. *Br. J. Pharmacol.*, **64**, 565–580.

FINNEY, D.J. (1978). *Statistical Method in Biological Assay*. 3rd edn. London: Charles Griffin Ltd.

FRIGO, G.M. & LECCHINI, S. (1970). An improved method for studying the peristaltic reflex in the isolated colon. *Br. J. Pharmacol.*, **39**, 346–356.

FRIGO, G.M., LECCHINI, S., MARCOLI, M., TONINI, M., D'ANGELO, L. & CREMA, A. (1984). Changes in sensitivity to the inhibitory effects of adrenergic agonists on intestinal motor activity after chronic sympathetic denervation. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **325**, 145–152.

GLOSSMANN, H., FERRY, D.R., GOLL, A., STRIESSNIG, J. & ZERNIG, G. (1985). Calcium channels: introduction into their molecular pharmacology. In *Cardiovascular Effects of Dihydropyridine-type Calcium Antagonists and Agonists* ed. Fleckenstein, A., Van Breemer, C., Grob, R. & Hoffmeister, F., pp. 113–139. Berlin, Heidelberg: Springer Verlag.

GLOSSMANN, H., FERRY, D.R., LUBBECKE, F., MEWES, R. & HOFMANN, F. (1983). Identification of voltage operated calcium channels by binding studies: differentiation of subclasses of calcium antagonist drugs with 3H-nimodipine radioligand binding. *J. Recept. Res.*, **3**, 177–190.

GODFRAIND, T., MILLER, R. & WIBO, M. (1986). Calcium antagonisms and calcium entry blockade. *Pharmacol. Rev.*, **38**, 321–416.

GODFRAIND, T. & WIBO, M. (1985). Subcellular localization of [^{3}H]-nitrendipine binding sites in guinea-pig ileal smooth muscle. *Br. J. Pharmacol.*, **85**, 335–340.

GOLENHOFEN, K. (1981). Differentiation of calcium activation processes in smooth muscle using selective agonists. In *Smooth muscle: an Assessment of Current Knowledge*. pp. 157–170. ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. London: Arnold.

HEDNER, T. (1986). Calcium channel blockers: spectrum of side effects and drug interaction. *Acta Pharmacol. Toxicol.*, **58** (suppl 2), 119–136.

HIRNING, L.D., FOX, A.P., McCLESKEY, E.W., PLIVERA, B.M., THAYER, S.A., MILLER, R.J. & TSIEN, R.W. (1988). Dominant role of N-type Ca^{2+} channels in evoked release of norepinephrine from sympathetic neurons. *Science*, **239**, 57–61.

HURWITZ, L. (1986). Pharmacology of calcium channels and smooth muscle. *Annu. Rev. Pharmacol. Toxicol.*, **26**, 225–258.

JANIS, R.A. & SCRIABINE, A. (1983). Sites of action of Ca^{2+} channel inhibitors. *Biochem. Pharmacol.*, **32**, 3499–3507.

KATSURAGI, T., SHIRAKABE, K., OGAWA, S., SOEJIMA, O. & FURUKAWA, T. (1990). Involvement of dihydropyridine-sensitive Ca^{2+} channels in adenosine-evoked inhibition of acetylcholine release from guinea pig ileal preparation. *J. Neurochem.*, **55**, 363–369.

LECCHINI, S., DEL TACCA, M., SOLDANI, G., FRIGO, G.M. & CREMA, A. (1969). The actions of atropine, tropenziline and N-butyl hyosacine bromide on the isolated distal colon of guinea-pig: a comparison of their activities and mechanisms of action. *J. Pharm. Pharmacol.*, **21**, 662–667.

LEE, K.S. & TSIEN, R.W. (1983). Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendimine in single dialysed heart cells. *Nature*, **302**, 790–794.

MIDDLEMISS, D.N. & SPEDDING, M. (1985). A functional correlate for dihydropyridine binding site in rat brain. *Nature*, **314**, 94–96.

MILLER, R.J. (1987). Multiple calcium channels and neuronal function. *Science*, **235**, 46–52.

MILLER, R.J., EWALD, D.A., FOX, A.P., HIRNING, L.D., McCLESKEY, E.W., PERNEY, T.M., STUREK, M., THAYER, S.A., TSIEN, R.W. & WALKER, M.W. (1988). The effect of calcium channel antagonists on peripheral neurones. *Ann. N.Y. Acad. Sci.*, **521**, 269–277.

MILLER, R.J. & FREEDMAN, S.B. (1984). Are dihydropyridine binding sites voltage sensitive calcium channels? *Life Sci.*, **34**, 1205–1221.

NORDSTROM, O., BRAESCH-ANDERSEN, S. & BARTFAI, T. (1986). Dopamine release is enhanced while acetylcholine release is inhibited by nimodipine (Bay e 9736). *Acta Physiol. Scand.*, **126**, 115–119.

PATON, W.D.M. & VIZI, E.S. (1969). The inhibitory action of noradrenaline and adrenaline on acetylcholine output by guinea-pig longitudinal muscle strips. *Br. J. Pharmacol.*, **35**, 10–28.

PRIOR, A., HARRIS, S.H. & WHORWELL, P.J. (1987). Reduction of colonic motility by intravenous nicardipine in irritable bowel. *Gut*, **28**, 1609–1612.

ROSENBERG, L.B., TICKU, M.K. & TRIGGLE, D.J. (1979). The effects of Ca^{2+} movements in guinea-pig ileal longitudinal muscle. *Can. J. Physiol. Pharmacol.*, **57**, 333–347.

STARKE, K., SPATH, L. & WICHMANN, T. (1984). Effects of verapamil, diltiazem and ryosidine on the release of dopamine and acetylcholine in rabbit caudate nucleus slices. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **325**, 124–130.

TERADA, K., KITAMURA, K. & KURIYAMA, H. (1987). Blocking actions of Ca^{2+} antagonists on the Ca^{2+} channels in the smooth muscle cells membrane of rabbit small intestine. *Pflügers Arch.*, **408**, 552–557.

THAYER, S.A., MURPHY, S.N. & MILLER, R.J. (1986). Widespread distribution of dihydropyridine-sensitive calcium channels in the central nervous system. *Mol. Pharmacol.*, **30**, 505–509.

TRAUBE, M. & McCALLUM, R.W. (1984). Calcium-channel blockers and the gastrointestinal tract. *Am. J. Gastroenterol.*, **79**, 892–896.

TSIEN, R.W., LIPSCOMBE, D., MADISON, D.V., BLEY, K.R. & FOX, A.P. (1988). Multiple types of neuronal calcium channel and their selective modulation. *Trends Neurosci.*, **11**, 431–437.

TURNER, T.J. & GOLDIN, S.M. (1985). Calcium channels in rat brain synaptosomes: identification and pharmacological characterization. *J. Neurosci.*, **5**, 841–849.

WOOD, J.D. (1987). Physiology of the enteric nervous system. In *Physiology of the Gastrointestinal Tract*, ed. Johnson, L.R. pp. 67–109. New York: Raven Press.

YOKOYAMA, K., SHIMIZU, M. & YAGASAKI, O. (1986). Effect of external Ca^{2+} on the spontaneous and the various stimuli-induced acetylcholine release from guinea-pig ileum myenteric plexus. *Jpn. J. Pharmacol.*, **40**, 194–198.

YOUSIF, F.B. & TRIGGLE, D.J. (1986). Inhibitory actions of a series of Ca^{2+} channel antagonists and K^+ depolarization induced responses in smooth muscle: an assessment of selectivity of action. *Can. J. Physiol. Pharmacol.*, **64**, 273–283.

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Modification of the rat uterine response to oestrogen and tamoxifen by thromboxane antagonists

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1 The thromboxane receptor antagonists EP092, AH23848 and BM 13.505 were used to investigate the role of thromboxane in the uterotrophic response to oestradiol and tamoxifen.

2 The parameters examined were uterine blood flow (measured by the microsphere technique), uterine wet and dry weights and the concentrations of cytosolic and nuclear oestrogen receptors.

3 Only EP092 potentiated the hyperaemic response to oestrogen but all three thromboxane antagonists inhibited oestradiol-stimulated uterine growth. This inhibition was accompanied by a decrease in nuclear oestrogen receptor concentration.

4 The uterotrophic response to tamoxifen was unaffected by the thromboxane antagonists.

5 The mechanism by which the thromboxane antagonists may be exerting their growth inhibitory effect is discussed, although, whether this effect can be attributed to blockade of thromboxane receptors or to some other mechanism is not clear from this study.

Introduction

As a time delay is apparent between the administration of oestradiol and the onset of the early uterotrophic response, a number of mediators have been implicated in the induction of the response to the steroid. Much experimental evidence points to a prostaglandin involvement in oestrogen-induced uterine events (Ryan *et al.*, 1974; Phaily & Senior, 1978). Various groups have reported increases in uterine prostaglandin synthesis after oestrogen exposure (Castracane & Jordan, 1975; Ham *et al.*, 1975; Poyser & Scott, 1980; Poyser, 1983); indeed uterine prostaglandin synthetase activity correlates with ovarian oestradiol output (Poyser & Scott, 1980). Since rat uterine homogenates are capable of generating both thromboxane A_2 and prostacyclin (Brown & Poyser, 1985), it is likely that the balance between the two is crucial to many reproductive processes.

In this study the contribution of thromboxane to the uterotrophic response induced by oestrogen and the non-steroidal anti-oestrogen, tamoxifen (which in the rat behaves as a partial agonist), was investigated by use of thromboxane receptor blocking drugs, namely EP092, AH23848 and the non-prostanoid antagonist BM 13.505. These compounds have proven receptor blocking activity (Armstrong *et al.*, 1984; 1985; Brittain *et al.*, 1985; Stegmeier *et al.*, 1986).

Methods

Mature virgin female rats of a CD-derived Sprague-Dawley random-bred strain from the Animal House, University of Bradford, were used throughout and were housed in light (07 h 00 min–19 h 00 min) and temperature (18°C) controlled rooms. Food and water were available *ad libitum*. All the animals in this study were bilaterally ovariectomized at least 21 days before further experimentation and were randomly assigned to groups. All the animals used were of similar body weight (around 330 g); there was no significant difference in weights within groups or between groups.

Measurement of blood flow

The microsphere technique (previously described by Phaily & Senior, 1978) was used to determine blood flow in the rat, anaesthetized with sodium pentobarbitone (60 mg kg^{-1}). This dose was selected to ensure anaesthetic control throughout the experimental procedure and minimal cardiovascular changes. The microspheres used were 15 μm in diameter (NEN-Trac, New England Nuclear, Boston, MA, U.S.A.), uniformly labelled with Sc^{46} and suspended in 10% (w/v) dextran containing 0.1% (w/v) Tween 80. Approximately 100,000 microspheres were injected into the left ventricle at a steady rate for 10 s. Uterine blood flows were calculated from wet weight of tissue. In all cases blood flow between the adrenals and kidneys was balanced, indicating a uniform distribution of microspheres. In the control group cardiac output was 113 ± 13 ml min^{-1} and was unaffected by any of the treatments as was heart rate (375 ± 15 beats min^{-1}). When dry weights are quoted the tissue was dried until the weight remained constant.

Measurement of cytoplasmic and nuclear oestrogen receptors by [^{3}H]-oestradiol exchange

Homogenization, cytosol and nuclear pellet preparation and determination of oestrogen receptors therein was carried out by the method of Marshall & Senior (1987). Briefly, animals were killed by cervical dislocation and the uteri were rapidly removed and homogenized in PE buffer (10 mM phosphate buffer, pH 7.4, containing 1.5 mM EDTA and 2 mM mercaptoethanol). All procedures were performed at 4°C unless otherwise stated. The exchange conditions employed were found to be optimal with respect to maximal exchange with minimal loss of binding sites. Homogenates were centrifuged at 100,000 *g* for 1 h. Aliquots of cytosol were incubated at 30°C for 1 h with 1–20 nM of [^{3}H]-oestradiol ([2,4,6,7-^{3}H]-oestradiol, 85–110 Ci mmol^{-1}) (Radiochemical Centre, Amersham, Bucks) plus or minus 100 fold excess of oestradiol. Separation of bound from free ligand was achieved by use of dextran-coated charcoal (0.5% dextran (BDH, Poole), 5% Norit A acid washed charcoal w/v in phosphate buffer). The radioactivity in the supernatant was determined by liquid scintillation counting. The assay of nuclear oestogen receptors involved the preparation of a low speed nuclear pellet (prepared by centrifugation at 800 *g* of homogenate). Aliquots of the nuclear suspension were treated in the same way as in the cytosolic assay. Separation was achieved with a 60% hydroxylapatite suspension in phosphate buffer. After ethanolic extraction of the pellet, radioactivity was determined by liquid scintillation counting (45% efficiency).

In this work the term cytoplasmic receptor is used: this refers to the receptor form that separates into the cytoplasmic cell fraction after homogenization. It represents the unoccupied inactive form of the receptor thought to be

located in the nucleus *in vivo* (King & Greene, 1984). The nuclear or activated form of the receptor remains in the nuclear compartment after fractionization. Although receptor localization is important, in terms of this study, receptor measurements made are used in a qualitative manner to assess the effect of drug treatments on the receptor population as a whole, rather than as an attempt to compartmentalize the oestrogen receptor.

Drug administration

Oestradiol (oestra-1,3,5-(10)-triene-3,17β-diol; BDH, Poole) was dissolved in a 10% (v/v) solution of propylene glycol and was administered intravenously (i.v.) at a dose level of 0.5 μg kg^{-1} into the tail vein of the conscious rat, 3 h before measurements were made. This dose of oestradiol was chosen as it gives maximal uterine blood flow 3 h after administration and is the minimum dose to produce this effect. Tamoxifen (ICI, Macclesfield, Cheshire) was dissolved in ethanol and dispersed in arachis oil before subcutaneous (s.c.) injection, 24 h before measurements were made, at a dose level of 1 mg kg^{-1} as previously described (Majid & Senior, 1982). Serum levels of tamoxifen have also been shown to peak at this time (Bowman *et al.*, 1982). The solvents used did not have any effect on the parameters measured and these results have been grouped as controls. EP092 ((±)-5-endo(6'-carboxyhex-2'Z-enyl)-6-exo-(1''-[N-(phenylthiocarbamoyl)-hydrazono]-ethyl-bicyclo[2,2,1] heptane) (Pharmacology Dept., University of Edinburgh) was dissolved in absolute alcohol (5 mg ml^{-1}) plus 2.5 μl M sodium hydroxide per mg EP092; the ethanol was then evaporated under nitrogen and the resultant sodium salt was dissolved in aqueous sodium hydroxide (M/400)/sodium chloride (0.9% w/v). EP092 was given 1 h 30 min and 3 h 10 min before measurements were made. AH23848 ([1α(Z),2β, 5α]-(±)-7-[5-[[(1,1'-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxo-cyclopentyl]-4-heptenoic acid) (Glaxo Group Research) was dissolved in 1% w/v sodium bicarbonate in sodium chloride (0.9% w/v). BM 13.505 (4-(2-(4-chlorobenzenesulphonamide)-ethyl)-benzene acetic acid) (Boehringer Mannheim, GmbH) was obtained in a stock solution of BM 13.505 100 mg, meglumin 65 mg; mannitol 164 mg; Tritriplex III 1 mg in water for injection to 5 ml, all subsequent dilutions were made in water for injection. Both AH23848 (10 mg kg^{-1}) and BM 13.505 (20 mg kg^{-1}) were administered intravenously 3 h 10 min before measurements were made. The doses of the thromboxane antagonists were chosen after preliminary studies which indicated that at these dosage levels the uterotrophic response was significantly altered.

Statistical analysis

The results (expressed as mean ± s.e.mean) were compared by analysis of variance and Student's *t* test (two tailed) (Snedecor & Cochran, 1979).

Results

In the absence of any exogenous oestradiol the thromboxane antagonists had no significant effect on the parameters studied.

Effect of EP092, AH23848 and BM 13.505 on oestradiol stimulated blood flow (Table 1)

The vehicles used for the thromboxane antagonists had no effect upon the oestradiol response. The addition of EP092 to the oestradiol regimen significantly enhanced the blood flow response. AH23848 and BM 13.505 had no effect on the oestradiol-induced hyperaemia. At these doses the antagonists had no effect on cardiovascular function or blood flow to other organs.

Effect of EP092, AH23848 and BM 13.505 on oestradiol-stimulated uterine weight (Table 1)

BM 13.505 reduced the uterine water imbibition response to oestradiol. All three antagonists effected a significant decrease in oestradiol-induced uterine dry weight.

Effect of EP092, AH23848 and BM 13.505 on tamoxifen-stimulated blood flow (Table 2)

No effect upon tamoxifen-stimulated uterine blood flow was observed in the presence of the thromboxane antagonists or their vehicles, nor did the antagonists affect the cardiovascular and regional organ blood flow in response to tamoxifen.

Effect of EP092, AH23848 and BM 13.505 on tamoxifen-stimulated uterine weight (Table 2)

The increases in tamoxifen-stimulated wet and dry uterine weight were unaffected by the presence of the thromboxane antagonists.

Table 1 The effect of EP092 (10 mg kg^{-1} i.v., 2 doses) given 10 min before and 1 h 30 min after oestradiol, AH23848 (2.5 mg kg^{-1} i.v., single dose) and BM 13.505 (20 mg kg^{-1} i.v., single dose) given 10 min before oestradiol on uterine blood flow and weight

Pretreatment	*No. of rats*	*Uterine blood flow* (ml min^{-1} 100 g^{-1})	*Uterine weight* (mg) *Wet*	*Dry*
None (control)	7	37 ± 10	75 ± 8	17 ± 1
Oestradiol alone	8	536 ± 102†††	99 ± 5†††	27 ± 1†††
Oestradiol + EP092 vehicle	7	540 ± 96	100 ± 5	26 ± 1
Oestradiol + EP092	12	889 ± 119*	92 ± 12	22 ± 1*
Oestradiol + AH23848 vehicle	8	542 ± 90	97 ± 6	27 ± 1
Oestradiol + AH23848	7	624 ± 67	91 ± 5	20 ± 1***
Oestradiol + BM 13.505 vehicle	6	560 ± 13	99 ± 4	24 ± 1
Oestradiol + BM 13.505	4	562 ± 85	81 ± 5*	16 ± 1.5**

Measurements were made 3 h after oestradiol (0.5 μg kg^{-1} i.v., single dose) administration.
Values are mean ± s.e.mean; ††† $P < 0.001$ values are significantly different from those of control group; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$ values are significantly different from those of oestradiol + vehicle group.

Table 2 The effect of EP092 (10 mg kg^{-1} i.v., 2 doses) given 1 h 30 min and 3 h 10 min before tamoxifen, and AH23848 (2.5 mg kg^{-1} i.v., single dose) and BM 13.505 (20 mg kg^{-1} i.v., single dose) given 3 h 10 min before tamoxifen on uterine blood flow and weight

Pretreatment	No. of rats	Uterine blood flow (ml min^{-1} 100 g^{-1})	Uterine weight (mg) Wet	Dry
None (control)	7	37 ± 10	75 ± 8	17 ± 1
Tamoxifen alone	7	123 ± 26†	136 ± 7††	24 ± 1†
Tamoxifen + EP092 vehicle	7	105 ± 16	129 ± 8	23 ± 2
Tamoxifen + EP092	9	138 ± 14	138 ± 12	25 ± 2
Tamoxifen + AH23848 vehicle	7	120 ± 13	140 ± 6	24 ± 1
Tamoxifen + AH23848	6	112 ± 17	143 ± 11	25 ± 2
Tamoxifen + BM 13.505 vehicle	6	107 ± 11	131 ± 7	23 ± 1
Tamoxifen + BM 13.505	6	122 ± 15	133 ± 6	24 ± 1

Measurements were made 24 h after tamoxifen (1 mg kg^{-1} s.c., single dose) administration.
Values are mean ± s.e.mean; † $P < 0.05$; †† $P < 0.005$ values are significantly different from those of control group.

Effect of EP092, AH23848 and BM 13.505 on oestrogen receptor levels (Figure 1)

The vehicles used had no effect upon oestrogen receptor levels. Three hours after administration of oestradiol alone there was a decrease in cytosolic receptor concentration which was accompanied by an increase in nuclear receptor concentration compared to control. EP092 pretreatment induced a slight increase in cytosolic oestrogen receptor whilst causing a reduction in the number of oestrogen receptors in the nuclear compartment on comparison with oestradiol alone. Pretreatment with AH23848 had a similar effect but the increase in cytosolic receptor concentration was significant when compared with the oestradiol alone response. BM 13.505 had little effect on the oestradiol response with respect to cytosolic receptor, but this compound caused a marked reduction in nuclear receptor content. As the thromboxane antagonists had no apparent effect on the uterotrophic response to tamoxifen, equivalent receptor measurements were not made.

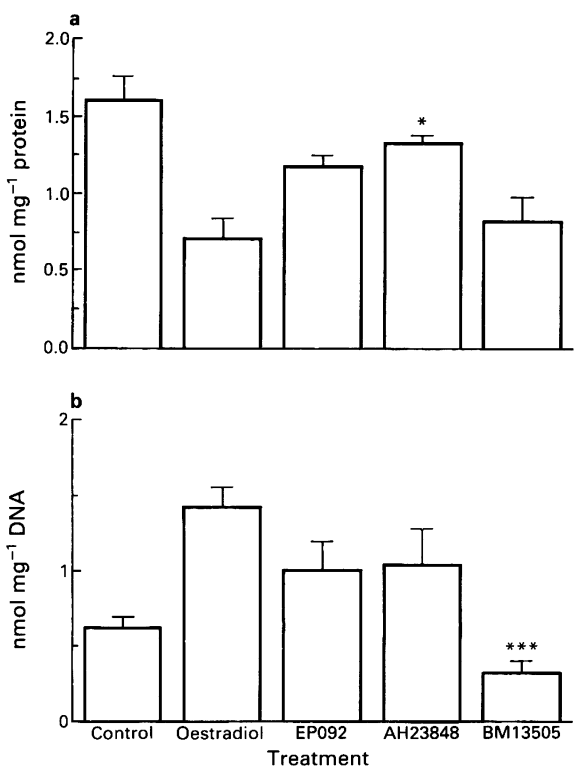

Figure 1 The effect of EP092 (10 mg kg^{-1}, i.v., 2 doses) given 10 min before and 1 h 30 min after oestradiol, AH23848 (2.5 mg kg^{-1}, i.v., single dose) and BM 13.505 (20 mg kg^{-1}, i.v., single dose) given 10 min before oestradiol on cytosolic (a) and nuclear (b) oestrogen receptor distribution in mature ovarectomized rats. Measurements were made 3 h after oestradiol (0.5 μg kg^{-1}, i.v., single dose) administration. Each column represents mean of 3–6 determinations, with 6 uteri per determination; vertical bars show s.e.mean. * $P < 0.05$, *** $P < 0.001$ values are significantly different from those of oestradiol alone group.

Discussion

Stimulation of prostaglandin synthesis by oestradiol has been widely accepted (Ramwell *et al.*, 1977). An oestradiol-induced rise in the thromboxane content of the ovariectomized rat uterus has also been reported (Jouanen *et al.*, 1985). Ramwell *et al.* (1977) suggested that much of the apparent stimulation of thromboxane synthesis by oestradiol may occur indirectly as a result of hyperaemia. Hyperaemia increases the blood volume in uterine tissues which consequently elevates the uterine platelet population. A numerical rise in platelet content alone may be sufficient to explain the enhanced thromboxane synthesis. However, direct oestrogen-stimulated thromboxane production by uterine myometrial and, to a greater extent, uterine endometrial tissue of ovariectomized rats has been reported (Brown & Poyser, 1985).

The cited evidence which suggests that oestradiol increases uterine thromboxane content, whether by *de novo* synthesis, increased uptake from other sources, enhanced release or diminished metabolic degradation, suggests that the thromboxane antagonists would enhance the oestradiol-induced hyperaemia through an ability to antagonize the powerful vasoconstrictor activity of thromboxane. The doses of the antagonists used in this work were selected as it has been demonstrated that they lie within the range of thromboxane antagonist activity (Armstrong *et al.*, 1984; 1985; Brittain *et al.*, 1985; Stegmeier *et al.*, 1986). However, in this study only EP092 was observed to elicit what would be expected as the classical effect of a thromboxane antagonist. Neither AH23848 nor BM 13.505 had any impact on the oestrogen-induced hyperaemia; this may be attributable to the fact that both these compounds display partial agonist activity (Brittain *et al.*, 1985; Patscheke *et al.*, 1987).

Only BM 13.505 caused a significant reduction in oestradiol-stimulated uterine wet weight although a similar trend was apparent with EP092 and AH23848. It has been suggested (Tchernitchin, 1979; 1983) that water imbibition is the result of eosinophilia. Therefore, it may be possible that thromboxane plays an effector role in the oestrogen induced cascade of events initiated by eosinophils; alternatively it may exert an anti-chemotactic influence on the eosinophils so as to prevent the initial migration to the uterus.

All the thromboxane antagonists caused a reduction in oestradiol-induced uterine growth. There is much evidence to substantiate a role for thromboxane in cell proliferation. Thromboxane A_2 is thought to be involved in initiating liver regeneration after partial hepatectomy in the rat (Kanzaki *et al.*, 1979), a tissue in which oestrogen receptors have been detected (Eisenfeld & Aten, 1979). Thromboxane A_2 has also been reported to enhance proliferation of B_{16} melanoma cells (Mahmud *et al.*, 1984); Honn *et al.* (1982) suggested a bidirectional control of tumour cell proliferation by the endogenous or exogenous thromboxane/prostacyclin balance; they demonstrated that thromboxane A_2 and its metabolites and analogues, stimulate tumour growth. It is possible that the stimulatory effect of thromboxane A_2 on cell growth may be attributed to an inhibitory action on adenylate cyclase. (Thromboxane B_2 causes a reduction in the basal level of intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) in B16a tumour cells: Honn *et al.*, 1982). However, cyclic AMP-independent inhibition of DNA synthesis has been observed by Wiley *et al.* (1983).

In the present study it could be presumed that the inhibitory effect of all three compounds on oestradiol-stimulated uterine dry weight was by virtue of thromboxane antagonism. However, no conclusive evidence can be presented to substantiate this, except that all three compounds have been shown to exhibit thromboxane receptor blocking activity on other smooth muscle preparations (vascular and non-vascular) and on platelets. In this instance, doubt is cast as to the exact nature of the dry weight-inhibitory effect on uterus since the classical vascular response to thromboxane antagonism, namely vasodilatation, was not uniformly observed with all three compounds.

Assuming that the mitogenic effects of oestrogen are at least partly dependent on the classical genomic response, the decreases in uterine dry weight seen in the presence of the thromboxane antagonists should be reflected by decreased nuclear receptor binding. This was in fact seen. This may be due to a direct interaction with the oestrogen receptor; alternatively, it could be an indirect effect, i.e. oestradiol was prevented from binding to its receptor in the presence of the thromboxane antagonists. Unfortunately, the quality of binding taking place cannot be assessed by the methodology employed in this work. The apparent loss of receptor observed as a result of pretreatment with BM 13.505 is suggestive of receptor processing which is associated with irreversible degradation of the activated receptor resulting in a diminution of functional receptor number (Horwitz *et al.*, 1978).

It is well-established that tamoxifen exhibits partial agonist properties in the rat uterus (Harper & Walpole, 1967). The agonist effect of tamoxifen on rat uterus has been demonstrated in this laboratory (Marshall & Senior, 1987), as have differences between the oestradiol- and tamoxifen-evoked responses (Marshall & Senior, 1986; 1989). In these experiments the thromboxane antagonists did not significantly alter the tamoxifen response. Tamoxifen is able to bind to the oestrogen receptor, although the resulting complex is thought to be in some way aberrant, which affects the interaction with the genome. It is possible that the conformational differences between the oestrogen/oestrogen receptor complex and the tamoxifen/oestrogen receptor complex may make the latter less susceptible to the effects of thromboxane receptor antagonists, hence the lack of modification of the uterine response. Alternatively, this discordance may be due to non-genomic mechanisms, as cell proliferation is also regulated by various growth factors such as platelet derived growth factor. Oestrogen and antioestrogen have opposing effects on peptide secretion (Dickson & Lippman, 1987). Therefore, it is possible that EP092, AH23848 and BM 13.505 are able to influence the factors produced as a result of oestrogen stimulation (hence the modification of the uterine weight response) but the antioestrogen evoked factors are not susceptible to their action.

In conclusion, the results of this study again point to diverging mechanisms of action between oestrogen and tamoxifen. From the results obtained in the oestradiol-stimulated uterus, it would seem that thromboxane may be a mediator of the early uterotrophic response. The susceptibility of the oestrogen weight response to inhibition by thromboxane antagonists is interesting as is the mechanism by which they exert this effect. Could the results of this work be extrapolated to other tissues where growth is hormone-dependent? We are accumulating evidence which indicates that the thromboxane antagonists inhibit growth of human derived hormone-dependent cell lines *in vitro*.

References

ARMSTRONG, R.A., JONES, R.L., PEESAPATI, U., WILL, S.G. & WILSON, N.H. (1984). Effect of the thromboxane receptor antagonist EP092 on early phase endotoxic shock in sheep. *Br. J. Pharmacol.*, **81**, 72P.

ARMSTRONG, R.A., JONES, R.L., PEESAPATI, U., WILL, S.G. & WILSON, N.H. (1985). Competitive antagonism at thromboxane receptors in human platelets. *Br. J. Pharmacol.*, **84**, 595–607.

BOWMAN, S.P., LEAKE, A. & MORRIS, I.D. (1982). Hypothalamic, pituitary and uterine cytoplasmic and nuclear oestrogen receptors and their relationship to the serum concentration of tamoxifen and its metabolite 4-hydroxytamoxifen, in the ovariectomised rat. *J. Endocr.*, **94**, 167–175.

BRITTAIN, R.T., BOUTAL, L., CARTER, M.C., COLEMAN, R.A., COLLINGTON, E.W., GEISCOW, H.P., HALLETT, P., HORNBY, J. & HUMPHREY, P.P. (1985). AH23848: a thromboxane receptor blocking drug that can clarify the pathophysiological role of thromboxane A_2. *Circulation*, **72**, 1208–1218.

BROWN, C.G. & POYSER, N.L. (1985). Further studies on prostaglandin and thromboxane production by the rat uterus during the oestrous cycle. *J. Reprod. Fert.*, **73**, 391–399.

CASTRACANE, V.D. & JORDAN, V.C. (1975). The effect of estrogen and progesterone on uterine prostaglandin biosynthesis in the ovariectomised rat. *Biol. Reprod.*, **13**, 587–596.

DICKSON, R.B. & LIPPMAN, M.E. (1987). Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. *Endocrine Rev.*, **8**, 29–43.

EISENFELD, A.J. & ATEN, R.F. (1979). Estrogen receptor in the mammalian liver. *Pharmacol. Steroid Biochem.*, **7**, 91–117.

HAM, E.A., CIRILLO, V.J., ZANETTI, M.E. & KUEHL, F.A. Jr. (1975). Estrogen-directed synthesis of specific prostaglandins in uterus. *Proc. Natl. Acad. Sci., U.S.A.*, **72**, 1420–1424.

HARPER, M.J.K. & WALPOLE, A.L. (1967). A new derivative of triphenylethylene. Effect on important action and mode of action in rats. *J. Reprod. Fert.*, **13**, 101–119.

HONN, K.V., DUNN, J.R. & MEYER, J. (1982). Thromboxanes and prostacyclin positive and negative modulators of tumor cell proliferation. In *Prostaglandins and Cancer:* First International Conference, pp. 375–379. New York: Alan R. Liss.

HORWITZ, K.B., KOSEKI, Y. & McGUIRE, W.L. (1978). Estrogen control of progesterone receptor in human breast cancer: Role of estradiol and antiestrogen. *Endocrinology*, **103**, 1742–1751.

JOUANEN, A., SAINTOT, M., THALER-DAO, H. & CRASTES DE PAULET, A. (1985). Prostaglandin synthesis from endogenous and exogenous arachidonic acid in the rat uterus. Effect of estradiol and progesterone. *Prostaglandins, Leukot. Med.*, **18**, 321–336.

KING, W.J. & GREENE, G.L. (1984). Monoclonal antibodies localise oestrogen receptor in the nuclei of target cells. *Nature*, **307**, 745–747.

KANZAKI, Y., MAHMUD, I., ASANAGI, M., FUKUI, N. & MIURA, Y. (1979). Thromboxane as a possible trigger of liver regeneration. *Cell. Mol. Biol.*, **25**, 147–152.

MAHMUD, I., FUKUI, N., MIURA, Y. & BRANDENSBERGER, H. (1984). Thromboxane synthesizing system in rat liver. *Prostaglandins, Leukot. Med.*, **16**, 371–377.

MAJID, E. & SENIOR, J. (1982). Anti-oestrogen modification of uterine responses to oestrogen in the rat. *J. Reprod. Fert.*, **66**, 79–85.

MARSHALL, K. & SENIOR, J. (1986). The effect of mepyramine and ranitidine on the oestrogen and antioestrogen stimulated rat uterus. *Br. J. Pharmacol.*, **89**, 251–256.

MARSHALL, K. & SENIOR, J. (1987). A study on the effect of a single dose of tamoxifen on uterine hyperaemia and growth in the rat. *Br. J. Pharmacol.*, **92**, 429–435.

MARSHALL, K. & SENIOR, J. (1989). The effect of a single dose of oestradiol on tamoxifen-induced uterine hyperaemia and growth in the rat. *Br. J. Pharmacol.*, **98**, 383–388.

PATSCHEKE, H. & STEGMEIER, K., HORNBERGER, W., STAIGER, C. & NEUGEBAUER, G. (1987). Inhibition of platelet activation by the novel thromboxane antagonist BM 13.505. *Thromb. Haemostas.*, **58**, 670P.

PHAILY, S.E. & SENIOR, J. (1978). Modification of oestrogen-induced hyperaemia by drugs in ovariectomised rats. *J. Reprod. Fert.*, **53**, 91–97.

POYSER, N.L. (1983). Differential stimulation of prostaglandins and thromboxane synthesizing capacities. *Prostaglandins, Leukot. Med.*, **10**, 163–177.

POYSER, N.L. & SCOTT, F.M. (1980). Prostacyclin and thromboxane production by the rat uterus and ovary *in vitro* during the oestrous cycle. *J. Reprod. Fert.*, **60**, 33–34.

RAMWELL, P.W., LEOVEY, E.M.K. & SINTENTOS, A.L. (1977). Regulation of arachidonic acid cascade. *Biol. Reprod.*, **16**, 70–87.

RYAN, M.J., CLARK, K.E., VAN ORDEN, D.E., FARLEY, D.B., EDVINSSON, L., SJOBERG, N-O., VAN ORDEN, L.S. III & BRODY, M.J. (1974). Role of prostaglandins in estrogen-induced uterine hyperaemia. *Prostaglandins*, **5**, 257–268.

SNEDECOR, G.W. & COCHRAN, W.G. (1979). *Statistical Methods*, (7th Edition). Ames, Iowa: Iowa State University Press.

STEGMEIER, K., PILL, J., MÜLLER-BECKMANN, B., SPONER, G. & PATSCHEKE, H. (1986). Comparison of the TX receptor blockers BM 13.177 and BM 13.505 *in vitro* and *in vivo*. *Proc. 6th Int. Conf. on Prostaglandins and Related Compounds*, p. 351, Florence, Italy: Fondazione Giovani Lorenzini.

TCHERNITCHIN, A.N. (1979). The role of eosinophil migration in the non-genomic response to oestrogens in the uterus. *J. Steroid Biochem.*, **11**, 417–442.

TCHERNITCHIN, A.N. (1983). Eosinophil-mediated non-genomic parameters of oestrogen stimulation – a separate group of responses mediated by an independent mechanism. *J. Steroid Biochem.*, **19**, 95–100.

WILEY, M.H., FEINGOLD, K.R., GRUNFELD, C., QUESNEY-HUNEEUS & WU, J.M. (1983). Evidence for cAMP-independent inhibition of S-phase DNA synthesis by prostaglandins. *J. Biol. Chem.*, **258**, 491–496.

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In vitro characterization of prostanoid EP-receptors in the non-pregnant human myometrium

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1 Prostaglandin receptors of the PGE type have been characterized in the non-pregnant human myometrium *in vitro* according to the scheme of Coleman *et al.* (1984) by use of the agonists PGE_2, sulprostone, rioprostil, AY23626, butaprost, misoprostol, 16,16-dimethylprostaglandin E_2, enprostil and iloprost, and, the antagonist AH6809.

2 All prostanoids tested were active in non-pregnant human myometrium either as stimulators and/or inhibitors of spontaneous activity or both. Biphasic responses to PGE_2 indicate that at least two receptor types of the EP-receptor exist, one mediating relaxation and the other mediating contraction.

3 Further evidence for the EP-receptor mediating excitation and relaxation was provided by the action of the EP_2-/EP_3-receptor selective prostanoids rioprostil, AY23626 and misoprostol, and the EP_1-/EP_2-receptor selective agonist 16,16-dimethylprostaglandin E_2.

4 Butaprost, an EP_2-receptor selective agonist, produced potent inhibition of spontaneous activity in the tissue which was generally longer-lasting than that evoked by the natural prostanoid PGE_2.

5 The EP_1-/EP_3-receptor selective agonist sulprostone and the EP_3-receptor agonist enprostil produced potent contractile responses supporting the presence of contractile EP_3-receptors in the non-pregnant human myometrium *in vitro*.

6 The EP_1-/IP-receptor selective agonist, iloprost, produced mixed responses in non-pregnant human myometrium. The contractile response was inhibited by the EP_1-receptor antagonist AH6809. However, responses to the EP_1-/EP_3-receptor selective agonist sulprostone were unaffected by AH6809 which may indicate that only a small population of EP_1-receptors is present.

7 Therefore it would seem that a heterogeneous population of EP-receptors is present in the non-pregnant human myometrium.

Introduction

Prostanoids appear to be involved in most aspects of uterine physiology and have been implicated in a multitude of pathophysiological conditions such as dysmenorrhoea, menorrhagia, preeclampsia and preterm labour. Indeed they have found their major therapeutic application in obstetrics and gynaecology. However, wide use of prostanoid medicines is limited by a high incidence of side-effects. Side-effect liability can be reduced by the development of drugs which have a selective action at a given receptor mediating the beneficial but not the deleterious effects. It is for this reason that the classification of prostanoid receptors is important.

Evidence for the existence of prostanoid receptors is abundant. They are highly potent in many biological systems and both their potency and profile of action are sensitive to small changes in chemical structure. In addition, the profile of activity is often species-, tissue-, or prostanoid-specific and in many cases, may be altered selectively by use of antagonists.

There are distinct receptors for the naturally-occurring prostanoids prostaglandin D (PGD), PGE (Jones, 1976), PGF, PGI and thromboxane which are designated 'P-receptors' (Kennedy *et al.*, 1982; 1983; Coleman, 1983; Coleman *et al.*, 1984). Furthermore the EP-receptor can be arbitrarily subdivided into EP_1-, EP_2- and EP_3-receptors on the basis of studies with selective agonists and antagonists (Coleman, 1987; Coleman *et al.*, 1985; 1987a,b). The classification is based on agonist and antagonist activity in both functional and binding studies. There are now many selective agonists and antagonists for the EP-receptor but there are few which show absolute selectivity for the individual EP_1-, EP_2- and EP_3-subtypes. In general, conclusions must be drawn on the basis of results obtained from various combinations of receptor selective compounds, and a process of elimination. To date, butaprost (TR4979) (Gardiner *et al.*, 1986) appears to be the most selective agonist for the study of EP-receptors in that it shows high potency and selectivity at the EP_2-receptor subtype, with little or no activity for the EP_1- or EP_3-receptors. Other PGE analogues such as sulprostone, rioprostil and misoprostol are useful but show activity at two EP-receptor types.

Thus sulprostone (Hess *et al.*, 1979), a stable acyl sulphonamide analogue of PGE_2 shows selectivity for both EP_1- and EP_3-receptors (Coleman *et al.*, 1987a,b; 1988), and rioprostil (Kluender & Woesser, 1979) shows selectivity for the EP_2- and EP_3-receptors (Reeves *et al.*, 1988; Coleman *et al.*, 1988).

Misoprostol is a potent anti-secretory prostaglandin, structurally related to PGE_1 (Collins *et al.*, 1985). It is believed that it exerts its gastric anti-secretory effects via EP_3-receptors (Reeves *et al.*, 1988). Some degree of EP_2-receptor activity has also been reported with misoprostol (Coleman *et al.*, 1988). 16,16-dimethyl prostaglandin E_2 has a selectivity of action within EP-receptors, being at least 10 times more potent on EP_1- than on EP_2-receptors (Coleman, 1987). Some action via the EP_3-receptor has also been recorded (Reeves *et al.*, 1988). Enprostil, a synthetic dehydroprostaglandin E_2 has activity at the EP_3-receptor and also at the FP- and TP-receptors (Eglen *et al.*, 1989) and EP_1-receptor (Coleman *et al.*, 1988). The combined use of these compounds in conjunction with EP_1-receptor blocking drugs can yield useful information about receptor populations.

Iloprost, which displays potent EP_1-, but negligible EP_2- or EP_3-receptor activity (Sheldrick *et al.*, 1988) may then corroborate evidence obtained with other EP-receptor selective agonists. Iloprost is also an IP-receptor agonist.

SC19920 (Sanner, 1972), though a weak antagonist at the EP_1-receptor, does appear to be selective in its prostanoid receptor blocking activity. AH6809, has a similar profile of EP_1-receptor blocking activity though it is approximately

10–40 times more potent with demonstrated pA_2 values ranging between 6.5–6.8 (Coleman *et al.*, 1985). AH6809 does have weak action at other receptors including the DP- and TP-receptors (Keery & Lumley, 1988) yielding pA_2 values at each site of 5.4 and 4.4 respectively. In this study AH6809 was used at a concentration of 10 μM to eliminate effectively any EP_1-receptor involvement.

The selectivity profiles of the agonists can be summarised as follows:

rioprostil/AY23626/misoprostol	$-EP_3 > EP_2 > EP_1$
16,16-dimethyl PGE_2	$-EP_3 > EP_1 > EP_2$
sulprostone	$-EP_3 \approx EP_1 \gg EP_2 \approx 0$
enprostil	$-EP_3 > EP_1 \gg EP_2$
butaprost	$-EP_2 \gg EP_1$ & $EP_3 > 0$
iloprost	$-EP_1(=IP) \gg EP_2$ & EP_3

In addition, 16,16-dimethyl PGE_2 and enprostil are known to have a similar level of FP-receptor agonist activity.

In vitro PGE_2 predominantly inhibits spontaneous myometrial activity in corporal specimens (Bygdeman & Eliasson, 1963; Bygdeman, 1964) though other workers have observed mild excitation (Pickles *et al.*, 1965) and more recent evidence has reported a biphasic response (Wiqvist *et al.*, 1983; Maigaard *et al.*, 1986) consisting of stimulation of spontaneous myometrial activity followed by inhibition at higher doses.

In non-pregnant human myometrium *in vivo* early work has shown a potent stimulatory effect after intravenous, intramuscular or subcutaneous administration of PGE_2 (Karim *et al.*, 1971) which originally suggested that a discrepancy existed between the effect of PGE_2 on the non-pregnant myometrium *in vitro* as compared to *in vivo*. However, later work demonstrated an inhibitory effect after intrauterine administration of PGE_2 (Toppozada *et al.*, 1974) which coincides with *in vitro* results. We have previously reported a biphasic response with PGE_2 in the spontaneously active human myometrium *in vitro* (Clayton *et al.*, 1986a). Generally, these effects are consistent with a physiological role for prostanoids in the regulation of uterine motility throughout the menstrual cycle and the identification of the EP-receptors will confer clinical advantages in treatment based upon PGE_2 and its analogues.

Methods

Non-pregnant human myometrium was obtained from premenopausal patients undergoing hysterectomy for the treatment of various benign disorders such as menorrhagia and fibroids. Patients with underlying malignancy were excluded from the studies. Samples of non-pregnant human myometrium were taken from the anterior wall of the corpus uteri. The phase of the menstrual cycle was assessed by histological examination of the endometrium by the pathology department of St. Luke's Hospital, Bradford. After surgery, human tissue samples were placed in Krebs solution at room temperature and transported to the laboratory where experimental preparations were completed within a 60 min post-operative period.

Samples of human myometrium were cut in the direction of muscle fibres to produce strips (20 × 3 × 3 mm) of predominantly longitudinal muscle. Care was taken to ensure that each strip was free from either endometrium or serosa. The tissues were placed under an initial passive tension of 2 g and superfused at a rate of 2 ml min^{-1} with Krebs solution aerated with 95% O_2/5% CO_2 at 37°C as previously described (Massele & Senior, 1981). Preparations were allowed to equilibrate for a period of not less than 2 h and at least until spontaneous activity had become regular. The Krebs solution passed through a section of silicon tubing which served as a drug injection site, just prior to passing over the tissue contained within the organ chamber.

Measurement of responses

Responses were measured via isometric transducers (Dynamometer UF1) linked first to a Grass Polygraph (Model 7D) chartpen recorder and then to a BBC Master microcomputer running integration software which allows measurement of area of tension and peak height of a response (Eltec Computer Ltd., Bradford).

Analysis of results

All agonists were injected directly into the flow of the superfusion fluid as a bolus dose and dose-effect curves constructed sequentially. Agonists were injected immediately after a spontaneous contraction to avoid superimposing responses on this activity.

The profile of spontaneous activity and sensitivity to agonists often changed markedly throughout the course of the experiments. For this reason, agonist dose-effect curves were not repeated, and comparisons were made between preparations in a non-paired manner. Where antagonists were used, they were dissolved in the Krebs superfusion solution and were in contact with tissues for a minimum contact period of not less than 30 min before agonist dose-effect curves were constructed.

The quantification of agonist responses in non-pregnant human myometrium presented several problems in this study, partly because of the variability of intrinsic spontaneous activity which occurs throughout the menstrual cycle. From examination of traces it appeared that the greater the magnitude of background contractions, the greater the magnitude of the excitatory response. Thus the results were expressed as T/B ratios of 'test' (T) agonist responses as a ratio of background (B) contractions. The inhibitory actions of prostanoids in non-pregnant human myometrium were expressed in terms of how they modified the periodicity of spontaneous activity, i.e., for how long (min) they extended the period between contractions. The time period between spontaneous contractions was measured in addition to the periods from the point of agonist addition to the re-occurrence of a spontaneous contraction of at least 80% of the peak height of the background contraction. The period of inhibition was then corrected for background frequency and expressed in absolute terms. Responses were often mixed, consisting of initial excitatory responses followed by inhibition. When this occurred, both aspects of the response were treated independently.

The use of T/B ratios to express agonist responses, provides a convenient method of deriving their potencies in individual preparations. As these values are effectively multiples of background activity it is possible to express potency in terms of the dose of agonist required to attain a response which reaches a predetermined value. For the purposes of this study, agonist potency was determined as the dose required to cause a response of equal magnitude to background activity, i.e. at the level where T/B ratio equalled 1. It must be made clear that a T/B value of 1 does actually represent an agonist response, and not simply the occurrence of the next spontaneous contraction. The T/B ratio of 1 was chosen as most agonists reach this level of response. The dose of agonist required to attain this level of response was defined as its ED_1 (Excitatory Dose) value. ED_1 values were obtained from individual dose-effect curves of T/B ratio against agonist concentration, and expressed as geometric means with 95% confidence limits. For agonist-induced inhibition of spontaneous activity, potencies were expressed as ID_4 values i.e. the dose of agonist required to extend the normal periodicity of spontaneous contractions by 4 min. This 4 min period was chosen as this represents an extension of approximately two times the normal periodicity of spontaneous activity in non-pregnant human myometrium,

as derived from pooled data (i.e. grouping data from all stages of the menstrual cycle). ID_4 values were obtained from individual dose-effect curves of inhibition (min) against agonist concentration and expressed as geometric means with 95% confidence limits.

Statistical comparisons were made by an unpaired Student's *t* test (Snedecor & Cochran, 1979).

Drugs

Compounds used, their vehicles and sources of supply are outlined in Table 1 (all final dilutions were in 0.9% sodium chloride solution (normal saline)).

Results

The spontaneous activity of non-pregnant human myometrium was not influenced by the presence of indomethacin (2.79 μM) in the Krebs solution, hence it was included to inhibit endogenous prostanoid synthesis in all experiments. We have previously shown that human myometrium contains thromboxane receptors (Clayton *et al.*, 1983; 1986b). In order to antagonize any TP-receptor activity of the synthetic analogues, GR32191 was used at a concentration of 1 μM (which is approximately 1000 times its pA_2 in human uterine artery (Baxter *et al.*, 1989)) in all experiments.

Agonists

Agonist potencies (ED_1 and ID_4) and maximal responses together with group sizes are given in Table 2.

PGE_2 had a dose-related activity in non-pregnant human myometrium which was not affected by the presence of GR32191 (Figure 1). The response comprised both excitation and inhibition. The excitatory component of the biphasic response was generally phasic in nature. The inhibitory response ranged from a reduction in peak height of spontaneous contractions at lower concentrations, to a complete inhibition of activity for periods in excess of 20 min.

Sulprostone was a potent agonist, and caused purely excitatory responses (ED_1 = 0.03 nmol) (Figure 2a). Responses developed rapidly and consisted of an increase in baseline tonus upon which frequent phasic contractions were superimposed. Like sulprostone, rioprostil was a potent excitatory agonist (ED_1 = 0.02 nmol) (Figure 2a). However, over the range 1.4–140 nmol, it caused dose-related inhibition of spontaneous activity (ID_4 = 1.4 nmol) (Figure 3). The profile of response was qualitatively similar to that produced by PGE_2 except that at lower doses there was a time lag between agonist injection and onset of the excitatory response. This time lag disappeared as the dose increased. As an excitatory agonist, rioprostil showed considerable variability in potency;

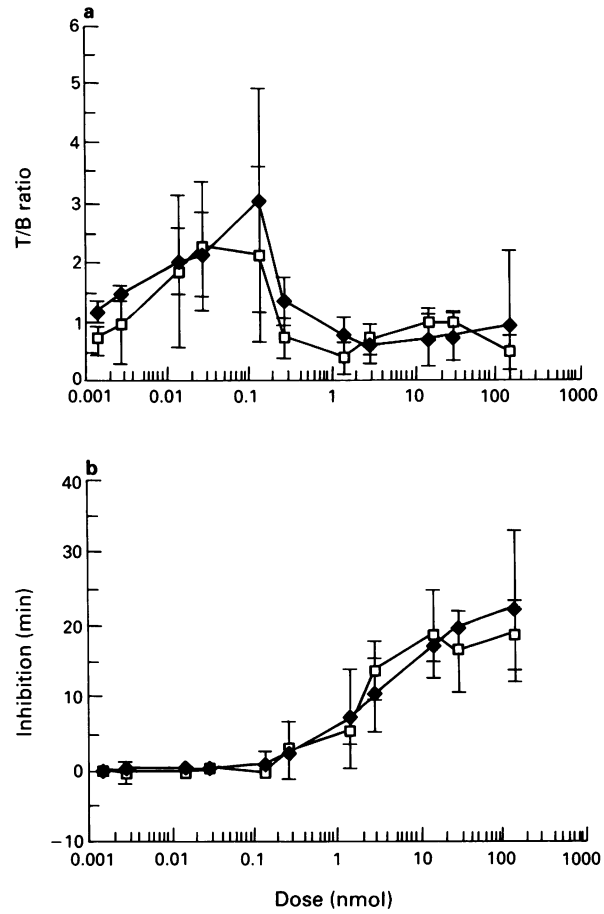

Figure 1 Mean dose-effect curves to stimulant (a) and inhibitory (b) responses to prostaglandin E_2 (PGE_2) in the absence (□) n = 8, and in the presence (◆) n = 8 of GR32191 (1 μM). Data are expressed as arithmetic means and vertical bars represent s.e.mean.

Table 1 Compounds used

Compound	*Stock vehicle*	*Source*
PGE_2	1% $NaHCO_3$	Upjohn
Dinoprostone		
Sulprostone	3% ethanol 0.01% tween	Schering
Rioprostil	Ethanol	Ortho
AY23626	1% $NaHCO_3$	Ayerst
11-desoxy-PGE_0		
Butaprost TR4979	Ethanol	Bayer
Iloprost ZK36 374	0.9% normal saline	Schering
Misoprostol	Ethanol	Searle
16,16-dimethyl PGE_2	1% $NaHCO_3$	Glaxo
Enprostil	Ethanol	Syntex
GR32191 ([1R-[[1α(z),2β,3β,5α]] -(+)-7-[5[[1,1-biphenyl)-4-yl) methoxy]-3-hydroxy-2- (1-piperidinyl)cyclopentyl]-4- heptanoic acid hydrochloride	Distilled water	Glaxo
AH6809 6-isopropoxy-9-oxaxanthene-2-carboxylic acid	1% $NaHCO_3$	Glaxo
Indomethacin	1% $NaHCO_3$	Sigma

Table 2 Mean ED_1, ID_4 and maximum responses for excitation and inhibition by prostanoids

	Excitation			*Inhibition*		
Prostanoid	ED_1 (95% CL) (*s.e.mean*)	*max* (*s.e.mean*)	n	ID_4 (95% CL)	*max*	n
PGE_2	0.02 (0.001–0.58)	3.34 (1.69)	8	0.8 (0.2–2.9)	21.3 (4.2)	8
Sulprostone	0.03 (0.006–0.16)	4.2 (0.83)	6	>110	NR	6
Rioprostil	0.02 (0.001–0.46)	2.17 (0.83)	6	1.4 (0.54–3.6)	33.9 (3.4)	6
AY23626	0.16 (0.07–0.83)	1.7 (0.5)	6	6.2 (1.3–30.0)	13.1 (2.5)	8
Butaprost	>120	NR	6	0.43 (0.06–2.9)	88.0 (21.4)	6
Misoprostol	0.07 (0.005–0.134)	2.2 (0.63)	6	2.11 (1.04–3.64)	24.0 (3.0)	7
16,16-dmPGE_2	0.02 (0.004–0.34)	1.8 (0.5)	6	3.53 (2.97–4.26)	5 (1.0)	7
Enprostil	0.02 (0.0125–0.025)	7.0 (1.0)	12	—	—	—
Iloprost	2.74 (1.03–7.3)	2.3 (0.3)	7	0.94 (0.2–4.9)	7.6 (2.3)	7

NR = no response.
ED_1 and ID_4 values are expressed as geometric means (nmol) with 95% CL in parentheses.
Maximum responses for excitation are expressed as multiples of background activity (i.e. maximum T/B ratios) and maximum responses for inhibition are expressed in min.

a similar variability was observed with PGE_2 as we have previously reported (Clayton *et al.*, 1989). Like rioprostil, AY23626, also demonstrated biphasic activity (Figures 2b and 3). Inhibitory responses produced an ID_4 value of 6.2 nmol, with a mean maximum inhibitory response of 13.1 min.

Butaprost did not cause excitation at any dose as reported in earlier work (Clayton *et al.*, 1989), but was a potent inhibitor of spontaneous activity (ID_4 = 0.43 nmol) producing a mean maximum inhibition of approximately 90 min (Figure 3).

Misoprostol, at low doses, is a potent excitatory agonist (Figure 2b). However, at higher doses misoprostol produced a dose-related inhibition of spontaneous activity with maximum mean inhibition of 24 min (ID_4 = 2.11 nmol). 16,16-dimethylprostaglandin E_2 provoked a response qualitatively similar to misoprostol i.e. at the high doses employed the inhibitory response was seen with little excitation, however the maximum inhibition was lower at 5 min (ID_4 = 3.53 nmol) (Figures 2b and 3). Enprostil produced a dose-related contractile response reaching a maximum at 2.5 nmol (Figure 2a).

The EP_1-/IP-receptor selective prostanoid, iloprost, evoked a biphasic response comprising an initial excitation (ED_1 =

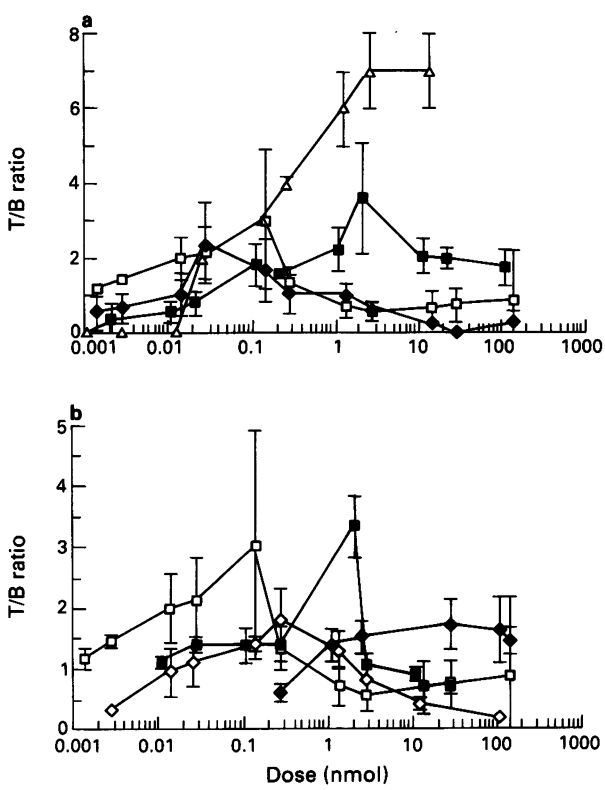

Figure 2 Mean stimulatory dose-effect curves to: (a) prostaglandin E_2 (PGE_2), (□) n = 8, rioprostil (◆) n = 6, sulprostone (■) n = 6 and enprostil (△) n = 12; (b) PGE_2 (□) n = 8, AY23626 (◆) n = 6, misoprostol (■) n = 6 and 16,16-dimethylPGE_2 (◇) n = 6. Data are expressed as arithmetic means and vertical bars represent s.e.mean.

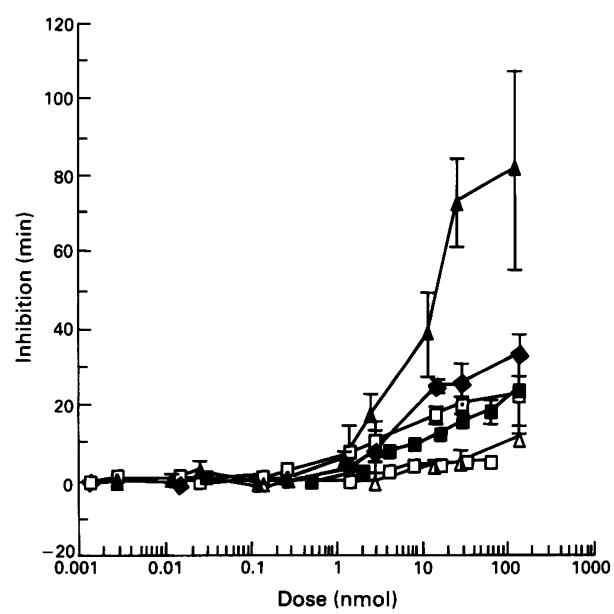

Figure 3 Mean inhibitory dose-effect curves to prostaglandin E_2 (PGE_2) (□) n = 8, rioprostil (◆) n = 6, butaprost (▲) n = 6, AY23626 (△) n = 8, misoprostol (■) n = 7 and 16,16-dimethyl PGE_2 (□) n = 7. Data are expressed as arithmetic means and vertical bars represent s.e.mean.

2.74 nmol, max. 2.3 × background) followed by an inhibition of spontaneous activity (ID_4 = 0.94 nmol), to maximum of 7.6 min. These biphasic responses were qualitatively similar to biphasic responses produced by PGE_2, although iloprost was considerably less potent as an excitatory agonist (Figure 4).

Antagonists

The effect of the EP_1- receptor blocking drug AH6809 on responses to sulprostone and iloprost was also investigated. At a concentration of 10 μM, approximately 30–80 times its reported pA_2 on EP_1-receptor containing preparations (Coleman *et al.*, 1985), AH6809 failed to affect dose-effect curves to sulprostone (Figure 5), but it did cause a rightward shift of excitatory dose-effect curves to iloprost (control ED_1 (95% CL) = 2.74 (1.03–7.3) nmol and in the presence of AH6809 = 22.8 (19–27) nmol). This shift in the excitatory response appears to be associated with a slight potentiation of the inhibitory component, although this was not statistically significant (Figure 4). Similar effects were found with the less potent EP_1-receptor antagonist SC19220 at a concentration of 10^{-5} M.

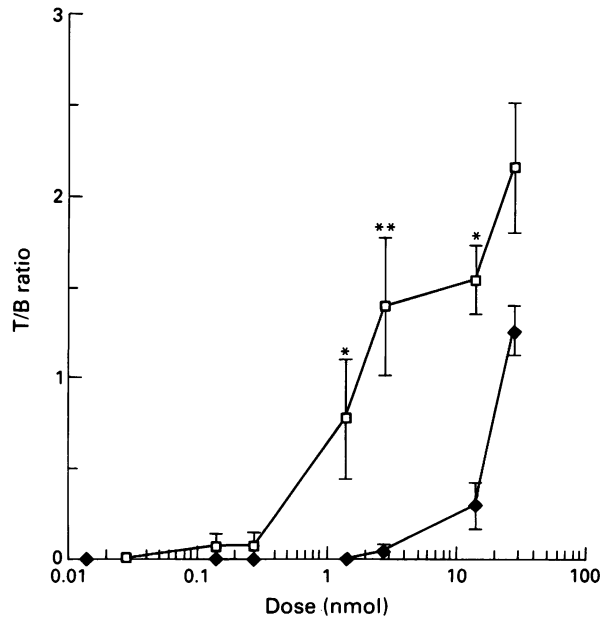

Figure 4 Mean stimulatory dose-effect curves to iloprost in the absence (□) n = 7 and in the presence (◆) n = 5 of AH6809 (10 μM). Data are expressed as arithmetic means and vertical bars represent s.e.mean. * $P < 0.05$; ** $P < 0.005$.

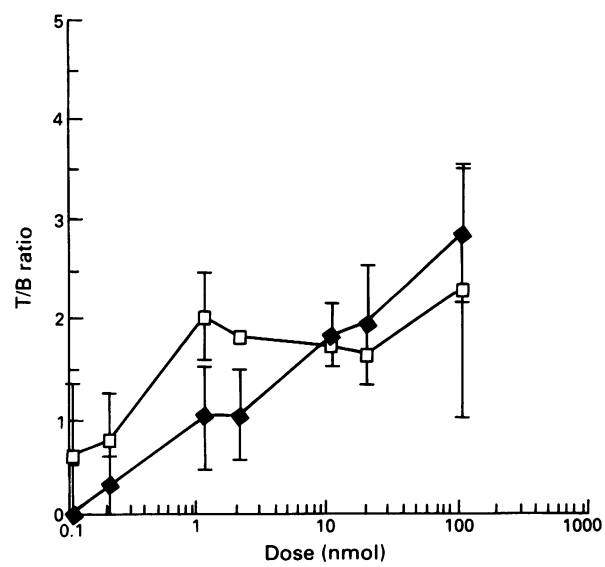

Figure 5 Mean stimulatory dose-effect curves to sulprostone in the absence (□) n = 5 and in the presence (◆) n = 5 of AH6809 (10 μM). Data are expressed as arithmetic means and vertical bars represent s.e.mean.

Discussion

All prostanoids tested were active on non-pregnant human myometrium, either as excitatory agonists, inhibitors of spontaneous activity or both. The existence of EP-receptors mediating excitation was supported by data obtained with the selective EP_1-/EP_3-receptor agonist sulprostone, which caused excitation (ED_1 = 0.03 nmol) over a similar range to PGE_2. The observation that the mean maximum response obtained with sulprostone was greater than that obtained with PGE_2 may be explained by the functional antagonism between dual inhibitory/excitatory response to PGE_2. It is possible that the excitatory component is opposed by the inhibitory component resulting in a lower maximum response. Sulprostone, on the other hand, did not cause inhibition at any concentration used and therefore reached its full excitatory potential.

Further evidence for EP-receptors mediating excitation, and additionally for EP-receptor-mediated inhibition in non-pregnant human myometrium is provided by the action of the EP_2-/EP_3-receptor selective prostanoid, rioprostil. Rioprostil, with an ED_1 value of 0.02 nmol, was of a similar potency to both PGE_2 and sulprostone as an excitatory agonist, and of a similar potency to PGE_2 as an inhibitor of spontaneous activity (ID_4 = 1.4 and 0.8 nmol respectively). Like PGE_2, rioprostil produced a lower mean excitatory response than sulprostone which may result from a functional antagonism of excitation by the inhibitory component of its response. The mean maximum inhibition however, was greater with rioprostil than PGE_2 (max inhibition = 33.9 and 21.3 min respectively) and why this should be so is not clear. It is possible that rioprostil is simply more lipophilic than PGE_2 and remains in the vicinity of the receptor for a longer time.

AY23626 which is also an EP_2-/EP_3-receptor selective compound had a qualitatively similar profile of activity to both PGE_2 and rioprostil. As an inhibitor of spontaneous activity AY23626 was approximately 3 times weaker than both PGE_2 and rioprostil. The observed inhibitory response of AY23626 in non-pregnant human myometrium is in line with that found in other tissues where it has been shown to be only 14 or 1.2 times less potent than PGE_2 at EP_2-receptors in guinea-pig ileum (circular muscle) and cat trachea respectively (Coleman *et al.*, 1987c).

The EP_2-/EP_3-receptor agonist misoprostol showed a biphasic response in this study; at high doses the inhibitory component of this response appeared dose-related (ID_4 = 2.11 nmol). At lower doses misoprostol is a potent EP_3-receptor agonist (Reeves *et al.*, 1988). The EP_2-receptor mediated response has been found in cat trachea where misoprostol is approximately 3 to 4 times weaker than PGE_2 (Coleman *et al.*, 1988).

The compound 16,16-dimethyl PGE_2 had a response qualitatively similar to misoprostol but as an inhibitor of spontaneous activity it was found to be weaker (ID_4 = 3.53 nmol). 16,16-dimethyl PGE_2 is a potent agonist at the EP_1-receptor where it has been shown on guinea-pig fundus to be approximately twelve times more potent than PGE_2 (Coleman *et al.*, 1988). In addition to its EP_1-/EP_2-receptor activity 16,16-dimethyl PGE_2 also has activity at the EP_3-receptor (Reeves *et al.*, 1988).

Enprostil, which is a potent EP_3-receptor agonist produced purely excitatory responses in non-pregnant human myometrium as previously reported (Clayton *et al.*, 1987). This compound has been reported to mediate action via EP_1-receptors also in the guinea-pig fundus preparation where it is approximately two times weaker than PGE_2. In addition to its EP-receptor activity, enprostil has moderate

action on the FP- and TP-receptors which has been observed in dog iris and rat aorta respectively (Coleman *et al.*, 1988). With respect to TP-receptors, we have observed some action with enprostil using selective TP-receptor antagonists (unpublished observation).

With the data obtained with sulprostone, rioprostil, misoprostol, enprostil and to a lesser extent with AY23626 and 16,16-dimethyl PGE_2, it appears that EP_3-receptors may mediate excitation in non-pregnant human myometrium, as activity at this site is common to all three compounds. Sulprostone and rioprostil have been shown to be 0.16 and 1.1 times as potent as PGE_2 in guinea-pig vas deferens, and AY23626 approximately 5.5 times as potent in chick ileum, preparations thought to contain a homogeneous population of EP_3-receptors (Coleman *et al.*, 1987c; Reeves *et al.*, 1988). However, sulprostone and 16,16-dimethyl PGE_2 also have activity at the EP_1-receptor and an additional contribution of this site to the excitatory response is possible. An involvement of EP_1-receptors is indicated by the excitatory action of the EP_1-/IP-receptor selective agonist, iloprost. This compound caused mixed responses in non-pregnant human myometrium of excitatory (ED_1 = 2.74 nmol, max. 2.3 × background) and inhibitory (ID_4 = 0.94 nmol, max. = 7.6 min) effects most probably mediated via EP_1- and IP-receptors respectively. The involvement of EP_1-receptors is further supported by the action of the EP_1-receptor blocking drug, AH6809 (10 μM), which caused an apparent 10 fold shift of dose-effect curves to iloprost. The data obtained with AH6809 and the EP-selective agonists suggest that EP_1- and EP_3-receptors may co-exist in non-pregnant human myometrium. Iloprost has been shown to be a partial agonist at EP_1-receptors on the bullock iris, guinea-pig trachea and rat fundus, but it is approximately equipotent with PGE_2 (Dong *et al.*, 1986).

Therefore, the rather low potency of iloprost as an excitatory agonist in non-pregnant human myometrium may indicate that only a small population of EP_1-receptors are present. Further support for this finding lies in the observation that dose-effect curves to sulprostone were unaffected by AH6809 (10 μM). Although this prostanoid possesses potent EP_1-receptor activity (Coleman *et al.*, 1988), this does not appear essential for its action in non-pregnant human myometrium.

The inhibitory actions of rioprostil, AY23626, misoprostol and 16,16-dimethyl PGE_2 which show selectivity for the EP_2-receptors and the lack of inhibitory effect with sulprostone and enprostil, which do not possess EP_2-receptor activity (Reeves *et al.*, 1988), suggest that EP_2-receptors may mediate the observed inhibition of spontaneous activity. The effect of the selective EP_2-receptor agonist, butaprost (Gardiner, 1986) also supports the proposal that the inhibitory effect of PGE_2 on non-pregnant human myometrium was through the EP_2-receptor.

The data presented in this paper are consistent with the presence in non-pregnant human myometrium of a heterogeneous population of prostanoid receptors of the EP-type mediating both excitation and relaxation of spontaneous activity. Based on data obtained by use of the receptor selective agonists and the antagonist AH6809, it is suggested that the prostanoid receptor subtypes present on non-pregnant human myometrium, which mediate excitation, are EP_1 and EP_3-receptors with a greater proportion of the EP_3-subtype being present in this tissue, and those which mediate inhibition are of the EP_2-receptor subtype.

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References

BAXTER, G.S., CLAYTON, J.K., COLEMAN, R.A., MARSHALL, K.M. & SENIOR, J. (1989). Prostanoid receptors mediating contraction and relaxation of human isolated uterine artery. *Br. J. Pharmacol.*, **98**, 649P.

BYGDEMAN, M. (1964). The effect of different prostaglandins on human myometrium *in vitro*. *Acta Physiol. Scand.*, **63**, Suppl. 242, 1–78.

BYGDEMAN, M. & ELAISSON, R. (1963). A comparative study on the effect of different prostaglandin compounds on the motility of the isolated human myometrium. *Med. Exp.* (Basel), **9**, 409–415.

CLAYTON, J.K., MARSHALL, K.M. & SENIOR, J. (1987). Effect of indomethacin on human myometrial responses to prostaglandins $F_{2\alpha}$, E_2 and E analogues. *Br. J. Pharmacol.*, **90**, 150P.

CLAYTON, J.K., MASSELE, A.Y. & SENIOR, J. (1983). The effects of prostaglandins and an endoperoxide analogue (U46619) on lower segment myometrium *in vitro*. *Br. J. Pharmacol.*, **78**, 53P.

CLAYTON, J.K., MARSHALL, K.M. & SENIOR, J. (1986a). A comparison of the responses of prostaglandins F, E and E analogues on human myometrium *in vitro*. In *Abstracts for the 6th International Conference on Prostaglandins and Related Compounds, Florence*, p. 107. Forvdazione Giovanni Lorenzini.

CLAYTON, J.K., MARSHALL, K.M., MASSELE, A.Y. & SENIOR, J. (1986b). Prostanoid receptors in the human myometrium *in vitro*. In *Abstracts for the 6th International Conference on Prostaglandins and Related Compounds, Florence*. p. 107. Forvdazione Giovanni Lorenzini.

CLAYTON, J.K., GARDINER, P.J., MARSHALL, K.M., SENIOR, J. & TUFEAK, N. (1989). The effect of rioprostil and butaprost (TR4979) on human myometrium and uterine artery. *Br. J. Pharmacol.*, **97**, 540P.

COLEMAN, R.A. (1983). Studies towards a classification of prostanoid receptors. *PhD Thesis, CNAA*.

COLEMAN, R.A. (1987). Methods in prostanoid receptor classification. In *Prostaglandins and Related Substances, a Practical Approach*. ed. Bennedeto, C., McDonald-Gibson, R.G., Nigam, S. & Slater, T.F. pp. 267–303. Oxford: IRL Press.

COLEMAN, R.A., HUMPHREY, P.P.A., KENNEDY, I. & LUMLEY, P. (1984). Prostanoid receptors – the development of a working classification. *Trends Pharmacol. Sci.*, **5**, 303–306.

COLEMAN, R.A., HUMPHRAY, J.M., SHELDRICK, R.L.G. & WHITE, B.P. (1988). Gastric anti-secretory prostanoids: actions at different prostanoid receptors. *Br. J. Pharmacol.*, **95**, 724P.

COLEMAN, R.A., KENNEDY, I. & SHELDRICK, R.L.G. (1985). AH6809, a prostanoid EP_1-receptor blocking drug. *Br. J. Pharmacol.*, **85**, 273P.

COLEMAN, R.A., KENNEDY, I. & SHELDRICK, R.L.G. (1987a). Further evidence for the existence of three subtypes of PGE_2-sensitive (EP-) receptors. *Br. J. Pharmacol.*, **91**, 323P.

COLEMAN, R.A., KENNEDY, I. & SHELDRICK, R.L.G. (1987b). New evidence with selective agonists and antagonists for the subclassification of PGE_2-sensitive (EP-) receptors. *Adv. Prostaglandin, Thromboxane, Leukotr. Res.*, **17**, 467–470.

COLEMAN, R.A., KENNEDY, I., SHELDRICK, R.L.G. & TOLOWINSKA, I.Y. (1987c). Evidence for the existence of three subtypes of PGE_2-sensitive (EP-) receptors. *Br. J. Pharmacol.*, **85**, 273P.

COLLINS, P.W., RAPPO, R. & DAJANI, E.Z. (1985). Chemistry and synthetic development of misoprostol. *Dig. Dis. Sci.*, **30**, (suppl.) 114S.

DONG, Y.J., JONES, R.L. & WILSON, N.H. (1986). Prostaglandin E receptor subtypes in smooth muscle: agonist activities of stable prostacyclin analogues. *Br. J. Pharmacol.*, **87**, 97–107.

EGLEN, R.M., CORNETT, C.M., DUBIQUE, L.K., JACOBSON, L.L., MONTGOMERY, W.W. & WHITING, R.L. (1989). Prostanoid receptor profile of enprostil and isomers *in vitro*. *Br. J. Pharmacol.*, **96**, 175P.

GARDINER, P.J. (1986). Characterisation of prostanoid relaxant/inhibitory receptors (ψ) using a highly selective agonist, TR4979. *Br. J. Pharmacol.*, **87**, 45–56.

HESS, H.J., SCHAAF, T.K., BINDRA, J.S., JOHNSON, M.R. & CONSTANTINE, J.W. (1979). Structure-activity considerations leading to sulprostone. In *International Sulprostone Symposium*. ed. Friebel, K., Schneider, A. & Wurfel, H. pp. 29–37. Berlin and Bergkamen: Schering.

JONES, R.L. (1976). Cardiovascular actions of prostaglandins D and E in the sheep: evidence for two distinct receptors. *Adv. Prostaglandin Thromboxane Res.*, **2**, 221–230.

KARIM, S.M.M., HILLIER, K., SOMERS, K. & TRUSSELL, R.R. (1971). The effect of prostaglandin E_2 and $F_{2\alpha}$ administered by different

routes on uterine activity and the cardiovascular system in pregnant and non-pregnant women. *J. Obstet. Gynaec. Br. Commonw.*, **78,** 172–179.

KEERY, R.L. & LUMLEY, P. (1988). AH6809, a prostaglandin DP-receptor blocking drug on human platelets. *Br. J. Pharmacol.*, **94,** 745–754.

KENNEDY, I., COLEMAN, R.A., HUMPHREY, P.P.A., LEVY, G.P. & LUMLEY, P. (1982). Studies on the characterisation of prostanoid receptors: a proposed classification. *Prostaglandins*, **24,** 667–689.

KENNEDY, I., COLEMAN, R.A., HUMPHREY, P.P.A. & LUMLEY, P. (1983). Studies on the characterisation of prostanoid receptors. *Adv. Prostaglandin Thromboxane Leukotr. Res.*, **11,** 327–332.

KLUENDER, H.C. & WOESSER, W. (1979). The synthesis of dimethylphosphonoprostaglandin analogues. *Prostaglandins Med.*, **2,** 439–447.

MAIGAARD, S., FORMAN, A. & ANDERSSON, K.E. (1986). Relaxant and contractile effects of some amines and prostanoids in myometrial and vascular smooth muscle within the human uteroplacental unit. *Acta Physiol. Scand.*, **49,** Suppl. 5, 33–40.

MASSELE, A.Y. & SENIOR J. (1981). Responses of the human myometrium *in vitro* to prostaglandins and bradykinin. *Br. J. Clin. Pharmacol.*, **12,** 285P.

PICKLES, V.R., HALL, W.J., BEST, F.A. & SMITH, G.N. (1965). Prostaglandins in endometrium and menstrual fluid from normal and dysmenorrhoeic subjects. *Br. J. Obstet. Gynaecol.*, **72,** 185–192.

REEVES, J.J., BUNCE, K.T., SHELDRICK, R.L.G. & STABLES, R. (1988). Evidence for the PGE receptor subtype mediating inhibition of acid secretion in the rat. *Br. J. Pharmacol.*, **95,** 805P.

SANNER, J.H. (1972). Dibenzoxazepine hydrazides as prostaglandin antagonists. *Intrascience Chem. Rep.*, **6,** 1–8.

SHELDRICK, R.L.G., COLEMAN, R.A. & LUMLEY, P. (1988). Iloprost – a potent EP_1- and IP-receptor agonist. *Br. J. Pharmacol.*, **94,** 334P.

SNEDECOR, G.W. & COCHRAN, W.G. (1979). *Statistical Methods*, (7th Edition). Ames, Iowa; Iowa State University Press.

TOPPOZADA, M., GAAFAR, A. & SHAALA, S. (1974). *In vivo* inhibition of the human non-pregnant uterus by prostaglandin E_2. *Prostaglandins*, **8,** 401–411.

WIQVIST, N., LINDBLOM, M., WIKLAND, M. & WILHELMSON, L. (1983). Prostaglandins and uterine contractility. *Acta Obstet. Gynaec. Scand. Suppl.*, **113,** 23–29.

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Regional heterogeneity in the contractile and potentiating effects of neuropeptide Y in rat isolated coronary arteries: modulatory action of the endothelium

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1 Neuropeptide Y (NPY) induced a concentration-dependent contraction of isolated rings of proximal epicardial (PC) and distal intramural (DC) coronary arteries of the rat, with an EC_{50} of ca. 1×10^{-7} M. The NPY-induced contraction at 3×10^{-7} M was significantly smaller in PC than DC arteries: 34% vs. 55% of the 125 mM K^+-induced response, respectively.

2 NPY (2×10^{-8} M) increased the sensitivity to noradrenaline (NA) and 5-hydroxytryptamine (5-HT) more in PC (4.2 and 2.8 fold, respectively) than in DC arteries (2.2 and 1.4 fold, respectively). The maximal contractile response to NA and 5-HT was increased more in DC (43% and 29%, respectively) than in PC arteries (20% and 12%, respectively).

3 Removal of the endothelium increased the sensitivity and maximal response to NPY as well as the spontaneous myogenic tone in PC but not in DC arteries. NPY had no relaxing effect on PC and DC arteries submaximally contracted with 10^{-6} M prostaglandin $F_{2\alpha}$, suggesting that spontaneous rather than stimulated release of endothelium-derived relaxing factor (EDRF) depresses the contractile action of NPY in PC arteries.

4 The results indicate a heterogeneity in the contractile and potentiating action of NPY in rat coronary arteries depending on size or location in the coronary circulation.

Introduction

Neuropeptide Y (NPY) is one of the most abundant peptides in the cardiac nerves (Gu *et al.*, 1983; 1984) and it is contained and released from sympathetic nerves together with noradrenaline (NA) (Lundberg *et al.*, 1983; 1984). NPY is a potent vasoconstrictor both *in vivo* and *in vitro* in the coronary vascular bed of several species (Aizawa *et al.*, 1985; Rudehill *et al.*, 1986; Franco-Cereceda & Lundberg, 1987; Han & Abel, 1987; Tseng *et al.*, 1988; Franco-Cereceda, 1989; Maturi *et al.*, 1989), and it potentiates catecholamine- and histamine-induced coronary vasoconstriction (Han & Abel, 1987; Macho *et al.*, 1989).

Although there is little information about the actions of NPY on resistance vessels (Owen & Taphorn, 1988; Andriantsitohaina & Stoclet, 1988), a correlation between the vascular effects of NPY and the diameter of the vessels has been proposed: it induces a stronger contraction in small arteries whereas the potentiation of the responses induced by other vasoconstrictors seems to be restricted to larger arteries where NPY has little or no direct contractile effect (Pernow, 1988; Franco-Cereceda, 1989).

There is some controversy about the role of endothelium in the vascular actions of NPY. NPY-induced contractions in human skeletal and pig splenic arteries seem to be endothelium-independent (Pernow, 1988), as is the potentiation of noradrenaline (NA)-induced contractions in rabbit ear artery (Budai *et al.*, 1989). However, Hieble *et al.* (1989) found that NPY had an endothelium-dependent potentiating effect in the same vascular bed.

The aims of the present study: (i) to determine whether or not NPY possesses a direct contractile effect on rat isolated proximal epicardial, and distal intramyocardial, coronary arteries *in vitro*; (ii) to examine how the NPY-induced response in these arteries is modulated by the endothelium; and (iii) to examine the ability of this peptide to enhance NA- and 5-hydroxytryptamine (5-HT)-induced contractions in the coronary arteries.

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Methods

Dissection and mounting

Segments of the proximal (PC) and distal (DC) part of the left coronary artery in hearts from 3 month old male Wistar were isolated as previously described (Nyborg, 1990) and mounted as ring preparations on two 40 μm diameter stainless steel wires on a double myograph (Mulvany & Nyborg, 1980) which allows direct determination of wall tension while the internal circumference of the vessels is controlled.

After equilibration in oxygenated (5% CO_2 in O_2) physiological saline solution (PSS) for 30 min, pH 7.4, 37°C, the internal circumference, L_0, of the vessels were set to 0.9 times the circumference they would have if relaxed and subjected to transmural pressure of 100 mmHg (Nyborg *et al.*, 1987). The effective normalized lumen diameter, $l_0 = L_0 \pi^{-1}$, ranged between 204 and 300 μm for the DC arteries and between 350 and 525 μm for the PC arteries.

The vessels were subsequently stimulated repetitively with K-PSS until reproducible responses were recorded.

Pharmacology

The direct contractile effect of NPY on the coronary arteries was tested in cumulative concentration-response experiments. The maximal obtainable concentration of NPY was 3×10^{-7} M. The arteries could be used only once because of development of tachyphylaxis to NPY.

The potentiating effect of NPY was studied on cumulative NA and 5-HT concentration-response curves. The vessels were washed in drug-free PSS for 30 min and NPY (2×10^{-8} M) was added. When the response to the peptide had stabilized (5–8 min), cumulative additions of NA or 5-HT were repeated. NPY (2×10^{-8} M) induced an initial contraction of the arteries that was less than 10–15% of that induced by K-PSS. NA-induced contractions were elicited in the presence of 10^{-5} M propranolol.

The role of the endothelium in the contractile effect of NPY was studied on consecutive arterial segments. One was taken as a control and the other had its endothelium removed by guiding a horse hair (PC arteries) or a 40 μm steel stainless

wire (DC arteries) through the vessel lumen and gently pushing the hair/wire back and forth. The function of the endothelium was tested by measuring the relaxation induced by 10^{-5} M acetylcholine in vessels contracted with 10^{-5} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

Solutions and drugs

The vessels were, unless otherwise stated, dissected and kept relaxed in PSS with the following composition (mM): NaCl 119, NaH_2CO_3 25, KCl 4.7, $CaCl_2$ 1.5, KH_2PO_4 1.18, $MgSO_4$ 1.17, EDTA 0.026 and glucose 11. K-PSS was similar to PSS except that NaCl was exchanged with KCl on an equimolar basis.

Drugs used were neuropeptide Y (NPY) (Peptides, U.K.), (−)-noradrenaline HCl (Sigma), 5-hydroxytryptamine creatinine sulphate complex (Sigma), propranolol (Sigma) and prostaglandin $F_{2\alpha}$ (Dinoprost, UpJohn). Drugs were dissolved in distilled water for stock solutions and they were added in volumes of not more than 0.3% to give the required concentrations.

Statistics

Vessel responses are expressed as either active tension, Nm^{-1}, calculated as increase in vessel wall force divided by twice the vessel segment length, or as percentage of the response elicited by K-PSS. In the potentiation experiments, NA- and 5-HT-induced responses are given as the percentage of the maximal response of the control concentration-response curve. The initial NPY-induced contractions were subtracted from those induced by NA and 5-HT. Sensitivities to the agonist are expressed as pD_2-values. Differences between mean values were analysed by Student's t test for paired and unpaired values where appropriate. A probability less than 0.05 was considered significant for both tests.

Results

Contractile effect of neuropeptide

NPY induced concentration-dependent contractions of both PC and DC arteries (Figure 1) with a threshold concentration between 10^{-10} M and 10^{-9} M for both types of arteries. The contractions were slow in onset and long-lasting compared to those induced by NA, 5-HT and K-PSS. The responses to NPY (3×10^{-7} M) were $0.95 \pm 0.20\,Nm^{-1}$ ($n = 6$) and $0.90 \pm 0.27\,Nm^{-1}$ ($n = 7$) in PC and DC arteries, respectively. The maximal contractions elicited by 5-HT (3×10^{-5} M) and NA (10^{-4} M) in PC arteries were $3.12 \pm 0.30\,Nm^{-1}$ ($n = 9$) and $0.68 \pm 0.10\,Nm^{-1}$ ($n = 7$), respectively (Figure 1a). The maximal contractions evoked by 5-HT and NA were $1.02 \pm 0.16\,Nm^{-1}$ ($n = 8$) and $0.65 \pm 0.19\,Nm^{-1}$ ($n = 7$), respectively in DC arteries (Figure 1b). NPY was the most potent of the 3 constrictor agents in boths kind of vessel. The order of potency: NPY > 5-HT > NA was similar in both PC and DC arteries, the pD_2-values being 6.94 ± 0.25 ($n = 6$), 6.06 ± 0.07 ($n = 9$) and 4.89 ± 0.13 ($n = 7$), respectively in PC arteries and 6.93 ± 0.27 ($n = 7$), 5.54 ± 0.07 ($n = 8$) and 4.91 ± 0.14 ($n = 7$), respectively, in the DC arteries. There was no difference in the sensitivities of PC and DC arteries except to 5-HT ($P < 0.01$).

Effect of neuropeptide Y on noradrenaline- and 5-hydroxytryptamine-induced contractile responses

NPY (2×10^{-8} M) caused a significant leftward shift ($\Delta pD_2 = 0.62 \pm 0.16$, $n = 7$, $P < 0.01$) of the NA concentration-response curve in PC arteries (Figure 2a). The maximal response increased by $20 \pm 8\%$ ($n = 7$, $P < 0.05$). NPY also caused a leftward shift ($\Delta pD_2 = 0.34 \pm 0.10$, $n = 7$, $P < 0.02$) of the NA concentration-response curve in DC arteries (Figure 2b); however, the maximal response was increased by $43 \pm 16\%$ ($n = 7$, $P < 0.05$).

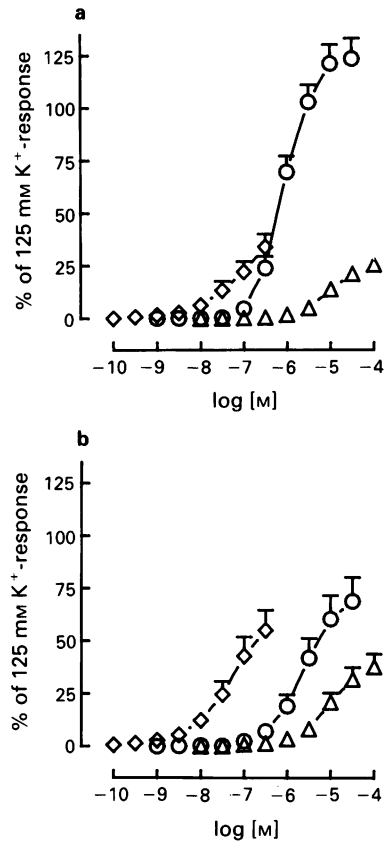

Figure 1 Contractile effects of neuropeptide Y (◇), 5-hydroxytryptamine (○) and noradrenaline (△) in rat PC (a) and DC (b) arteries. Responses are expressed as percentage of the 125 mM K^+-induced response in each vessel. Points represent mean values of 6–9 vessels. Vertical bars indicate s.e.mean where this value exceeds the size of the symbol.

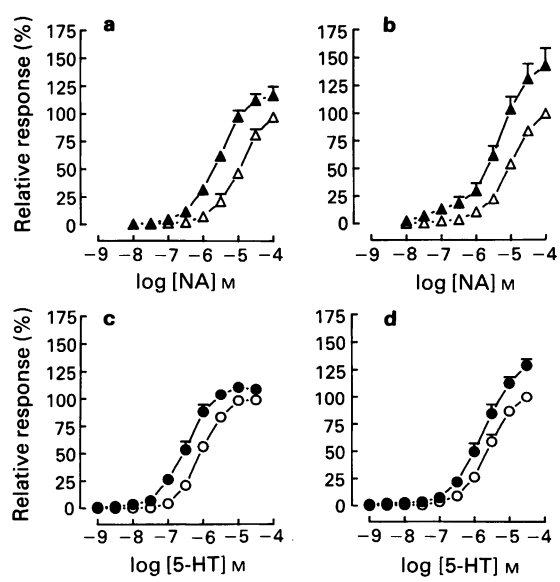

Figure 2 Effects of pretreatment with neuropeptide Y (NPY, ▲) on the noradrenaline (NA, a,b) and 5-hydroxytryptamine (5-HT, c,d) concentration-response curve in rat PC (a,c) and DC (b,d) arteries. NPY (2×10^{-8} M) was added 5–8 min before and was present throughout the construction of the concentration-response curves. Points show mean values from 7–9 observations. Vertical bars indicate s.e.mean where this value exceeds the size of the symbol.

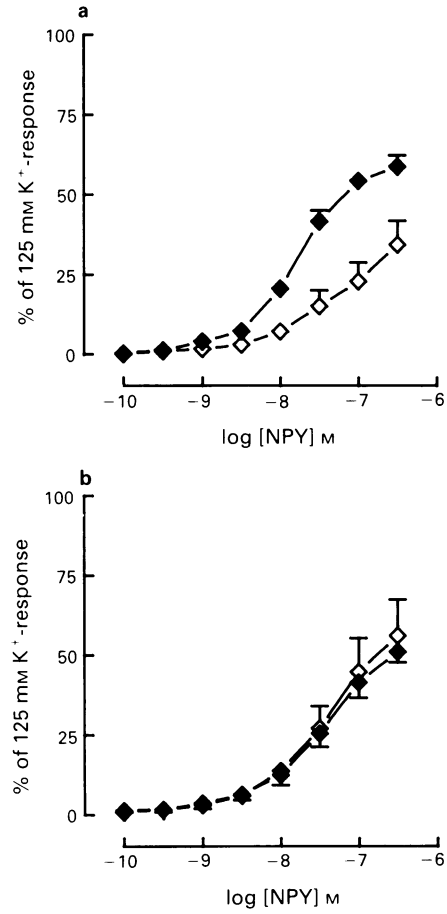

Figure 3 Concentration-response curves showing the contractile effects on neuropeptide Y (NPY) in rat PC (a) and DC (b) arteries with endothelium (◇) and after removal of the endothelium (◆). The contractile effect is expressed as a percentage of that induced by 125 mM K^+.

NPY (2×10^{-8} M) also had a potentiating effect on the 5-HT concentration-response curve in PC arteries ($\Delta pD_2 = 0.44 \pm 0.05$, $n = 9$, $P < 0.001$) (Figure 2c). The maximal response was slightly but significantly increased by $12 \pm 3\%$ ($n = 9$) ($P < 0.01$). In contrast, NPY had no potentiating effect on the 5-HT concentration-response curve in DC arteries ($\Delta pD_2 = 0.16 \pm 0.07$, $n = 8$) (Figure 2d), although the maximal response was increased by $29 \pm 6\%$ ($n = 8$) ($P < 0.01$).

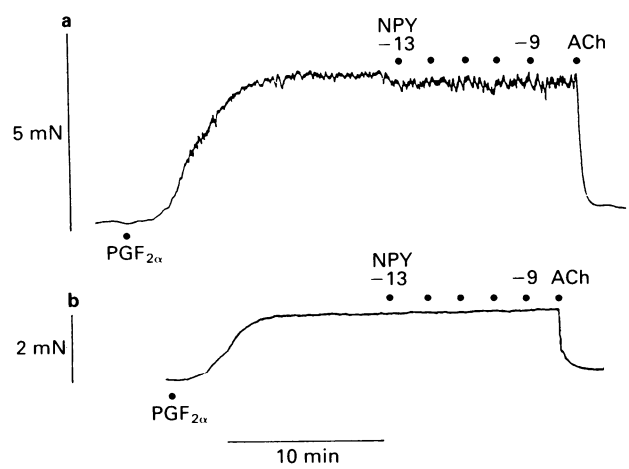

Figure 4 Tracings showing the effect of neuropeptide Y (NPY, 10^{13}–10^{-9} M) on a 390 μm PC (a) and a 282 μm DC (b) artery, submaximally contracted with 10^{-6} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Presence of endothelium is demonstrated by the relaxation induced by acetylcholine (ACh) 10^{-5} M. Vertical and horizontal scales show force (mN) and time, respectively.

Effects of neuropeptide Y on endothelium-denuded arteries

The sensitivity to NPY was significantly greater in PC arteries without than with endothelium (Figure 3a), the pD_2 value being 6.93 ± 0.30 ($n = 5$) and 7.70 ± 0.14 ($n = 5$) ($P < 0.05$) in endothelium-intact and denuded arteries, respectively. The response to NPY (3×10^{-7} M) was also significantly greater in the endothelium-denuded arteries than in the control arteries: $59 \pm 4\%$ ($n = 5$) vs. $34 \pm 7\%$ ($n = 5$) ($P < 0.02$) of the K-PSS-induced response. Removal of the endothelium in DC arteries did not change the pD_2-value or the maximal response to NPY (3×10^{-7} M) (Figure 3b).

Relaxation of PC and DC arteries with 10^{-5} M acetylcholine was $85 \pm 5\%$ ($n = 5$) and $97 \pm 2\%$ ($n = 6$) in endothelium-intact arteries and $8 \pm 4\%$ and $7 \pm 3\%$ in endothelium-denuded arteries, respectively. The K-PSS-induced responses were not significantly different, $1.67 \pm 0.24\,Nm^{-1}$ ($n = 6$) and $1.70 \pm 0.49\,Nm^{-1}$ ($n = 6$), in DC arteries with and without endothelium, respectively. However, there was a decrease in the responses to K-PSS in PC arteries from $2.88 \pm 0.35\,Nm^{-1}$ ($n = 5$) before to $1.91 \pm 0.22\,Nm^{-1}$ ($n = 5$, $P < 0.01$) after removal of the endothelium. The lower response to K-PSS was compensated by a greater spontaneous tone, which increased $0.57 \pm 0.15\,Nm^{-1}$ ($n = 5$) after the endothelium was removed, giving a total active tension (spontaneous tone plus K-PSS-induced) of $2.48 \pm 0.21\,Nm^{-1}$ ($n = 5$) after removal of the endothelium. The increase in spontaneous tone in the distal arteries following removal of endothelium was not significant: $0.17 \pm 0.10\,Nm^{-1}$ ($n = 6$).

Cumulative addition of NPY (10^{-13} M–10^{-9} M) did not induce relaxation in any of 6 PC and DC arteries with intact endothelium submaximally contracted with 10^{-6} M $PGF_{2\alpha}$ (Figure 4).

Discussion

NPY induced a potent contraction both in large epicardial and small intramural arteries of the rat left coronary vascular bed. These findings are consistent with previous results obtained in rabbit (Han & Abel, 1987) and human (Franco-Cereceda & Lundberg, 1987; Tseng *et al.*, 1989) isolated epicardial coronary arteries, as well as with *in vivo* studies in the dog (Allen *et al.*, 1986; Macho *et al.*, 1989; Martin & Patterson, 1989; Maturi *et al.*, 1989) and pig (Rudehill *et al.*, 1986).

The contraction induced by the highest obtainable concentration of NPY (3×10^{-7} M) was similar in PC and DC arteries. However, NPY induced a greater relative contraction at all concentrations in DC arteries than PC arteries indicating that NPY would increase the vascular resistance more in DC than in PC arteries *in vivo*. This is in agreement with perfusion studies in the dog coronary (Macho *et al.*, 1989; Maturi *et al.*, 1989) and rabbit renal vasculature (Owen & Taphorn, 1988). The direct constrictor effect of NPY in PC arteries contrasts with findings in human epicardial arteries (Franco-Cereceda, 1989) where NPY has no direct contractile effect. The origin of this discrepancy is unknown but may be attributed to differences in basic properties of the arteries, NPY receptor density, or level of spontaneous EDRF release.

NPY was about 50–100 fold more potent than NA and 5-HT; NPY also induced contractions that were sustained following washout with drug-free PSS in contrast to those induced by 5-HT or NA. These results are in agreement with those reported for human (Franco-Cereceda & Lundberg, 1987) and dog (Macho *et al.*, 1989) coronary arteries and also

for other vascular preparations, such as cat cerebral (Edvinsson *et al.*, 1984) and human skeletal muscle (Pernow, 1988) arteries. NPY-induced contractions were of the same magnitude as those evoked by NA or 5-HT in DC arteries but 5-HT evoked much larger responses in rat PC than in DC arteries as previously demonstrated (Nyborg & Mikkelsen, 1988).

NPY potentiates NA-induced smooth muscle contraction in several vascular beds (Edvinsson *et al.*, 1984; Wahlestedt *et al.*, 1985; Neild 1987; Owen & Taphorn, 1988; Pernow, 1988; Oshita *et al.*, 1989), including rabbit epicardial arteries (Han & Abel, 1987) and resistance arteries in the guinea-pig (Franco-Cereceda & Lundberg, 1985) and dog isolated hearts (Macho *et al.*, 1989). NPY has also been shown to potentiate NA-induced contractions of rat mesenteric resistance arteries (Andriantsitohaine & Stoclet, 1988), although NPY has no direct contractile effect in these vessels. Despite the latter data, there is a general tendency for the potentiation of NA-induced vasoconstriction by NPY to be more pronounced in larger arteries, where it has a weak direct contractile action (Pernow *et al.*, 1987; Pernow, 1988) than in smaller arteries (Owen & Taphorn, 1988; Macho *et al.*, 1989; Maturi *et al.*, 1989). Our results showing a 2 fold greater increase in NA sensitivity of PC and DC arteries are thus consistent with this view; however, the maximal response was increased more in the small than in the large arteries. It must be noted that β-adrenoceptor function dominates in rat small flow-regulating coronary arteries under normal physiological conditions (Nyborg & Mikkelsen, 1985; Nyborg, 1990). The potentiating effect of NPY was not restricted to NA, but occurred also with 5-HT. This supports the idea that NPY sensitizes vascular smooth muscle in a non-specific way (Edvinsson *et al.*, 1984; Wahlestedt *et al.*, 1985; Neild, 1987; Han & Abel, 1987; Andriantsitohaina & Stoclet, 1988; Oshita *et al.*, 1989). The sensitization may be mediated through a partial smooth muscle cell membrane depolarization and an increased calcium entry through voltage-dependent calcium channels (Neild, 1987; Andriantsitohaina & Stoclet, 1988).

Studies concerning the specific role of the endothelium in mediating the vascular effects of NPY seem to be contradictory; both endothelium-dependence (Daly & Hieble, 1987; Hieble *et al.*, 1989) and endothelium-independence (Andriantsitohaina & Stoclet, 1988; Budai *et al.*, 1989) have been described. The direct contractile effect of NPY on human skeletal and pig splenic arteries, both regarded as small arteries, occurs via an endothelium-independent mechanism (Pernow & Lundberg, 1988; Pernow, 1989), but coronary constriction may involve formation of a cyclo-oxygenase product (Martin & Patterson, 1989). NPY seems also capable of releasing EDRF in human coronary arteries (Tseng *et al.*, 1988) and perhaps also in the rat kidney vascular bed (Carter & Halliday, 1989). Our results exclude a major involvement of endothelium-derived substances in the overall vascular response to NPY in the distal part of the rat coronary circulation. The increased responses and sensitivity of endothelium-denuded PC arteries to NPY would indicate a NPY-induced EDRF release in these vessels. However, NPY in low concentrations did not relax endothelium-intact PC arteries suggesting that EDRF release is unlikely to have occurred. Furthermore, PC arteries are characterized by a high basal spontaneous release of EDRF which suppresses spontaneous myogenic tone (Nyborg, 1990; present study). The potentiation of NPY-induced contraction after endothelium removal is probably explained by suppression of basal release of EDRF rather than by stimulation of EDRF release.

In summary, this work shows that NPY can regulate rat coronary vascular tone directly and indirectly. NPY caused a greater contraction in the smaller DC than larger PC arteries whereas NPY increased the sensitivity to NA and 5-HT more in PC than DC arteries. The contractile responses to NPY were differentially affected by the presence of vascular endothelium: it had no effect on NPY responses in DC arteries but depressed contractions to NPY in PC arteries through basal release of EDRF. The present results therefore confirm that the coronary vascular bed is highly heterogeneous in its response to vasoactive substances (Miwa & Toda, 1984; Nyborg & Mikkelsen, 1988; Maturi *et al.*, 1989; Nyborg, 1990).

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References

ANDRIANTSITOHAINA, R. & STOCLET, J.C. (1988). Potentiation by neuropeptide Y of vasoconstriction in rat resistance arteries. *Br. J. Pharmacol.*, **95**, 419–428.

AIZAWA, Y., MURATA, M., HAYASHI, M., FUNAZAKI, T., ITO, S. & SHIBATA, A. (1985). Vasoconstrictor effect of neuropeptide Y (NPY) on canine coronary artery. *Jpn Circ. J.*, **49**, 584–588.

ALLEN, J.M., GJÖRSTRUP, P., BJÖERKMAN, J.-A., EK, L., ABRAHAMSSON, T. & BLOOM, S.R. (1986). Studies on cardiac distribution and function of neuropeptide Y. *Acta Physiol. Scand.*, **126**, 405–411.

BUDAI, D., VU, H.Q. & DUCKLES, S.P. (1989). Endothelium removal does not affect potentiation by neuropeptide Y in rabbit ear artery. *Eur. J. Pharmacol.*, **168**, 97–100.

CARTER, D.V. & HALLIDAY, J. (1989). Does NPY release EDRF in the perfused rat kidney? *Br. J. Pharmacol.*, **97**, 518P.

DALY, R.N. & HIEBLE, P.J. (1987). Neuropeptide Y modulates adrenergic neurotransmission by an endothelium dependent mechanism. *Eur. J. Pharmacol.*, **138**, 445–446.

EDVINSSON, L., EKBLAD, E., HÅKANSON, R. & WAHLESTEDT, C. (1984). Neuropeptide Y potentiates the effect of various vasoconstrictor agents on rabbit blood vessels. *Br. J. Pharmacol.*, **83**, 519–525.

FRANCO-CERECEDA, A. (1989). Endothelium- and neuropeptide Y-induced vasoconstriction of human epicardial coronary arteries *in vitro*. *Br. J. Pharmacol.*, **97**, 968–972.

FRANCO-CERECEDA, A. & LUNDBERG, J.M. (1985). Neuropeptide Y and sympathetic control of heart contractility and coronary vascular tone. *Acta Physiol. Scand.*, **124**, 361–369.

FRANCO-CERECEDA, A. & LUNDBERG, J.M. (1987). Potent effects of neuropeptide Y and calcitonin gene-related peptide on human coronary vascular tone *in vitro*. *Acta Physiol. Scand.*, **131**, 159–160.

GU, J., ADRIAN, T.E., TATEMOTO, K., POLAK, J.M., ALLEN, J.M. & BLOOM, S.R. (1983). Neuropeptide tyrosine (NPY) – a major cardiac neuropeptide. *Lancet*, **i**, 1008–1010.

GU, J., POLAK, J.M., ALLEN, J.M., HUANG, W.M., SHEPPARD, M.N., TATEMOTO, K. & BLOOM, S.R. (1984). High concentrations of a novel peptide, neuropeptide Y, in the innervation of mouse and rat heart. *J. Histochem. Cytoch.*, **32**, 467–472.

HAN, C. & ABEL, P.W. (1987). Neuropeptide Y potentiates contraction and inhibits relaxation of rabbit coronary arteries. *J. Cardivasc. Pharmacol.*, **9**, 675–681.

HIEBLE, J.P., DUESLER, J.G. & DALY, R.N. (1989). Effects of neuropeptide Y on the response of isolated blood vessels to norepinephrine and sympathetic field stimulation. *J. Pharmacol. Exp. Ther.*, **250**, 523–528.

LUNDBERG, J.M., TERENIUS, L., HÖKFELT, L., HÖKFELT, T. & GOLDSTEIN, M. (1983). High levels of neuropeptide Y (NPY) in peripheral noradrenergic neurons in various mammals including man. *Neurosci. Lett.*, **42**, 167–172.

LUNDBERG, J.M., HUA, X. & FRANCO-CERECEDA, A. (1984). Effects of neuropeptide Y (NPY) on the mechanical neurotransmission in the heart, vas deferens and urinary bladder. *Acta Physiol. Scand.*, **121**, 325–332.

MACHO, P., PEREZ, R., HUIDOBRO-TORO, P. & DOMENECH, R. (1989). Neuropeptide Y (NPY): a coronary vasoconstrictor and potentiator of catecholamine-induced coronary vasoconstriction. *Eur. J. Pharmacol.*, **167**, 67–74.

MARTIN, S.E. & PATTERSON, R.E. (1989). Coronary constriction due to neuropeptide Y: alleviation with cyclooxygenase blockers. *Am. J. Physiol.*, **3**, H927–H934.

MATURI, M.R., GREENE, R., SPEIR, E., BURRUS, C., DORSEY, L.M.A., MARKLE, D.R., MAXWELL, M., SCHMIDT, W., GOLDSTEIN, S.R. &

PATTERSON, R.E. (1989). Neuropeptide-Y. A peptide found in human coronary arteries constricts primarily small coronary arteries to produce myocardial ischaemia in dogs. *J. Clin. Invest.*, **83**, 1217–1224.

MIWA, K. & TODA, N. (1984). Regional differences in the response to vasoconstrictor agents of dog and monkey isolated coronary arteries. *Br. J. Pharmacol.*, **82**, 295–301.

MULVANY, M.J. & NYBORG, N.C.B. (1980). An increased sensitivity of mesenteric resistance vessels in young and adult spontaneously hypertensive rats. *Br. J. Pharmacol.*, **71**, 585–596.

NEILD, T.O. (1987). Actions of neuropeptide Y on innervated and denervated rat tail arteries. *J. Physiol.*, **386**, 19–30.

NYBORG, N.C.B. (1990). Action of noradrenaline on isolated rat proximal and distal coronary arteries: selective release of endothelium-derived relaxing factor in proximal arteries. *Br. J. Pharmacol.*, **100**, 552–556.

NYBORG, N.C.B. & MIKKELSEN, E.O. (1985). *In vitro* studies on responses to noradrenaline, serotonin, and potassium of intramyocardial and mesenteric resistance vessels from Wistar rats. *J. Cardiovasc. Pharmacol.*, **7**, 417–423.

NYBORG, N.C.B. & MIKKELSEN, E.O. (1988). Correlation between serotonin receptor occupancy, serotonin response and diameter of isolated rat coronary arteries. In *Resistance Arteries*. ed. Halpern, W., Pegram, P.L., Brayden, J.E., Mackey, K., McLaughlin, M.K. & Osol, G. pp. 99–104. Ithaca, New York: Perinatology Press.

NYBORG, N.C.B., BAANDRUP, U., MIKKELSEN, E.O. & MULVANY, M.J. (1987). Active, passive and myogenic characteristics of isolated rat intramural coronary resistance arteries. *Pflügers Arch./Eur. J. Pharmacol.*, **410**, 664–670.

OSHITA, M., KIOSHI, S. & MURAMATSU, I. (1989). Selective potentiation of extracellular Ca^{2+}-dependent contraction by neuropeptide Y in rabbit mesenteric arteries. *Gen. Pharmacol.*, **20**, 363–367.

OWEN, M.P. & TAPHORN, M.C. (1988). Segmental heterogeneity of vasoconstrictor effects to neuropeptide Y displayed in resistance arteries of the renal vasculature. In *Resistance Arteries*. ed. Halpern, W., Pegram, P.L., Brayden, J.E., Mackey, K., McLaughlin, M.K. & Osol, G. pp. 74–84. Ithaca, New York: Perinatology Press.

PERNOW, J. (1988). Co-release and functional interactions of neuropeptide Y and noradrenaline in peripheral sympathetic vascular control. *Acta Physiol. Scand.*, Suppl. 568, 1–56.

PERNOW, J. (1989). Actions of constrictor (NPY and endothelin) and dilator (substance P, CGRP and VIP) peptides on pig splenic and human skeletal muscle arteries: involvement of the endothelium. *Br. J. Pharmacol.*, **97**, 983–989.

PERNOW, J. & LUNDBERG, J.M. (1988). Neuropeptide Y induces potent contraction of arterial vascular smooth muscle via an endothelium-independent mechanism. *Acta Physiol. Scand.*, **134**, 157–158.

PERNOW, J., SWENBERG, T. & LUNDBERG, J.M. (1987). Actions of calcium antagonists on pre- and postjunctional effects of neuropeptide Y on human peripheral blood vessels *in vitro*. *Eur. J. Pharmacol.*, **136**, 207–218.

RUDEHILL, A., SOLLEVI, A., FRANCO-CERECEDA, A. & LUNDBERG, J.M. (1986). Neuropeptide Y (NPY) and the pig heart: release and coronary vasoconstrictor effects. *Peptides*, **7**, 821–826.

TSENG, C.-J., ROBERTSON, D., LIGHT, R.T., ATKINSON, J.R. & ROBERTSON, R.M. (1988). Neuropeptide Y is a vasoconstrictor of human coronary arteries. *Am. J. Med. Sci.*, **296**, 11–16.

WAHLESTEDT, C., EDVINSSON, L., EKBLAD, E. & HÅKANSON, R. (1985). Neuropeptide Y potentiates adrenaline-evoked vasoconstriction: Mode of action. *J. Pharmacol. Exp. Ther.*, **234**, 735–741.

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The role of nitric oxide as an endogenous regulator of platelet and neutrophil activation within the pulmonary circulation of the rabbit

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1 Intravenous (i.v.) administration of adenosine diphosphate (ADP), platelet activating factor (PAF) and thrombin induced a dose-related accumulation of ^{111}indium-labelled platelets within the thoracic region of anaesthetized rabbits.

2 I.v. administration of the inhibitor of NO biosynthesis, L-N^G-nitro arginine methyl ester (L-NAME; 10 mg kg^{-1}) significantly potentiated the peak platelet accumulation induced by ADP, PAF and thrombin. Additionally L-NAME prolonged the disaggregation of platelets in comparison to D-NAME (10 mg kg^{-1}). Such changes were reversible by the administration of L-arginine (900 mg kg^{-1}).

3 I.v. administration of PAF induced a small accumulation of ^{111}indium-labelled neutrophils within the pulmonary circulation which could be greatly potentiated by pretreatment of the animals with L-NAME. In contrast, thrombin administration did not cause significant accumulation of ^{111}indium-labelled erythrocytes in the pulmonary circulation of anaesthetized rabbits.

4 Intracarotid (i.c.) administration of thrombin induced a marked accumulation of radiolabelled platelets within the cranial vasculature which was not potentiated by the prior administration of L-NAME (at either 10 mg kg^{-1} or 100 mg kg^{-1}).

5 These results suggest that endogenous NO may regulate platelet and polymorphonuclear leukocyte activation within the pulmonary but not the cerebral circulation of rabbits.

Introduction

Endothelium-derived nitric oxide (NO) is widely believed to play an important role in vascular haemostasis. Thus, NO is a potent vasodilator (Furchgott, 1980) and inhibits both platelet adhesion (Radomski *et al.*, 1987) and platelet/neutrophil aggregation *in vitro* (Azuma *et al.*, 1986; McCall *et al.*, 1989). Stimuli of NO release (e.g. carbachol) also reduce platelet aggregation in intact animals (Hogan *et al.*, 1987; Bhardwaj *et al.*, 1988). Nonetheless, the possible physiological and pathophysiological roles of NO as a regulator of platelet/neutrophil aggregation and disaggregation *in vivo* remain largely unknown. The present experiments were therefore performed to test the hypothesis that NO is an endogenous regulator of the function of formed blood elements within the vasculature. To this end, use has been made of L-N^G-nitro arginine methyl ester (L-NAME), a potent and selective inhibitor of vascular and non-vascular NO biosynthesis (Gibson *et al.*, 1990; Moore *et al.*, 1990) on *in vivo* platelet and neutrophil aggregation in the pulmonary (Page *et al.*, 1982; Thiemermann *et al.*, 1990) and cerebral (May *et al.*, 1990) vascular beds of anaesthetized rabbits; a non-invasive technique for the determination of *in vivo* platelet aggregation with ^{111}indium-labelled platelets was used.

Methods

Animal selection and exclusion criteria

The study was carried out on male rabbits (New Zealand White, Froxfield, Petersfield, Hampshire) weighing 2.5 to 3.5 kg. Animals received a standard diet and water *ad libitum*.

^{111}Indium-labelling of platelets

Details of this procedure for rabbit platelets have been described elsewhere (May *et al.*, 1990; Thiemermann *et al.*, 1990). Briefly, blood (10 ml) was obtained from the marginal ear vein and collected into 3.8% (w/v) trisodium citrate and centrifuged (200 *g*, 10 min) to obtain platelet-rich plasma (PRP). The platelets were then washed by centrifugation (640 *g*, 15 min) in a calcium-free Tyrode solution containing prostaglandin E_1 (PGE_1 300 ng ml^{-1}) (CFTP). The washed platelet pellet was resuspended in 1.5 ml of CFTP and the platelets incubated for 90 s with ^{111}indium oxine (25–50 μCi). After a further centrifugation (640 *g*, 15 min) to remove any excess, unbound ^{111}indium, the platelet pellet was resuspended in CFTP (1 ml/recipient rabbit).

^{111}Indium-labelling of erythrocytes

Blood was taken as described above and PRP obtained as before and then discarded. A 1 ml sample of erythrocytes was removed from the remaining blood and washed by centrifugation (200 *g*, 10 min) in 10 ml CFTP. Erythrocytes were pelleted by centrifugation (640 *g*, 10 min) and the supernatant discarded. The erythrocyte pellet was carefully washed with 1 ml CFTP and resuspended in 1 ml of the same solution by gentle shaking. This erythrocyte solution was incubated with ^{111}indium oxine (25–50 μCi) for 1.5 min at 37°C. The volume was made up to 10 ml with CFTP and centrifuged (640 *g*, 10 min) and the excess ^{111}indium oxine removed. Erythrocytes were then resuspended in CFTP (1 ml per recipient rabbit) and administered to previously anaesthetized recipient rabbits as described for radiolabelled platelets.

^{111}Indium-labelling of neutrophils

Blood was taken from a conscious donor rabbit from the marginal ear vein into 3.8% w/v trisodium citrate (30 ml blood: 10 ml citrate). Of this, 15 ml was taken for the preparation of platelet-poor plasma (PPP) for Percoll gradients and resuspending neutrophils. To the remaining 25 ml blood, 8 ml dextran (6% in saline) was added and the volume made up to 50 ml with 0.9% saline. Leukocyte-rich plasma (LRP) was then obtained by sedimentation for 30 min and centrifuged (275 *g*, 10 min) to obtain a leukocyte pellet. (The supernatant

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was used to obtain PPP (Percoll) for the washing stages). The leukocyte pellet was resuspended in PPP (without Percoll), layered onto a 40:60% Percoll gradient and centrifuged at 275 g for 10 min. The neutrophil band produced was removed, centrifuged at 275 g without washing and resuspended in 2 ml PPP (without Percoll) for labelling with ^{111}indium mercaptopyridine.

^{111}Indium mercaptopyridine was prepared by adding 250 μl of 2-mercaptopyridine-N-oxide (40 μg ml^{-1} in 50 mM phosphate buffered saline) to 50–200 μCi ^{111}indium chloride and incubating for 5 min at room temperature before adding to the neutrophil suspension and incubating for 15 min (room temperature). The cells were washed twice in PPP (Percoll) before being resuspended in PPP (without Percoll) for administration to recipient animals (1 ml per recipient).

Measurement of in vivo *platelet and neutrophil activation by the automated isotope monitoring system*

Animals were anaesthetized with a combination of diazepam (5 mg kg^{-1}, i.p.) and Hypnorm (0.4 ml kg^{-1}, i.m. containing fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg ml^{-1}). ^{111}Indium-labelled platelets were administered via the marginal ear vein and allowed to equilibrate in the circulation for 1 h (platelets and erythrocytes) or 90 min (neutrophils). Circulating ^{111}indium-labelled platelets, erythrocytes or neutrophils were continuously monitored in the thoracic and cerebral vascular beds by one inch crystal scintillation detectors placed over the thorax (to measure changes in the pulmonary circulation) and head (to measure changes in the cerebral circulation). Counts were estimated with a dual channel spectrometer (Nuclear Enterprises NE 461) and logged with the aid of a special application interface by a microcomputer (Dell System 200). *In vivo* platelet or neutrophil aggregation in response to either i.v. or i.c. administration of agonists was measured as an increase in radioactivity in either the pulmonary circulation (i.v. agonists) or the cerebral circulation (i.c. agonists). Changes in radioactivity were corrected for blood pool ^{111}indium radioactivity (baseline value) and expressed as % increase in counts. The area under the curve of the agonist-induced % increase in counts was calculated.

Administration of platelet/neutrophil agonists and drugs

Platelet agonists were administered either via the marginal ear vein (i.v. route) or via the carotid artery (i.c. route). Submaximal doses of ADP (10 μg kg^{-1}), PAF (0.05 μg kg^{-1}) and thrombin (10 iu kg^{-1}) were chosen for this study from dose-response data obtained in a previous study (May *et al.*, 1990). An initial dose of ADP (10 μg kg^{-1}, i.v. or 100 μg kg^{-1}, i.c.) was administered to each rabbit prior to the start of each experiment in order to determine whether the platelets were functioning normally and also to eliminate any platelets that may have been damaged during the labelling procedure. For ADP (10 μg kg^{-1}, i.v.) and PAF (0.05 μg kg^{-1}, i.v.) where it is possible to obtain reproducible responses in one animal, the platelet agonist was administered prior to and 5 min after L-NAME (10 mg kg^{-1}, i.v.) with an hour between successive agonist doses. In some groups of animals receiving PAF as the agonist, the animals received L-arginine (300 or 900 mg kg^{-1}, i.v.) 2 min before the L-NAME, or D-NAME alone (10 mg kg^{-1}, i.v.) in place of L-NAME. For animals receiving thrombin (10 iu, kg^{-1}, i.v.) as the platelet agonist, separate groups of animals were used since reproducible responses to thrombin cannot necessarily be obtained. To one group, L-NAME (10 mg kg^{-1}, i.v.) was administered 5 min before the administration of thrombin (10 iu kg^{-1}, i.v.) and to the second group an equivalent volume of saline (0.5 ml) was administered in the place of L-NAME. In a second and third group of rabbits this protocol was repeated except that the thrombin was administered via the carotid artery instead of the marginal ear vein. In this case, two doses of L-NAME were used 10 mg kg^{-1} and 100 mg kg^{-1}. In addition, the experiments with i.v. thrombin were repeated with radiolabelled erythrocytes instead of platelets. In one group of animals receiving radiolabelled neutrophils, L-NAME (10 mg kg^{-1}, i.v.) was administered 5 min before PAF (0.3 μg kg^{-1}, i.v.). In a second group an equivalent volume of saline was given in place of the L-NAME.

Drugs

The following compounds were used: ADP bovine serum albumin (BSA), L-arginine, L-N^G-nitro arginine methyl ester (L-NAME). Tyrode salt solution and thrombin (Sigma, Poole, Dorset); D-N^G-nitro arginine methyl ester (D-NAME) (Bachem Ltd.); diazepam (Valium, Roche, Welwyn Garden City); Hypnorm (Janssen Pharmaceutica Ltd., Oxford); ^{111}indium oxine, ^{111}indium chloride (Amersham International plc); PAF (Novabiochem, Nottingham). PAF was dissolved in 0.25% BSA. All other compounds were prepared in physiological saline.

Statistical comparison

The area under the curve (AUC) of the % increase in radioactive counts was calculated for each response. The numbers shown in the text are the mean ± s.e.mean of at least 5 observations. Results were analysed by means of an F test and then a Student's t test, Mann Whitney U test or Wilcoxon test as appropriate.

Results

Haemodynamic measurements

Treatment of the animals with L-NAME (10 mg kg^{-1}, i.v.) produced a sustained increase in blood pressure of approximately 20% (51.2 ± 2.7 mmHg to 61.5 ± 3.9 mmHg, $n = 15$ $P < 0.05$, Student's t test). Neither D-NAME (10 mg kg^{-1}, i.v.) or an appropriate volume of saline (0.5 ml) altered blood pressure.

Investigations with radiolabelled platelets in the pulmonary circulation

The accumulation of ^{111}indium-labelled platelets in the pulmonary vasculature in response to ADP (10 μg kg^{-1}, i.v., $n = 8$), PAF (0.05 μg kg^{-1}, i.v., $n = 8$) and thrombin (10 iu kg^{-1}, i.v., $n = 5$) was transient in nature only (Figure 1, Table 1). Pretreatment with L-NAME (10 mg kg^{-1}, i.v. 5 min before the administration of agonist) elicted an initial minimal accumulation of platelets (6.2 ± 0.6%) and significantly potentiated the platelet response induced by i.v. ADP, PAF and thrombin (Figure 1, Table 1) as assessed by the area under the curve of the thoracic accumulation of platelet-associated radioactivity. The addition of L-arginine (300–900 mg kg^{-1}, i.v.) 2 min prior to L-NAME produced a substantial but not significant reversal of the effect of L-NAME on the PAF-induced platelet response. However, the administration of L-arginine (900 mg kg^{-1}, i.v.) 2 min before the administration of L-NAME completely reversed ($P < 0.001$) the L-NAME-mediated augmentation of thrombin-induced platelet accumulation (Table 2).

Investigations with radiolabelled erythrocytes and neutrophils within the pulmonary circulation

The i.v. administration of PAF (0.3 μg kg^{-1}; $n = 6$) produced a small accumulation (7.64 ± 2.69 as assessed by AUC) of ^{111}indium-labelled polymorphonuclear leukocytes (PMNs) in the pulmonary vasculature that was smaller than the platelet accumulation induced by the same dose of PAF (data not shown) and this was transient in nature. Pretreatment with L-NAME (10 mg kg^{-1}, i.v.) 5 min before the administration of PAF significantly potentiated the peak response induced by i.v. PAF (see Figure 2). Furthermore, the extent of the neu-

Table 1 Responses to either two consecutive doses of adenosine diphosphate (ADP, 10 $\mu g kg^{-1}$, i.v.) or platelet activating factor (PAF, 0.05 $\mu g kg^{-1}$, i.v.) 60 min apart in rabbits with ^{111}indium-labelled platelets in pulmonary vasculature

Agonist	n	*Treatment*	*Response* (AUC) 1st	2nd	*% difference*
ADP (10 $\mu g kg^{-1}$)	8	L-NAME (10 $mg kg^{-1}$)	4.49 ± 0.47	7.19 ± 1.68*	34.97 ± 8.81
PAF (0.5 $\mu g kg^{-1}$)	8	L-NAME (10 $mg kg^{-1}$)	9.53 ± 1.37	17.22 ± 3.98*	80.06 ± 21.02
	5	D-NAME	9.81 ± 1.9	10.27 ± 2.9*	−2.15 ± 9.3
	5	L-Arg (300 $mg kg^{-1}$) + L-NAME	10.11 ± 0.9	17.79 ± 2.6*	72.85 ± 15.68
	5	L-Arg (900 $mg kg^{-1}$) + L-NAME	9.52 ± 0.39	13.26 ± 1.3*	47.53 ± 3.45

The treatments indicated were administered 5 min before the second dose of agonist. L-Arginine (L-Arg) where given, was administered 2 min prior to L-N^G-nitro arginine methyl ester (L-NAME). Responses are expressed as mean ± s.e.mean of the area under the curve (AUC) of the accumulation of platelet associated radioactivity in the pulmonary vasculature. After statistical analysis with an F test, results were analysed by means of a paired Student's *t* test or Wilcoxon test as appropriate, where * $P < 0.05$.

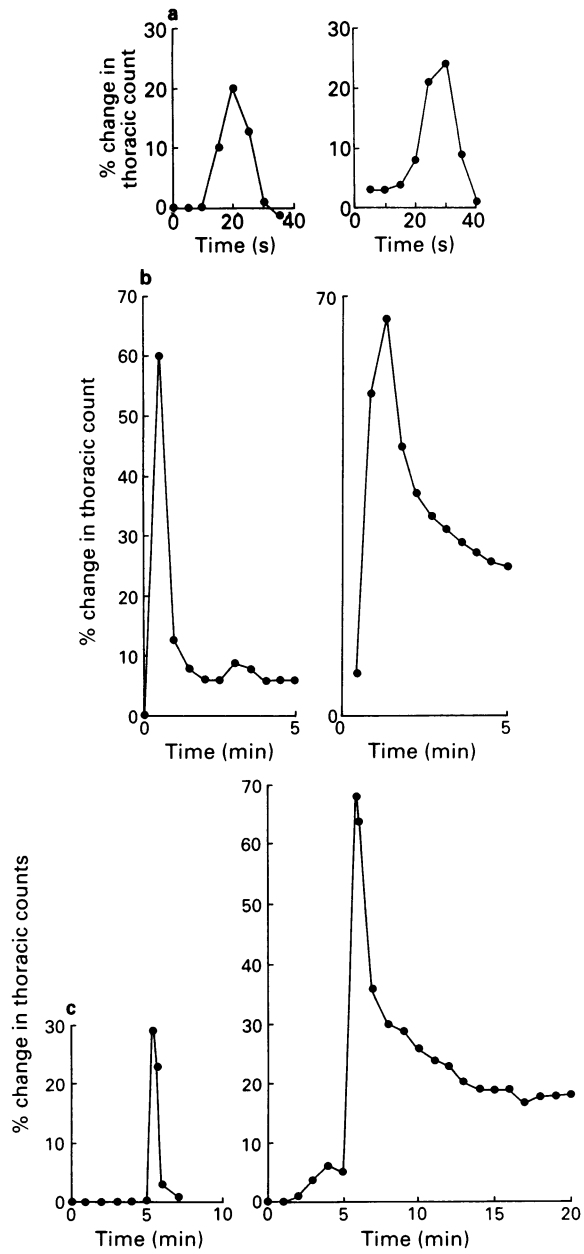

Figure 1 Examples of responses to adenosine diphosphate (ADP, 10 $\mu g kg^{-1}$, i.v.) (a), platelet activating factor (PAF, 0.05 $\mu g kg^{-1}$, i.v.) (b) and thrombin (10 $iu kg^{-1}$, i.v.) (c) before (left panel) and after (right panel) L-N^G-nitro arginine methyl ester (L-NAME 10 $mg kg^{-1}$, i.v.) in rabbits with ^{111}indium-labelled platelets. Thrombin was administered to separate groups of rabbits; to one group (left panel) saline was administered 5 min before agonist, to the other group (right panel) L-NAME (10 $mg kg^{-1}$, i.v.) was administered.

Table 2 Responses to intravenous thrombin (10 $iu kg^{-1}$) 5 min after treatment with either saline (0.5 $ml kg^{-1}$, i.v.) or L-N^G nitro arginine methyl ester (L-NAME, 10 $mg kg^{-1}$, i.v.) in the pulmonary vasculature; in addition, a third group of animals received L-arginine (900 $mg kg^{-1}$, i.v.) 2 min prior to L-NAME

Treatment	n	*Response* (AUC)
Saline	5	1.86 ± 0.77
L-NAME (10 $mg kg^{-1}$)	5	21.55 ± 11.91*
L-Arginine (900 $mg kg^{-1}$) + L-NAME	6	2.06 ± 0.33

Responses are expressed as mean ± s.e.mean of the area under the curve (AUC) of the accumulation of platelet-associated radioactivity in the pulmonary vasculature. After statistical analysis with an F test, results were analysed by means of an unpaired Student's *t* test or a Mann Whitney test as appropriate, where * $P < 0.05$.

trophil accumulation assessed as the AUC of the accumulation of neutrophil-associated radioactivity was also significantly potentiated (25.55 ± 5.75).

The administration of L-NAME (10 $mg kg^{-1}$, i.v.) 5 min before the administration of thrombin (10 $iu kg^{-1}$, i.v.) itself produced a small accumulation of ^{111}indium-labelled erythrocytes of similar magnitude and duration to that seen with ^{111}indium-labelled platelets. However, the administration of

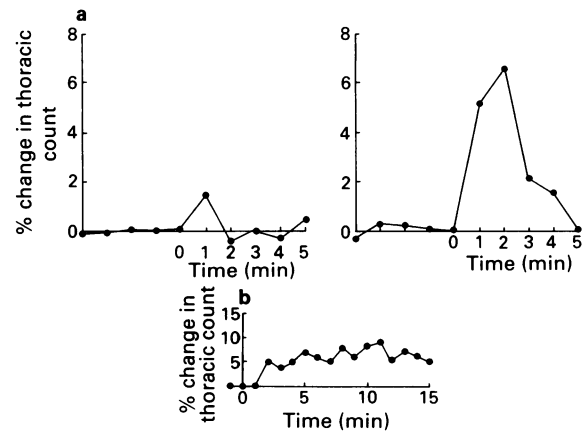

Figure 2 Examples of responses following platelet activating factor (PAF, 0.3 $\mu g kg^{-1}$, i.v.) administration to rabbits with ^{111}indium-labelled neutrophils are illustrated in (a). Separate groups of animals were used. To one group (left panel) saline was administered 5 min before the agonist, to the other group (right panel) L-N^G-nitro arginine methyl ester (L-NAME, 10 $mg kg^{-1}$, i.v.) was administered. In (b) is shown the response to thrombin (10 $iu kg^{-1}$, i.v.) after pretreatment with L-NAME in an animal with ^{111}indium-labelled erythrocytes.

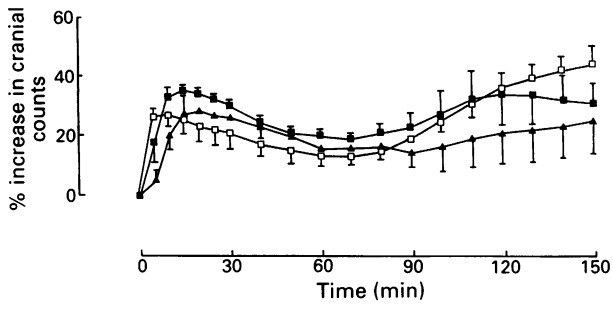

Figure 3 Changes in cranial platelet-associated radioactivity over time following intracarotid administration of thrombin (10 iu kg^{-1}) in animals pretreated with either saline (1 ml, i.v.) (□), L-N^{G}-nitro arginine methyl ester (L-NAME, 10 mg kg^{-1}, i.v.) (■) or L-NAME (100 mg kg^{-1}, i.v.) (▲) 5 min before the administration of thrombin.

thrombin 5 min later did not produce any accumulation of radiolabelled erythrocytes within the pulmonary vasculature.

Investigations with radiolabelled platelets within the cerebral circulation

Intracarotid administration of thrombin (10 iu kg^{-1}, $n = 5$) produced a sustained accumulation of platelet-associated radioactivity within the cerebral vasculature which is known not to be due to release of the radiolabel from the platelets (May *et al.*, 1990). Pretreatment of the animals with L-NAME (10 mg kg^{-1}, i.v.) 5 min before the administration of thrombin had no significant effect on either the peak or the AUC of the accumulation of radiolabelled platelets within the cerebral circulation (Figure 3). In addition pretreatment of the animals with a 10 fold larger dose of L-NAME (100 mg kg^{-1}, i.v.) 5 min prior to the administration of the thrombin also had no significant effect on the peak or AUC (Figure 3). Furthermore, intra-arterial administration of L-NAME (100 mg kg^{-1}) 5 min before administration of thrombin had no significant effect on the AUC (thrombin plus L-NAME; 43.15 ± 15, $n = 4$; thrombin plus saline; 34 ± 12, $n = 4$).

Discussion

Numerous techniques are available for studying platelet aggregation *in vitro*. Of these, platelet aggregometry, first described by Born (1962) has proved to be useful in elucidating the major pathways and mediators involved in the platelet aggregation response. However, such techniques cannot take into account the multitude of control mechanisms operative *in vivo* and thus are not predictive of platelet function in the intact organism. One characteristic of platelet activity which is difficult to reproduce accurately *in vitro* is the influence of the vessel wall in the cycle of aggregation/disaggregation which underlies the physiological significance of platelet function *in vivo*.

All three of the platelet agonists chosen for this study have been previously shown to produce dose-related increases in thoracic platelet-associated radioactivity following intravenous administration. Furthermore, intracarotid administration of thrombin has been shown to produce a dose-related increase in cranial platelet-associated radioactivity with this technique in the rabbit (May *et al.*, 1990). The intravenous doses of agonists used here all produced a submaximal increase in platelet-associated radioactivity, whilst only PAF caused a similar accumulation of radiolabelled neutrophils at doses that have previously been shown to induce thrombocytopaenia in this species (Honey *et al.*, 1986). No change in erythrocyte-associated radioactivity was observed following intravenous administration of thrombin implying that the changes detected using radiolabelled platelets were not secondary to changes in blood flow and therefore blood pooling within the pulmonary vasculature.

Treatment of animals with L-NAME (10 mg kg^{-1}) produced a rapid and sustained increase in blood pressure but neither D-NAME (10 mg kg^{-1}), the biologically inactive enantiomer of L-NAME, nor an appropriate volume of saline (0.5 ml) altered blood pressure suggesting (i) that a basal release of NO from either vascular endothelial cells or circulating neutrophils plays a physiological role in modulating vascular tone in this species, and (ii) L-NAME was present at sufficient local concentrations to inhibit NO biosynthesis.

L-NAME administration also produced a small but sustained accumulation of platelet-associated radioactivity in the thoracic region. Whilst this observation may, at first site, imply a basal anti-aggregatory role for NO in the pulmonary microvasculature, it is more likely to be secondary to a redistribution of blood, consequent upon an increase in blood pressure since the same dose of L-NAME elicited a very similar accumulation of erythrocyte-associated radioactivity under these conditions.

L-NAME but not D-NAME (both 10 mg kg^{-1}) administration significantly enhanced the increase in thoracic platelet accumulation following intravenous injection of either a submaximal dose of PAF, thrombin or ADP and additionally significantly increased and prolonged the accumulation of radiolabelled neutrophils in response to PAF. Furthermore, the normal disaggregation pattern was substantially prolonged in animals receiving L-NAME. This suggests that endogenously produced NO may play a role in inhibiting or limiting platelet aggregation.

L-NAME, at a dose that produced a significant effect on pulmonary platelet accumulation induced by intravenous thrombin, failed to have any significant effect on the accumulation of platelets in the cerebral vasculature induced by intracarotid thrombin. In addition, a 10 fold higher dose also failed to have any significant effect, even when administered by the intra-arterial route. It is possible that intravenous administration of L-NAME may have a preferential effect on pulmonary NO production compared to cerebral NO production, which may occur if the majority of L-NAME is taken up by the pulmonary endothelial cells, the first vascular bed encountered following intravenous administration. This would explain why inhibiting NO synthesis then appears to have a larger effect on the pulmonary compared with cerebral platelet accumulation. However, this seems unlikely as a 10 fold higher dose of L-NAME still had no significant effect. On the other hand, the characteristics of platelet accumulation observed in the cerebral vasculature are very different in profile from that seen in the pulmonary circulation. Pulmonary platelet accumulation is very transient compared with the cerebral platelet accumulation and probably represents simple platelet aggregation. Cerebral platelet accumulation appears to involve the coagulation cascade to a greater extent as shown by the incorporation of ^{125}I-fibrinogen and the susceptibility of the accumulation of platelets to reversal with fibrinolytic agents (May *et al.*, 1990). Thus, we propose that the release of NO in the cerebral vasculature is less likely to interfere in the response, thereby explaining the lack of effect of L-NAME in these experiments. In addition, there remains the possibility that NO produced in the pulmonary vasculature is relatively more important in regulating platelet function than in the cerebral vasculature in which the principal function of NO may be vasodilatation rather than inhibition of platelet function. Endothelium-dependent relaxations have been demonstrated in the cerebral microvasculature of the mouse (Rosenblum *et al.*, 1987) so it is unlikely that the cerebral vasculature is not important as a site of production of NO, but this may be preferentially abluminally released and be more important with respect to vascular smooth muscle relaxation than inhibition of platelet function.

NO is formed from L-arginine by the action of a specific deimidase enzyme system and much attention has been focussed on characterizing this enzyme (Taych & Marletta, 1989; Bojc & Fung, 1990; Knowles *et al.*, 1990). L-NAME is believed to inhibit NO biosynthesis via the competitive inhibi-

tion of this enzyme. Accordingly, the effect of this inhibitor in isolated blood vessels may be partially reversed by co-administration of precursor L-arginine (Moore *et al.*, 1990). In the present experiments, pretreatment of animals with L-arginine (300–900 mg kg^{-1}) produced a substantial but not statistically significant ($P > 0.05$) partial reversal of the effect of L-NAME on the PAF-induced platelet response. In contrast, L-arginine (900 mg kg^{-1}) administration completely reversed ($P < 0.001$) the L-NAME-mediated augmentation of thrombin-induced platelet accumulation in these experiments. Although L-NAME is believed to inhibit NO biosynthesis by competitive inhibition of the enzyme, very large concentrations of L-arginine are required to achieve a reversal of this, not only in the present study, but also in other systems (Moore *et al.*, 1991). From the present data we clearly cannot exclude the possibility that mediators other than NO may have a minor role to play in the effect of L-NAME on pulmonary platelet accumulation *in vivo* induced, in particular, by PAF. However, NO seems to be important in modulating the extent of platelet aggregation induced by thrombin and may also be of major importance in regulating the disaggregation of platelets and neutrophils within the pulmonary vasculature.

Recent research has highlighted the important part played by the vascular endothelium in the control of platelet function and haemostasis. Endothelial cells synthesize and release prostacyclin (PGI_2) (Moncada *et al.*, 1976), a potent inhibitor of platelet function and tissue plasminogen activator (tPA) (Loskutoff *et al.*, 1977) which promotes clot dissolution. The results of the present study provide evidence that NO produced either from vascular endothelial cells, circulating neutrophils (McCall *et al.*, 1989; Salvemini *et al.*, 1989) or platelets themselves (Radomski *et al.*, 1990) makes a major contribution to the control of platelet and neutrophil aggregation and disaggregation *in vivo*. Decreases in nitric oxide production have been observed in atherosclerotic human coronary arteries (Chester *et al.*, 1990) and this suggests that NO biosynthesis and activity serves as an important protective mechanism. Disorders of this protective mechanism may therefore contribute to or even underlie thrombotic disorders.

References

AZUMA, H., ISHIKAWA, M. & SEKIZAKI, S. (1986). Endothelium-dependent inhibiton of platelet aggregation. *Br. J. Pharmacol.*, **88**, 411–415.

BHARDWAJ, R., PAGE, C.P., MAY, G.R. & MOORE, P.K. (1988). Endothelium derived relaxing factor inhibits platelet aggregation in human whole blood and in the rat *in vivo*. *Eur. J. Pharmacol.*, **157**, 83–92.

BOJC, K.M. & FUNG, H.-L. (1990). Endothelial nitric oxide generating enzyme(s) in the bovine aorta: subcellular location and metabolic characterization. *J. Pharmacol. Exp. Ther.*, **253**, 20–25.

BORN, G.V.R. (1962). Quantitative investigations into the aggregation of blood platelets. *Proc. Physiol. Soc.*, **162**, 67P.

CHESTER, A.H., O'NEIL, G.S., MONCADA, S., TADJKARIMI, S. & YACOUB, M.H. (1990). Low basal and stimulated release of nitric oxide in atherosclerotic epicardial coronary arteries. *Lancet*, **336**, 897–900.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GIBSON, A., MIRZAZADEH, S., HOBBS, A. & MOORE, P.K. (1990). L-N^G-monomethyl arginine and L-N^G-nitro arginine inhibit non-adrenergic, non-cholinergic relaxation of the mouse anococcygeus muscle. *Br. J. Pharmacol.*, **99**, 602–606.

HOGAN, J.C., LEWIS, M.J. & HENDERSON, A.H. (1988). *In vivo* EDRF activity influences platelet function. *Br. J. Pharmacol.*, **94**, 1020–1022.

HONEY, A.C., LAD, N. & TUFFIN, D.P. Effect of indomethacin and dazoxiben on intravascular platelet aggregation in the anaesthetized rabbit. *Thromb. Haemostas.*, **56**, 80–85.

KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1990). Kinetic characterisation of nitric oxide synthase from rat brain. *Biochem. J.*, **269**, 207–210.

LOSKUTOFF, D.J. & EDGINTON, T.S. (1977). Synthesis of a fibrinoyltic activator and inhibitor by endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 3903–3907.

MAY, G.R., HERD, C.M., BUTLER, K.D. & PAGE, C.P. (1990). Radioisotopic model for investigating thromboembolism in the rabbit. *J. Pharmacol. Meth.*, **24**, 19–35.

McCALL, T.B., BOUGHTON-SMITH, N.K., PALMER, R.M.J., WHITTLE, B.J.R. & MONCADA, S. (1989). Synthesis of nitric oxide from L-arginine by neutrophils: release and interaction with superoxide anion. *Biochem. J.*, **261**, 293–296.

MONCADA, S., GRYGLEWSKI, R., BUNTING, S. & VANE, J.R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*, **263**, 663–665.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitro arginine (L-NOARG), a novel L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.*, **99**, 408–412.

MOORE, P.K., OLUYOMI, A.O., BABBEDGE, R.C., WALLACE, P. & HART, S.L. (1991). L-N^G-nitro arginine methyl ester exhibits antinociceptive activity in the mouse. *Br. J. Pharmacol.* (in press).

PAGE, C.P., PAUL, W. & MORLEY, J. (1982). An *in vivo* model for studying platelet aggregation and disaggregation. *Thromb. Haemostas.*, **47**, 210–213.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987). Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* **ii**, 1057–1058.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 5193–5197.

ROSENBLUM, W.I., NELSON, G.H. & POVLISHOCK, J.T. (1987). Laser-induced endothelial damage inhibits endothelium-dependent relaxation in the cerebral microcirculation of the mouse. *Circ. Res.*, **60**, 169–176.

SALVEMINI, D., DE NUCCI, G., GRYGLEWSKI, R.J. & VANE, J.R. (1989). Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 6328–6332.

TAYCH, M.A. & MARLETTA, M.A. (1989). Macrophage oxidation of L-arginine to nitric oxide, nitrite and nitrate: tetrahydrobiopterin is required as a cofactor. *J. Biol. Chem.*, **264**, 19654–19658.

THIEMERMANN, C., MAY, G.R., PAGE, C.P. & VANE, J.R. (1990). Endothelin-1 inhibits platelet aggregation *in vivo*: a study with ^{111}-indium-labelled platelets. *Br. J. Pharmacol.*, **99**, 303–308.

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4-Aminopyridine-induced increase in basal and stimulation-evoked [^{3}H]-NA release in slices from rat hippocampus: Ca^{2+} sensitivity and presynaptic control

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1 We have examined the mechanisms by which the K^+-channel blocker 4-aminopyridine (4-AP) can dose-dependently increase both basal [^{3}H]-noradrenaline ([^{3}H]-NA) release and the [^{3}H]-NA release evoked by electrical stimulation, but not the release of [^{3}H]-acetylcholine ([^{3}H]-ACh), from slices of rat hippocampus.

2 Both the electrically evoked and the 4-AP-induced release were blocked by tetrodotoxin (TTX) (3 μM). The Ca^{2+}-dependence of the 4-AP-induced release (EC_{50} 0.15 mM) was, however, different from that of the electrically evoked [^{3}H]-NA release (EC_{50} 0.76 mM).

3 The 4-AP-induced release could be inhibited by $CdCl_2$(10 μM) and ω-conotoxin (30 nM), but not by nifedipine (1 μM).

4 Transmitter release evoked by 100 μM 4-AP could be blocked by the α_2-adrenoceptor agonist, UK14,304 (0.1 μM) and by the A_1-receptor agonist **R**-N^6-phenylisopropyl adenosine (**R**-PIA, 1 μM) and increased by the α_2-adrenoceptor antagonist, yohimbine (1 μM), both in 0.25 and 1.3 mM Ca^{2+}-containing medium. By contrast, the effect of α_2-adrenoceptor agonists and antagonists on transmitter release evoked by electrical stimulation was markedly reduced in the presence of 4-AP (100 μM).

5 The results suggest that 4-AP can depolarize some nerve endings in the central nervous system, leading to transmitter release that is dependent on nerve impulses and Ca^{2+}. Furthermore, the fact that α_2-receptors and adenosine A_1 receptor agonists can influence the release of NA evoked by 4-AP suggests that these drugs may have actions that are independent of blockade of aminopyridine-sensitive K^+-channels.

Introduction

Noradrenaline (NA) release from rat and rabbit hippocampus can be inhibited by activation of adenosine A_1-receptors, opiate receptors, or α_2-adrenoceptors (Jonzon & Fredholm, 1985; Jackish *et al.*, 1985). The mechanisms underlying the inhibition are incompletely understood (Starke, 1987; Fredholm & Dunwiddie, 1988), but might involve an inhibition of Ca^{2+}-channels (Dunwiddie, 1985; Madison *et al.*, 1987) or an activation of K^+-channels (Trussell & Jackson, 1985; 1987). 4-Aminopyridine (4-AP), tetraethylammonium chloride (TEA) and dendrotoxin (DTX) can be used as tools to elucidate the role of K^+-channels (Rudy, 1988). 4-AP has been shown to increase the release of NA evoked by field stimulation (Schoffelmeer & Mulder, 1983; Huang *et al.*, 1988). This enhancement of the evoked transmitter release is generally explained by the ability of aminopyridines to block the delayed rectification and hence to prolong the action potential and the period of Ca^{2+} entry (Rudy, 1988). In a previous paper (Hu & Fredholm, 1989) we observed that 4-AP tended to stimulate [^{3}H]-NA release *per se* and it has been shown that 3,4-diaminopyridine causes release of [^{3}H]-NA from rabbit hippocampal slices (Huang *et al.*, 1989). An effect of 4-AP on spontaneous [^{3}H]-NA release from other tissues (Leander *et al.*, 1977; Schoffelmeer & Mulder, 1983) and on spontaneous release of ACh from striatal slices (Dolezal & Tucek, 1983) has been reported earlier. In this study we have examined the actions of 4-AP both on spontaneous transmitter release and on electrically evoked release, and compared these processes with regard to Ca^{2+}-dependence and presynaptic control.

Methods

Male Sprague-Dawley rats (150–200 g) were decapitated and the brains were rapidly removed and placed in ice-cold saline. The hippocampus was dissected out and cut into 0.4 mm thick slices with a McIlwain tissue chopper.

The release of [^{3}H]-NA was studied essentially as described by Jonzon & Fredholm (1985). Briefly, the slices were incubated with (−)-[^{3}H]-NA in the presence of pargyline chloride (0.025 mg ml^{-1}) and ascorbic acid (0.02 mg ml^{-1}), placed in chambers and superfused for 60 min before the experiment with gassed Krebs Ringer bicarbonate buffer of the following composition (in mM): NaCl 118, $MgSO_4$ 1.15, KH_2PO_4 1.15, $NaHCO_3$ 25, glucose 11.1 and variable amounts of $CaCl_2$ (0–1.3). Pulses of 10 V, 2 ms duration were delivered by two platinum electrodes placed 2.5 cm apart. In most experiments the buffer contained desipramine (1 μM) to inhibit noradrenaline reuptake.

In order to study electrically evoked [^{3}H]-NA release, two periods of stimulation for 5 min were given during the fourth and twelfth fractions and the release of tritium during the second stimulation (S_2, given during fraction 12) was divided by the amount released during the first (S_1, given during fraction 4), the basal outflow was subtracted. Drugs were added 15 min before stimulation.

In the experiments where we examined the 4-AP-induced release, only one 5 min stimulation was given (during the fourth fraction) and 4-AP solution was administered from the ninth fraction onwards. The release induced by 4-AP was calculated by summing the release during fractions 11–16. The background release was subtracted. In some experiments 4-AP was administered from fraction 9 onwards, and an S_2-stimulation period was given during fraction 12. In experiments performed to check whether TTX would affect basal release, the procedure was the same as above except that TTX was added at fraction 8. The basal release was calculated as % per min by summing the release during fractions 8–11. In a few experiments the release of ^{3}H-material from [^{3}H]-choline labelled hippocampal slices was also studied essentially as described by Jackisch *et al.* (1984). In brief, the slices were labelled with [^{3}H]-choline (0.1 μM; 80 Ci mmol^{-1}) for 30 min; the slices were thereafter transferred to superfusion chambers

(1 slice/chamber) and continuously superfused with gassed Krebs-Ringer bicarbonate buffer of the following composition (in mM): NaCl 118, KCl 4.85, $MgSO_4$ 1.15, KH_2PO_4 1.15, $CaCl_2$ 1.25, $NaHCO_3$ 25, glucose 11.1, containing hemicholinium-3 10 μM, at a flow rate of 0.5 ml min^{-1}.

After the experiment the radioactivity in the fractions and the remaining radioactivity in the slices from each chamber was counted and the fractional release was calculated by a microcomputer programme. The statistical analysis was carried out by microcomputer programme using 1-way ANOVA or Student's t test and a probability level of <0.05 was considered significant.

Chemicals

(−)-(7,8-[^{3}H])-noradrenaline (44 Ci $mmol^{-1}$; 1 mCi[37 MBq] ml^{-1}) and [^{3}H]-choline (80 Ci $mmol^{-1}$; 1 mCi[37 MBq] ml^{-1}) were obtained from the Radiochemical Centre, Amersham, U.K. R-N^6-phenylisopropyl adenosine (R-PIA) was obtained from Boehringer, Mannheim, Germany. Tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), tetrodotoxin, hemicholinium-3, and ascorbic acid were from Sigma, desipramine (DMI) from Ciba-Geigy and pargyline hydrochloride from Karolinska apoteket, Stockholm. Cadmium chloride was from Merck (Darmstadt, FRG). UK 14,304 (5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline) and yohimbine hydrochloride were gifts from Dr S.Z. Langer and Dr J.M. Lundberg. ω-Conotoxin was from Penninsula Laboratories, Inc., Belmont, Calif., U.S.A.

Results

Field stimulation induced a frequency- and time-dependent release of [^{3}H]-NA from rat hippocampus (Figures 1 and 2) confirming and extending earlier results (Jonzon & Fredholm, 1985). Under control conditions there was an essentially linear relationship between frequency stimulation and amount of released transmitter (Figure 1). Similarly, the amount of [^{3}H]-NA released was directly related to the number of pulses delivered at a given frequency of stimulation under control conditions (Figure 2). In agreement with our previous findings (Hu & Fredholm, 1989) the evoked release of transmitter was enhanced by 300 μM 4-AP. However, in the presence of such a large amount of 4-AP the evoked release was no longer linearly related to frequency or duration of stimulation (Figures 1 and 2). Thus, the apparent increase in transmitter release was much larger at low frequencies and at short duration of stimulation. It was, however, not possible to examine whether the release of transmitter was linear at lower stimulation frequencies and/or shorter periods of stimulation, since the magnitude of the control release was too low to allow certain determination. Furthermore, 300 μM 4-AP itself caused a large increase in transmitter overflow that precluded the study of all but the largest evoked release. However, using a lower concentration of 4-AP (30 μM) it was observed that there was an essentially linear relationship between frequency and amount of transmitter released, at least until some 10% of the total tissue transmitter content was released (Figure 1).

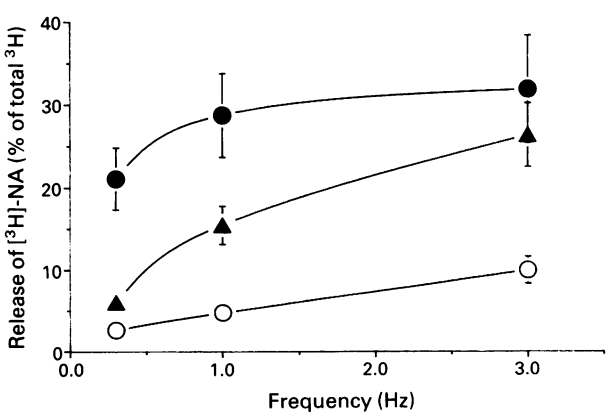

Figure 1 Frequency-dependence of the [^{3}H]-noradrenaline ([^{3}H]-NA) release induced by field stimulation of slices from rat hippocampus. Hippocampal slices were treated as described in methods. The buffer contained 1.3 mM Ca^{2+}. Three 5 min trains of pulse of 10 V and 2 ms duration at the rate of 0.3, 1.0 and 3.0 Hz were delivered. 4-Aminopyridine (4-AP) was added 15 min before the first stimulation. Results are expressed as the amount of radioactivity released by each stimulation (in % of the total radioactivity present in the slice at the time of the stimulation): (○) control; (▲) 30 μM and (●) 300 μM 4-AP. Mean (with s.e.mean shown by vertical bars) of five experiments.

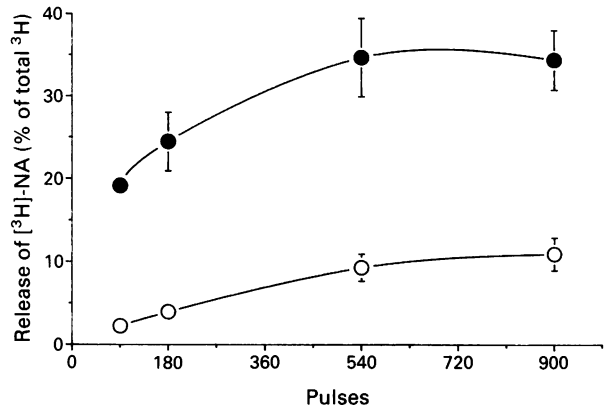

Figure 2 The effect of varying the number of pulses on the [^{3}H]-noradrenaline ([^{3}H]-NA) release induced by field stimulation of slices from rat hippocampus. Four stimulations (10 V, 3 Hz, 2 ms duration) were given at 0.5, 1.0, 3.0, and 5 min. 4-Aminopyridine (4-AP) was added 20 min before the first stimulation: (○) control and (●) 300 μM 4-AP. Mean (s.e.mean shown by vertical bars) of at least three experiments for each point. For further details see legend to Figure 1.

As shown in Figure 3 the basal [^{3}H]-NA release was increased by 4-AP itself in a concentration-dependent manner. There was a small delay between the time that 4-AP reached the slice and the time when the [^{3}H]-NA release was increased. This could partly represent the time it takes for the drug to penetrate into the slice. It can also be noted that the highest concentration of 4-AP caused a release that could not be maintained. This could represent an exhaustion of the releasable pool similar to the one indicated by the experiments shown in Figures 1 and 2. Under the same conditions, 3,4-diaminopyridine (3,4-DAP) could also induce [^{3}H]-NA

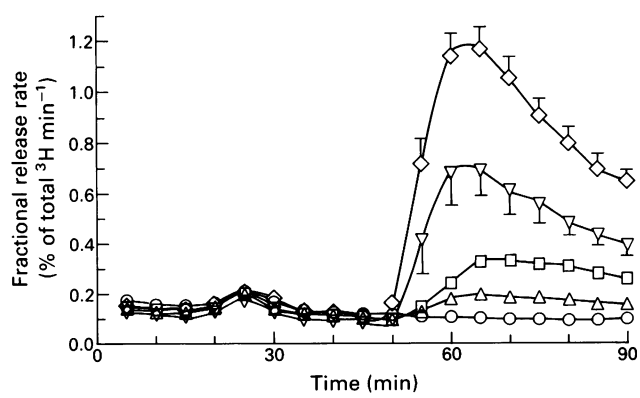

Figure 3 Dose-dependence of [^{3}H]-noradrenaline ([^{3}H]-NA) release induced by 4-aminopyridine (4-AP) in slices from rat hippocampus. The buffer contained 0.25 mM Ca^{2+}. First one stimulation with 10 V, 3 Hz and 2 ms duration was given during the 4th fraction. 4-AP was added at about 43 min (i.e. the 9th fraction) without field stimulation. (○) Control; (△) 10 μM 4-AP; (□) 30 μM 4-AP; (▽) 100 μM 4-AP and (◇) 300 μM 4-AP. Mean (s.e.mean shown by vertical bars) of 4 experiments at each dose.

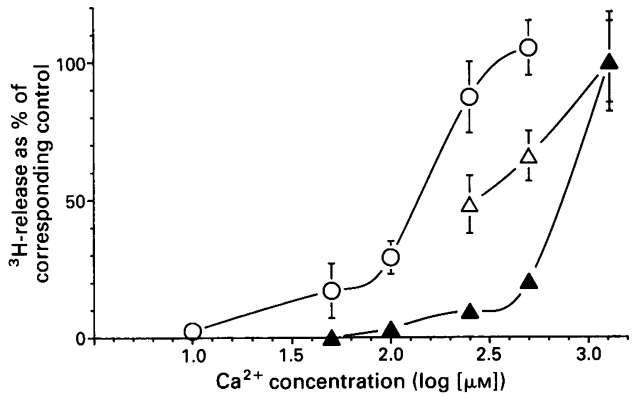

Figure 4 Ca^{2+} dependence of the [^{3}H]-noradrenaline ([^{3}H]-NA) release induced by 4-aminopyridine (4-AP, 100 μM) in slices from rat hippocampus. The experimental protocol is essentially the same as that in Figure 3. After an initial (S_1) field stimulation period, 100 μM 4-AP was added from fraction 9 onwards. Experiments with low Ca^{2+} concentration (under 0.25 mM) were carried out in buffers containing EGTA. The release of radioactivity during the five peak fractions in the second peak are summarized and expressed as a percentage of corresponding maximum release (i.e. at 1.3 mM Ca^{2+}). The results are expressed as means of at least 4 experiments at each point with s.e.mean shown by vertical bars: (○) 4-AP-induced release. In this series of experiments the EC_{50} for Ca was 0.15 ± 0.01 mM. In order to calculate the release of radioactivity induced by field stimulation in the presence of 4-AP (△) the release caused by 4-AP alone was subtracted. For this reason there were rather large errors in the determinations with low amounts of Ca^{2+} and a full concentration curve could not be determined. The release of radioactivity by field stimulation (▲) was calculated as described in Methods. The EC_{50} for Ca was 0.76 ± 0.07 mM. Results are given as mean of at least 4 determinations at each point; vertical bars show s.e.mean.

release. 3,4-DAP (30 μM) released about 8.8% of the total radioactivity in slices during 25 min. This is similar to the 9.7% released by 100 μM 4-AP. 3,4-DAP is also approximately three times more potent than 4-AP in enhancing the [^{3}H]-NA release induced by field stimulation (results not shown).

The Ca^{2+}-dependence of the field stimulation evoked release was compared to that of the release induced by 4-AP. Lowering the Ca^{2+} concentration from 1.3 mM to 0.25 mM was virtually without effect on the 4-AP-induced increase in basal [^{3}H]-NA release. By contrast, the field stimulation evoked release was reduced to less than one fourth. In order to examine the Ca^{2+}-dependence at lower levels of Ca^{2+}, EGTA was used to buffer the free Ca^{2+} concentration at 0.25, 0.1, 0.05, and 0.01 mM as described by Bartfai (1979). The results of these experiments which are shown in Figure 4 show that the 4-AP induced [^{3}H]-NA release exhibits a lower Ca^{2+}-dependence. Finally, we wanted to examine whether the evoked release of [^{3}H]-NA during a perfusion of 4-AP would exhibit a Ca^{2+}-dependence similar to that in 4-AP-induced release, and in field stimulation induced release in the absence of 4-AP. The results exhibit an intermediate behaviour.

In order to check whether cholinergic neurones are equally affected by 4-AP, we also checked the release of [^{3}H]-ACh. The basal efflux of [^{3}H]-ACh was 0.091 ± 0.005% of the tissue content per min (n = 29) under control conditions. In the presence of 100 μM 4-AP it was 0.100 ± 0.004 (n = 23) and in the presence of 300 μM 4-AP it was 0.078 ± 0.007 (n = 6). Thus, the lack of effect of 4-AP on acetylcholine release from

Table 1 Effect of drugs acting on the A_1-receptor, α_2-adrenoceptor and Ca^{2+} channels on the 4-aminopyridine (4-AP, 100 μM)-induced release in 0.25 mM Ca^{2+}

Drugs	*% of control*	P *values*
R-PIA (1 μM)	80 ± 5 (8)	**
UK (0.1 μM)	49 ± 6 (5)	***
Yoh (1 μM)	120 ± 18 (4)	*
Yoh + R-PIA[a]	69 ± 4 (4)	*
$CdCl_2$ (10 μM)	35 ± 4 (4)	***
ω-CgTX (30 nM)	61 ± 8 (4)	***
Nif (1 μM)	88 ± 10 (4)	NS

The release is expressed as fractional release calculated by averaging the release of radioactivity in five fractions after the addition of 4-AP, subtracting the basal release and dividing by the amount of radioactivity at the start of the stimulation. The effect of the drug is expressed as a percentage of the release in the absence of drugs (10.8 ± 1.6% of total tissue content, n = 17). Mean and s.e.mean. The number of the experiments is within the parentheses.

R-PIA = R-N_6-phenylisopropyl adenosine, ω-CgTx = ω-conotoxin, Nif = nifedipine, UK = UK14,304, Yoh = yohimbine, NS = not significant, * $P < 0.05$, ** $P < 0.01$, ** $P < 0.001$. [a]The effect of R-PIA in the presence of yohimbine is compared to the effect of yohimbine alone.

Table 2 Effect of drugs acting on A_1-receptor, α_2-adrenoceptor and Ca^{2+} channels on the 4-aminopyridine (4-AP, (100 μM)-induced [^{3}H]-noradrenaline ([^{3}H]-NA) release in 1.3 mM Ca^{2+}

Drugs	*% of control*	P *values*
R-PIA (1 μM)	70 ± 19 (8)	**
UK (1 μM)	53 ± 10 (6)	***
Yoh (1 μM)	134 ± 20 (4)	*
Yoh + R-PIA[a]	77 ± 10 (4)	**
$CdCl_2$ (10 μM)	47 ± 18 (4)	**
ω-CgTX (30 nM)	53 ± 6 (4)	**

The release is expressed as fractional release calculated by averaging the release of radioactivity in five fractions after the addition of 4-AP, subtracting the basal release and dividing by the amount of radioactivity at the start of the stimulation. The effect of the drugs is expressed as a percentage of the release in the absence of drugs (9.7 ± 2% of total tissue content, n = 12). Mean and s.e.mean and number of the experiments is within the parentheses.

The abbreviations of the drugs are listed in Table 1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. [a]The effect of R-PIA in the presence of yohimbine is compared to the effect of yohimbine alone.

Table 3 Effect of drugs acting on A_1-receptor, α_2-adrenoceptor and Ca^{2+} channels on [^{3}H]-noradrenaline ([^{3}H]-NA) release evoked by field stimulation in presence of 100 μM 4-aminopyridine (4-AP) in a buffer containing 1.3 mM Ca^{2+}

Drugs	*% of control*	P *values*
R-PIA (1 μM)	104 ± 6 (5)	NS
UK (1 μM)	78 ± 8 (4)	*
Yoh (1 μM)	136 ± 9 (6)	**
Yoh + R-PIA[a]	108 ± 5 (5)	*
$CdCl_2$ (10 μM)	73 ± 4 (5)	**
ω-CgTX(30 nM)	58 ± 4 (4)	***
Nif (1 μM)	93 ± 13 (5)	NS

The release is determined as fractional release calculated by averaging the release of radioactivity in five fractions after the addition of 4-AP, subtracting the basal release and dividing by the amount of radioactivity at the start of the stimulation. The effect of the drugs is expressed as % of the release in the absence of drugs (39 ± 1% of total tissue content, n = 30). Mean and s.e.mean and the number of the experiments is within the parentheses.

The abbreviations of the drugs are listed in Table 1. NS = not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. [a]The effect of R-PIA in the presence of yohimbine is compared to the effect of yohimbine alone.

hippocampus reported earlier (Fredholm, 1990a) was confirmed.

To check whether an interaction with the NA uptake system in nerve endings could account for the ability of 4-AP to release NA, we also carried out experiments in the absence of desipramine (DMI). Field stimulation evoked a [^{3}H]-NA release which was 9.1% of the total tissue content in the absence of DMI and 14.5% in its presence (1 μg ml^{-1}). Release of [^{3}H]-NA induced by 100 μM 4-AP was 6.2% in control and 8.5% in the presence of DMI. Thus, DMI increased electrically evoked and 4-AP-induced release in a similar manner. Hence, 4-AP-induced release does not require uptake inhibition.

The 4-AP-induced release was essentially abolished by TTX at 3 μM concentration, indicating that it is not simply due to leakage or to a tyramine-like action of 4-AP. TTX (3 μM) caused a small but significant ($P < 0.05$) decrease of basal [^{3}H]-NA release (about 12%). The basal release was 0.54 ± 0.02 in control conditions (10 experiments) and 0.47 ± 0.02 in the presence of TTX (8 experiments).

As it was shown in Table 1, in the presence of 0.25 mM Ca^{2+}, 10 μM $CdCl_2$ and 30 nM ω-conotoxin (blockers of both N- and L-type Ca^{2+}-channels) strongly reduced the 4-AP-induced release. Nifedipine, a dihydropyridine antagonist of L-type channels, was without effect. The release of [^{3}H]-NA induced by 4-AP in the absence of nerve impulses was inhibited to about 50% by 0.1 μM UK14,304, an α_2-adrenoceptor agonist, and it was enhanced about 20% by 1 μM yohimbine, an α_2-adrenoceptor antagonist. **R**-PIA, 1 μM, an adenosine receptor agonist, itself had a direct (21 ± 10%) inhibitory effect on the 4-AP induced release. When the α_2-receptor was blocked by 1 μM yohimbine the inhibitory effect of **R**-PIA was enhanced to 41 ± 4%. The same results were achieved in the presence of 1.3 mM Ca^{2+} in the buffer in the 4-AP-induced release (Table 2). Table 3 lists the effects of all the drugs in the presence of 100 μM 4-AP under field stimulation.

In particular we have confirmed that 4-AP leads to a reduction in the magnitudes of inhibition of transmitter release afforded by α_2-adrenoceptor stimulation (e.g. Schoffelmeer & Mulder, 1983; Hu & Fredholm, 1989). Despite this, we found that UK 14,304 could inhibit significantly the release of transmitter evoked by 4-AP alone. By contrast, inhibition of α_2-adrenoceptors with yohimbine was not very effective in increasing 4-AP-induced transmitter release (20% increase at 0.25 mM Ca^{2+}; 34% increase at 1.3 mM Ca^{2+}). The magnitude of this effect was similar to that seen for electrically evoked transmitter release in the presence of 4-AP (36%, Table 3), but markedly less than that seen with electrically evoked transmitter release in the absence of 4-AP (more than 150% increase; Hu & Fredholm, 1989).

Discussion

We have confirmed and extended the previous finding that 4-AP is able to increase the release of [^{3}H]-NA from brain slices (Schoffelmeer & Mulder, 1983; Hu & Fredholm, 1989; Huang *et al.*, 1989). Since 4-AP also may increase the spontaneous [^{3}H]-NA release from vascular preparations (Leander *et al.*, 1977) and the release of ACh from striatal slices (Dolezal & Tucek, 1983) the phenomenon is apparently not unique for the preparation we have used. Conversely, there are several instances where 4-AP does not increase spontaneous transmitter release (Thesleff, 1980). Indeed, in the present study we found that the basal release of ACh was not enhanced by 4-AP under exactly the same type of conditions as those used for [^{3}H]-NA release.

As reviewed by Rudy (1988) the delayed rectifier I_K and fast transient K^+ current (I_A) can be blocked by 4-AP. By contrast the Ca-activated K^+ current apparently is not blocked by 4-AP (Rudy, 1988) and may even be stimulated (Rogawski, 1989). It was also found that a slowly inactivating K^+-current (I_D) was blocked by 4-AP as well as by dendrotoxin (Stansfeld *et al.*, 1986). 3,4-Diaminopyridine is more potent as an inhibitor of K^+-channels (Thesleff, 1980) and it was also more potent in inducing [^{3}H]-NA release. This agrees with the results by Huang *et al.* (1989) in rabbit hippocampal slices. Since we have also found that TEA is able to increase the release of the transmitter (unpublished observations) it seems likely that the effect is related to inhibition of a K^+-channel. The type of channel involved is not known, but recently Storm (1988) has shown that the I_D current can be blocked by low concentrations of 4-AP (50 μM) whereas blockade of the I_A current requires high concentrations (5 mM).

The release of [^{3}H]-NA was essentially completely blocked by TTX. This indicates that the release depends on propagated nerve impulses. Since the noradrenergic cell bodies are not present in the slice preparation, these nerve impulses may be set up in the cut distal end of the noradrenergic neurones. TTX slightly affects the resting release of NA. Thus it seems that the nerve impulses must have been induced by 4-AP and not just enhanced. This conclusion agrees with that drawn by others (Huang *et al.*, 1989).

Even though both electrically evoked [^{3}H]-NA release and the release by induced 4-AP thus appear to depend on the initiation of electrical impulses and consequent release of transmitter, the two processes differ markedly in their Ca^{2+}-dependence. The transmitter release induced by 4-AP required far lower concentrations of Ca^{2+}. If it is assumed that the actual release process is essentially similar and hence that a similar concentration of intracellular Ca^{2+} is required, then there must be a marked difference in the operation of voltage-dependent Ca^{2+}-channels in the two situations.

One possible explanation could be that different types of voltage-dependent channels are involved in the two situations. However, the L-type selective channel inhibitor nifedipine was inactive in both situations, whereas ω-conotoxin, which predominantly acts on N-type channels, was effective. Instead, it is possible that the same type of channel is activated for longer periods of time, thereby providing a longer period for calcium ion entry into the nerve-endings. One reason for such a longer time of entry could be that 4-AP had blocked K^+-channels, terminating the action potential. However, this does not appear to be the entire explanation since the Ca^{2+}-dependence of transmitter release induced by nerve impulses superimposed upon a period of 4-AP infusion was intermediary between 4-AP and electrical stimulation rather than identical with the former. Instead it seems possible that for some reason the actual nerve impulses set up by 4-AP are different even though they do depend upon TTX.

During the preparation of this manuscript a paper by Heemskerk and co-workers (1990) was published. They found that 4-AP could increase [^{3}H]-noradrenaline release in parallel with the increase in phosphorylation of an identified protein kinase C substrate, GAP 43. The same group has also demonstrated that phosphorylation of GAP 43 is correlated to neurotransmitter release (Dekker *et al.*, 1989). The possibility then exists that 4-AP could lead to a general increase of protein kinase C activity. However, stimulation of protein kinase C leads not only to increased noradrenaline release in the rat hippocampus (Fredholm & Lindgren, 1988), but also to release of acetylcholine (Fredholm, 1990b). Since we could not demonstrate any change in acetylcholine release by 4-AP, such a generalized stimulatory effect seems unlikely.

Therefore, our tentative explanation for the effect of 4-AP on noradrenaline release is that the effect is indirect. For example, 4-AP-induced spontaneous bursting activity in the rat hippocampus *in vitro* (Buckle & Haas, 1982) could release glutamate that might be involved in the release of noradrenaline (Vezzani *et al.*, 1987; Schmidt & Taylor, 1988). However, direct evidence for such an interaction is lacking and, Huang *et al.* (1989) in fact provided evidence against it.

The release of transmitter induced by 4-AP could be blocked not only by Ca^{2+}-channel blocking drugs but also by different presynaptic agonists. We have previously shown that the presynaptic inhibitory effect of an adenosine analogue is

not antagonized by 4-AP (Hu & Fredholm, 1989). Therefore it is not surprising that the A_1-adenosine receptor agonist R-PIA was at least as effective in antagonizing 4-AP induced as electrically evoked [^{3}H]-NA release.

We previously found that 4-AP was able to antagonize the presynaptic control of [^{3}H]-NA release exerted via α_2-adrenoceptors (Hu & Fredholm, 1989). Therefore it was surprising that release of transmitter evoked by 4-AP could in fact be inhibited better by α_2-agonists than could electrically evoked release. This becomes even more surprising in view of the finding that 4-AP is able to block α_2-adrenoceptors (Hu & Fredholm, 1989; Drukarch *et al.*, 1989).

The present results include two important findings: (a) 4-AP can lead to a release of noradrenaline, but not acetylcholine, by a process that depends on nerve impulses and is extremely calcium-dependent; (b) stimulation of α_2-adrenoceptors and adenosine A_1-receptors may inhibit transmitter release via a mechanism that is completely independent of 4-AP sensitive K^+-channels.

References

BARTFAI, T. (1979). Preparation of metal-chelate complexes and the design of steady-state kinetic experiments involving metal nucleotide complexes. In *Advances in Cyclic Nucleotide Research*, Vol. 10. ed. Brooker, G., Greengard, P. & Robinson, G.A. pp. 219–241. New York: Raven Press.

BUCKLE, P.J. & HAAS, H.L. (1982). Enhancement of synaptic transmission by 4-aminopyridine in hippocampal slices of the rat. *J. Physiol.*, **326**, 109–122.

DEKKER, L.V., DE GRAAN, P.N.E., VERSTEEG, D.H.G., OESTREICHER, A.B. & GISPEN, W.H. (1989). Phosphorylation of B-50 (GAP43) is correlated with neurotransmitter release in rat hippocampal slices. *J. Neurochem.*, **52**, 24–30.

DOLEZAL, V. & TUCEK, S. (1983). The effects of 4-aminopyridine and tetrodotoxin on the release of acetylcholine from rat striatal slices. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **323**, 90–95.

DRUKARCH, B., KITS, K.S., LEYSEN, J.E., SCHEPENS, E. & STOOF, J.C. (1989). Restricted usefulness of tetraethylammonium and 4-aminopyridine for the characterization of receptor-operated K^+-channels. *Br. J. Pharmacol.*, **98**, 113–118.

DUNWIDDIE, T.V. (1985). The physiological role of adenosine in the central nervous system. *Int. Rev. Neurobiol.*, **27**, 63–139.

FREDHOLM, B.B. (1990a). Differential sensitivity to blockade by 4-aminopyridine of presynaptic receptors regulating [^{3}H]-acetylcholine release from rat hippocampus. *J. Neurochem.*, **54**, 1386–1390.

FREDHOLM, B.B. (1990b). The inhibitory effect of adenosine A_1-receptor stimulation on acetylcholine release in the rat hippocampus does not depend on protein kinase C. *Acta Physiol. Scand.*, (in press).

FREDHOLM, B.B. & DUNWIDDIE, T.V. (1988). How does adenosine inhibit transmitter release? *Trends Pharmacol. Sci.*, **9**, 130–134.

FREDHOLM, B.B. & LINDGREN, E. (1988). Protein kinase C activation increases noradrenaline release from the rat hippocampus and modifies the inhibitory effect of α_2-adrenoceptor and adenosine A_1-receptor agonists. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **337**, 477–483.

HEEMSKERK, F.M.J., SCHRAMA, L.H., GIANOTTI, C., SPIERENBURG, H., VERSTEEG, D.H.G., DE GRAAN, P.N.E. & GISPEN, W.H. (1990). 4-Aminopyridine stimulates B-50 (GAP43) phosphorylation and [^{3}H]-noradrenaline release in rat hippocampal slices. *J. Neurochem.*, **54**, 863–869.

HU, P.-S. & FREDHOLM, B.B. (1989). α_2-adrenoceptor agonist-mediated inhibition of [^{3}H]-noradrenaline release from rat hippocampus is reduced by 4-aminopyridine, but that caused by an adenosine analogue or ω-conotoxin is not. *Acta Physiol. Scand.*, **136**, 347–353.

HUANG, H.Y., ALLGAIER, C., HERTTING, G. & JACKISCH, R. (1988). Phorbol ester-mediated enhancement of hippocampal noradrenaline release: which ion channels are involved? *Eur. J. Pharmacol.*, **153**, 175–184.

HUANG, H.Y., HERTTING, G., ALLGAIEER, G. & JACKISH, R. (1989). 3,4-diaminopyridine-induced noradrenaline release from CNS tissue as a model for action potential-evoked transmitter release: effects of phorbol ester. *Eur. J. Pharmacol.*, **169**, 115–123.

JACKISH, R., STRITTMATTER, H., KASAKOV, L. & HERTTING, G. (1984). Endogenous adenosine as a modulator of hippocampal acetylcholine release. *Naunyn-Schmeiedebergs Arch. Pharmacol.*, **327**, 319–325.

JACKISH, R., FEHR, R. & HERTTING, G. (1985). Adenosine: An endogenous modulator of hippocampal noradrenaline release. *Neuropharmacol.*, **24**, 499–507.

JONZON, B. & FREDHOLM, B.B. (1985). Release of purines, noradrenaline, and GABA from rat hippocampal slices by field stimulation. *J. Neurochem.*, **44**, 217–224.

LEANDER, S., ARNER, A. & JOHANSSON, B. (1977). Effects of 4-aminopyridine on mechanical activity and noradrenaline release in the rat portal vein in vitro. *Eur. J. Pharmacol.*, **46**, 351–361.

MADISON, D.V., FOX, A.P. & TSIEN, R.W. (1987). Adenosine reduces an inactivating component of calcium current in hippocampal CA3 neurons. *Biophys. J.*, **51**, 30a.

ROGAWSKI, M.A. (1989). Aminopyridines enhance the opening of calcium-activated potassium channels in GH_3 anterior pituitary cells. *Mol. Pharmacol.*, **35**, 458–468.

RUDY, B. (1988). Diversity and ubiquity of K channels. *Neuroscience*, **25**, 729–747.

SCHMIDT, C.J. & TAYLOR, V.L. (1988). Release of [^{3}H]-norepinephrine from rat hippocampal slices by N-methyl-D-aspartate: comparison of the inhibitory effects of Mg^{2+} and MK-801. *Eur. J. Pharmacol.*, **156**, 111–120.

SCHOFFELMEER, A.N.M. & MULDER, A.H. (1983). Differential control of Ca^{2+} dependent [^{3}H]-noradrenaline from rat brain slices through presynaptic opiate receptors and α-adrenoceptors. *Eur. J. Pharmacol.*, **87**, 449–458.

STANSFELD, C.E., MARSH, S.J., HALLIWELL, J.V. & BROWN, D.A. (1986). 4-aminopyridine and dendrotoxin induce repetitive firing in rat visceral sensory neurons by blocking slowly inactivating outward current. *Neurosci. Lett.*, **64**, 299–304.

STARKE, K. (1987). Presynaptic α-autoreceptors. *Rev. Physiol. Biochem. Pharmacol.*, **107**, 73–146.

STORM, J.F. (1988). Temporal integration by a slowly inactivating K^+ current in hippocampal neurons. *Nature*, **336**, 379–381.

THESLEFF, S. (1980). Aminopyridines and synaptic transmission. *Neuroscience*, **5**, 1413–1419.

TRUSSELL, L.O. & JACKSON, M.B. (1985). Adenosine-activated potassium conductance in culture striatal neurons. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 4857–4861.

TRUSSELL, L.O. & JACKSON, M.B. (1987). Dependence of an adenosine-activated potassium current on a GTP-binding protein in mammalian central neurons. *J. Neurosci.*, **7**, 3306–3316.

VEZZANI, A., WU, H.-Q. & SAMANIN, R. (1987). [^{3}H]-norepinephrine release from hippocampal slices is an in vitro biochemical tool for investigating the pharmacological properties of excitatory amino acid receptors. *J. Neurochem.*, **49**, 1438–1442.

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Hoe 140 a new potent and long acting bradykinin-antagonist: *in vitro* studies

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1 Hoe 140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]bradykinin) is a new bradykinin (BK)-antagonist. It was tested in several *in vitro* assays and compared with D-Arg-[Hyp^2, Thi^{5,8},D-Phe^7]BK.

2 In receptor binding studies in guinea-pig ileum preparations, Hoe 140 showed an IC_{50} of 1.07×10^{-9} mol l^{-1} and a K_I value of 7.98×10^{-10} mol l^{-1}.

3 In isolated organ preparations Hoe 140 and D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK inhibited bradykinin-induced contractions concentration dependently, with IC_{50}-values in the guinea-pig ileum preparation of 1.1×10^{-8} mol l^{-1} and 3×10^{-5} mol l^{-1}, respectively. pA_2 values in this tissue were 8.42 and 6.18, respectively. In the rat uterus preparation the IC_{50} value was 4.9×10^{-9} mol l^{-1} for Hoe 140. D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK showed an IC_{50} of 4.0×10^{-6} mol l^{-1}. The IC_{50} values in the guinea-pig isolated pulmonary artery were 5.4×10^{-9} mol l^{-1} and 6.4×10^{-6} mol l^{-1}, respectively. In the rabbit aorta no inhibitory effects on Des-Arg^9-BK induced contractions were observed.

4 In cultured bovine endothelial cells, Hoe 140 antagonized ($IC_{50} = 10^{-8}$ mol l^{-1}) bradykinin-induced endothelium-derived relaxing factor (EDRF) release and the bradykinin-induced increase in cytosolic free calcium ($IC_{50} = 10^{-9}$ mol l^{-1}).

5 Hoe 140 (10^{-7} mol l^{-1}) totally suppressed the bradykinin-induced (10^{-8} to 10^{-4} mol l^{-1}) prostacyclin (PGI_2) release from cultured endothelial cells of bovine aorta. D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK (10^{-7} mol l^{-1}) showed a weaker antagonism.

6 Taken together these results show that Hoe 140 is a highly potent bradykinin antagonist. It was two to three orders of magnitude more potent than D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK.

Introduction

Bradykinin (BK) is a nonapeptide with a wide range of actions. Although the effects of exogenously applied BK to isolated organs or to animals have been extensively investigated (for review see Erdös, 1979 and Taylor *et al.*, 1989) its precise physiological role has not yet been identified. The actions of BK are mediated through at least two different types of receptor designated as BK_1 and BK_2 (Regoli & Barabé, 1980; Regoli *et al.*, 1990). Most biological actions seem to be mediated by BK_2-receptors. BK elicits contraction or relaxation of vascular smooth muscle depending on species and site. BK also produces contraction of smooth muscles of the respiratory and gastrointestinal tract and the uterus. In several species exogenously applied kinins have been shown to produce the cardinal symptoms of inflammation (Wilhelm, 1973). BK causes vasodilatation and increases vascular permeability, which lead to oedema. It releases prostaglandins and leukotrienes through activation of phospholipase A_2 (Griesbacher & Lembeck, 1987). By stimulating nociceptive afferent nerves, BK mediates pain and hyperalgesia (Juan & Lembeck, 1974; Steranka *et al.*, 1987).

Since the sequence of BK was determined in 1960, quite a number of analogues have been synthesized in an attempt to find effective antagonists. A breakthrough was achieved by Vavrek and Stewart who described the first sequence related competitive BK-antagonist (Vavrek & Stewart, 1985a,b). Since then many BK-antagonists have been synthesized in an effort to improve potency. These compounds have been tested *in vitro* and *in vivo*. Their antinociceptive (Steranka *et al.*, 1988), anti-inflammatory and antiexudative potential (Costello & Hargreaves, 1989) has been demonstrated by several investigators. These actions of BK are mainly mediated by the BK_2 receptor type (Regoli, 1984). However, the potency of these antagonists in smooth muscle preparations in terms of pA_2 values did not exceed a value of 7 (Steranka *et al.*, 1989).

Unlike the above mentioned class of antagonists, Hoe 140 (Figure 1) (Henke *et al.*, 1990) contains several unnatural amino acids which were also used during the development of potent ACE-inhibitors. Hoe 140 exceeds the potency of the hitherto known antagonists by two or three orders of magnitude and represents a new class of BK-antagonists. The following paper describes part of the *in vitro* pharmacology of Hoe 140.

Methods

Receptor binding

The specific binding of [^{3}H]-BK was analysed by the method of Manning *et al.* (1986) with minor modifications. In brief, crude membranes from guinea-pig ileum were incubated for 1 h at 25°C in a buffer solution with [^{3}H]-BK (1 nmol l^{-1}) and various concentrations of the test compounds (11 concentrations; range: 1×10^{-5} to 1×10^{-10} mol l^{-1}). Non specific binding was determined in the presence of 1 μmol l^{-1} unlabelled BK.

Incubations were done in a volume of 200 μl (50 μl [^{3}H]-BK, 50 μl test compound or unlabelled BK, 100 μl membrane suspension) on 96-well microtiter plates. At the end of the incubation period, samples were filtered under reduced pressure through a Whatman GF/B glass fibre filter mat using a Skatron (12 fold) cell harvester. Following 3 washes (36 ml each per 12 samples) with ice-cold 25 mmol l^{-1} TES buffer, the filters were punched out into miniscintillation vials, extracted overnight with 3 ml scintillation cocktail and measured in an LKB liquid scintillation spectrometer.

Specific, saturable binding was calculated by subtracting the nonspecific binding (determined in the presence of 1 μmol l^{-1} unlabelled BK) from total binding. IC_{50} and K_I values were calculated on an IBM PC using the programme EBDA (McPherson, 1986).

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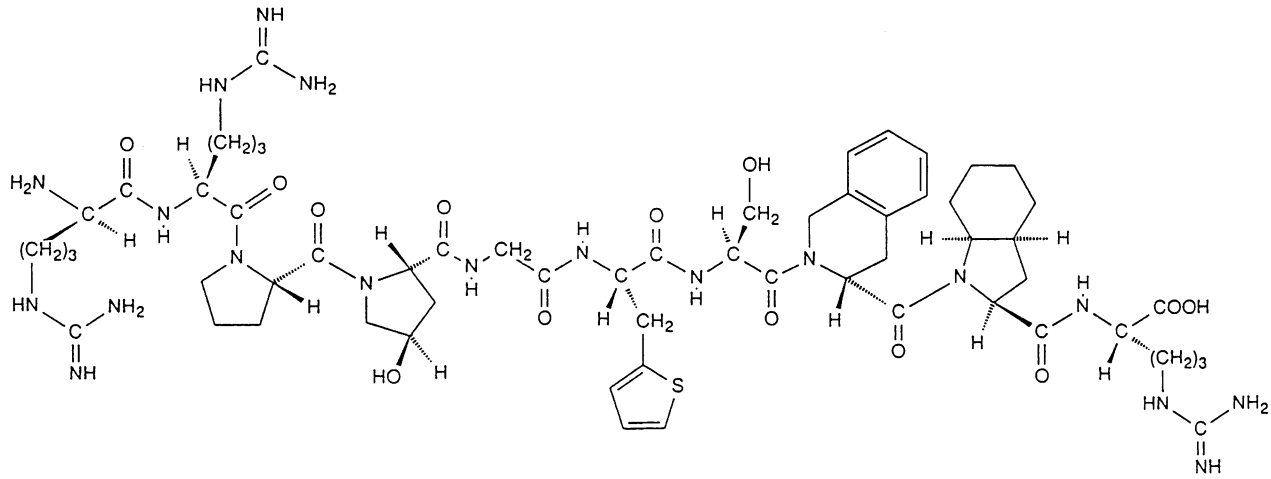

Figure 1 Hoe 140 structural formula and nomenclature: D-arginyl-L-arginyl-L-prolyl-L-[(4R)-4-hydroxyprolyl]-glycyl-L-[3-(2-thienyl)alanyl]-L-seryl-D-(1,2,3,4-tetrahydroisoquinolin-3-yl-carbonyl)-L-[(3a*S*,7a*S*)-octahydroindol-2-yl-carbonyl]-L-arginine; Molecular weight (base): 1304.59.

Smooth muscle preparations contracted by bradykinin

Guinea pig ileum Guinea pigs of either sex (250–300 g, Marioth strain, Hoechst breeding farm) were used. Segments of 1.5 cm length (unstretched) were suspended in 10 ml organ baths containing Tyrode solution (37°C). Contractions of the segments were recorded isometrically with a load of 1 g. After an equilibration period of about 30 min a stable baseline tone was reached and two or three contractions were obtained to BK (4×10^{-8} mol l^{-1}). After the contraction the isolated tissue was washed for 5 min. Following a rest of 10 min the segments had relaxed to baseline levels. Only segments producing reproducible responses were used. The last control response was taken as 100% and subsequent results obtained with BK-antagonists expressed as a percentage of this. The segments were incubated with the BK-antagonists for 10 min before BK was added.

In a second experiment the segments were contracted with BK in cumulative concentrations of 10^{-9} mol l^{-1} up to 10^{-6} mol l^{-1} to calculate the pA_2-values. Maximal responses were obtained to cumulative concentrations of BK in the presence of different concentrations (3×10^{-9}–10^{-6} mol l^{-1}) of Hoe 140. The antagonist activity expressed as the pA_2-values was calculated by the method of van Rossum *et al.* (1963).

Rat uterus Female Wistar rats (Moellegard, Skensved, DK) weighing 200–236 g were used. Segments (10–20 mm in length) were mounted in an organ bath with Tyrode solution (37°C). A tension of 1 g was applied. Contractions were recorded isotonically. BK (10^{-8} mol l^{-1}) induced submaximal contractions, which were repeated 10 min after rinsing the organ bath. The BK-antagonists were added 5 min prior to BK.

Guinea pig pulmonary artery The pulmonary artery was quickly removed from stunned guinea-pigs (450–500 g, Hoechst breeding farm) and immersed in Ringer solution at room temperature. Spirally cut strips (45°, 25 × 4 mm) were suspended with a preload of 0.5 g in a 10 ml bath containing Ringer solution (37°C, pH 7.4). Isotonic recordings were made with a lever displacement measuring system (Hugo Sachs Electronic KG, Freiburg, F.R.G.), which was connected to a chart recorder (SE 460 Goerz Metrawatt, BBC, Mannheim, F.R.G.).

After an equilibration period of 1 h, strips were contracted by BK at 2×10^{-7} mol l^{-1}. After a washout, the pulmonary arteries were incubated with Hoe 140 (10^{-9} to 5×10^{-8} mol l^{-1}) or D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK (10^{-6} to 5×10^{-5} mol l^{-1}) before BK was added.

Rabbit aorta The descending thoracic aorta, which contains the BK_1-receptor type (Regoli & Barabé, 1980), was excised from rabbits of either sex (Hoechst, breeding farm, 2.5–3.5 kg) and dissected free of connective tissue (Linz *et al.*, 1986). The aorta was divided into 2 mm wide rings and cut off in small strips. The strips were suspended in a 25 ml organ bath containing Krebs-bicarbonate solution at 37°C and pH 7.4. Contractions of the strips were recorded isometrically with a load of 2 g on the tissues. After an equilibrium period of about 5 h, Hoe 140 was tested at a concentration of 10^{-7} mol l^{-1} for its ability to inhibit BK_1 receptor-mediated contractions elicited by the BK_1 receptor agonist Des-Arg^9-BK (3×10^{-7} mol l^{-1}; Bachem, Heidelberg, F.R.G.). Control contractions with Des-Arg^9-BK alone performed 30 min before and 30 min following the contractions in the presence of Hoe 140 were similar in size and served as reference values.

Bradykinin-induced EDRF release and bradykinin-induced Ca^{2+} response in cultured endothelial cells from pig aorta

Simultaneous detection of EDRF by bioassay and guanylate cyclase activation Confluent endothelial cells from the aorta of the pig grown in 24-well plates were washed with 3 × 200 µl of HEPES-buffer (28.5 mmol l^{-1}; pH 7.35) containing per 100 ml 1.2 g DMEM/F12-medium (Gibco) and 5000 units penicillin and 500 µg streptocmycin (Biochrom). Then the cells were incubated with 200 µl HEPES-Tyrode-buffer (pH 7.35) containing 10^{-7} mol l^{-1} superoxide dismutase (SOD) and the respective drugs. Each well ($n = 4$) was incubated at 37°C for 1 min (control) and then 50 µl of the supernatant was quickly transferred to the guanylate cyclase assay (see below). The remaining supernatant was aspirated and the same well was incubated for 3.5 min with 200 µl HEPES-Tyrode-buffer containing BK 3×10^{-8} mol l^{-1}. The same procedure with the same well was repeated with BK 3×10^{-8} mol l^{-1} for 4.5 min. The respective supernatants were quickly transferred to the guanylate cyclase assay. When testing Hoe 140 the compound was preincubated for 5 min before the addition of BK.

Guanylate cyclase assay Soluble guanylate cyclase (GC) was isolated from bovine lung. GC-activity was determined in a total volume of 70 µl by measuring the formation of [^{32}P]-guanosine 3′:5′-cyclic monophosphate ([^{32}P]-cyclic GMP) from [α-^{32}P]-GTP by a modification of the method of Mülsch *et al.* (1987). The reaction was started by addition of 50 µl of quickly transferred supernatants (see above). The tubes were incubated for 1 min at 37°C under normal atomosphere, and

isolation of [^{32}P]-cyclic GMP and calculation of GC-activity were performed as described by Schultz & Böhme (1984).

Measurements of the intracellular free Ca-concentration Cultured endothelial cells from the aorta of the pig were seeded on quartz coverslips (35 × 14 × 0.8 mm^3) and grown to confluence. The cells were loaded with the fluorescent probe indo-1 by incubation (90 min, 38°C) with 2 μmol l^{-1} indo-1/AM and 0.025% (w/v) Pluronic F-127, a non-ionic detergent (Tsien *et al.*, 1982; Grynkiewicz *et al.*, 1985). Thereafter, the coverslips were washed and transferred into cuvettes, filled with HEPES buffer. Fluorescence was recorded in a temperature-controlled (37°C) spectrofluorometer (Schoeffel RRS 1000). The excitation wavelength was set to 350 nm; emission was simultaneously measured at 400 nm and 450 nm. The intracellular free calcium concentration ($[Ca^{2+}]_i$) was calculated on a second-to-second basis from the ratio of both fluorescence intensities (R) as $[Ca^{2+}]_i = 250\,\text{nmol l}^{-1}$. $(R - 1/9\ R_{max})/(R_{max} - R)$. R_{max} is the ratio of both fluorescence intensities when indo-1 is saturated with Ca^{2+} and was obtained by recording the fluorescence from a calibration solution. R is the ratio of both fluorescence intensities each of which was corrected for the background signal. The background was determined by adding the calcium ionophore ionomycin (1 μmol l^{-1}) and Mn^{2+} (2 mmol l^{-1}), a quencher of indo-1, to the cells at the end of each experiment. Peak $[Ca^{2+}]_i$ values after stimulation were obtained by averaging the $[Ca^{2+}]_i$ values of the 5 s period with the highest $[Ca^{2+}]_i$ value.

Bradykinin-induced prostacyclin release in cultured endothelial cells from bovine aorta

Measurement of PGI_2 release was performed in cultured primary endothelial cells grown in 6-well plates (Nunc, Wiesbaden, F.R.G.). Each well (ca. 200 μg protein) was washed with 3 × 1 ml of Krebs-Ringer-buffer (KRB). Then the cells were preincubated for 15 min at 37°C with 1 ml KRB without EDTA containing the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), 10^{-4} mol l^{-1}, prior to the addition of BK and SOD 3 × 10^{-7} mol l^{-1}. After the incubation of BK (10^{-8} to 10^{-4} mol l^{-1}) and SOD for 1 min an aliquot was removed and assayed for PGI_2 by specific radioimmunoassay (Amersham Buchler, Braunschweig, F.R.G.) for 6-keto $PGF_{1\alpha}$, the stable hydrolysis product of PGI_2. When testing Hoe 140 and D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK the compounds were preincubated for 5 min before addition of BK.

Drugs

Hoe 140 and D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK (Vavrek & Stewart, 1985a) were synthesized in the Department of Pharma Synthesis at Hoechst AG and freshly prepared for each experiment with physiological saline. BK (Bachem, Heidelberg, F.R.G.) was taken from a frozen stock solution and diluted in physiological saline. All other compounds were purchased from commercial sources.

The composition of the physiological salt solutions was (in mmol l^{-1}): Tyrode: NaCl 137, KCl 2.68, $NaHCO_3$ 11.9, NaH_2PO_4 0.47, $CaCl_2$ 0.68, $MgCl_2$ 0.49, D-glucose 5.05; Ringer: NaCl 154, KCl 5.6, $CaCl_2$ 1.9, $NaHCO_3$ 2.4, D-glucose 5.0. All solutions were gassed with a mixture of 95% O_2 and 5% CO_2.

The composition of the buffers was (in mmol l^{-1}): Receptor binding buffer: TES 25, pH 6.8 containing, 1,10-phenantroline 1, dithiothreitol 1.0, captopril 1, bacitracin 140 μg ml^{-1} and 0.1% bovine serum albumin (BSA); HEPES-Tyrode-buffer: HEPES 10, KCl 2.7, $MgCl_2$ 2, NaCl 137, $CaCl_2$ 1.8, NaH_2PO_4 0.36, D-glucose 5, pH 7.35; Krebs-Ringer-buffer: NaCl 126, KCl 4.75, $CaCl_2$ 1.27, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, EDTA 0.03, D-glucose 11.

Statistics

Paired and unpaired data were analyzed for statistical significance ($P < 0.05$) with the appropriate Student's t test or Dunnett's test (Sachs, 1984).

Results

Receptor binding

Hoe 140 and D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK showed distinct differences in their binding potency. The IC_{50} value of Hoe 140 was 1.07 × 10^{-9} mol l^{-1} and the K_I value was 7.98 × 10^{-10} mmol l^{-1} whereas the IC_{50} value of the D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK was 8.5 × 10^{-8} mol l^{-1} and the K_I value was 1.6 × 10^{-8} mol l^{-1} (Table 1).

Isolated smooth muscle preparations

Guinea-pig ileum Hoe 140 antagonized BK-induced contractions with an IC_{50} of 1.1 × 10^{-8} mol l^{-1}. D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK was less active with an IC_{50} of 3 × 10^{-5} mol l^{-1}. pA_2 values for Hoe 140 and D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK were 8.42 and 6.18, respectively. Partial agonist activity was observed for both compounds at a concentration of 10^{-4} mol l^{-1}. The effect of Hoe 140 was 16% and that of D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK was 75% of the response to BK (4 × 10^{-8} mol l^{-1}).

Rat uterus BK-induced contractions were inhibited by Hoe 140 with an IC_{50} of 4.9 × 10^{-9} mol l^{-1}. D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK showed an IC_{50} of 4.0 × 10^{-6} mol l^{-1}. No agonist activity was detected for Hoe 140 at a concentration of 10^{-4} mol l^{-1}.

Guinea-pig pulmonary artery Hoe 140 and D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK concentration-dependently inhibited BK-induced contractions with IC_{50} values of 5.4 × 10^{-9} and 6.4 × 10^{-6} mol l^{-1}, respectively. At a concentration of 1 × 10^{-4} mol l^{-1}, both substances exhibited intrinsic agonistic activity similar to the magnitude of the effect of BK (2 × 10^{-7} mol l^{-1}).

Rabbit aorta Hoe 140 (10^{-7} mol l^{-1}) was completely inactive when tested for inhibition of contractions induced by the

Table 1 Comparison between *in vitro* activities of Hoe 140 and D-Arg-[Hyp^2, Thi^{5,8},D-Phe^7/BK

Test	*Hoe 140* (mol l^{-1})	*D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK* (mol l^{-1})
Receptor binding	IC_{50} 1.07 × 10^{-9} K_I 7.98 × 10^{-10}	IC_{50} 8.5 × 10^{-8} K_I 1.6 × 10^{-8}
Isolated smooth muscle preparations		
Guinea-pig ileum	IC_{50} 1.1 × 10^{-8}	IC_{50} 3 × 10^{-5}
Rat uterus	IC_{50} 4.9 × 10^{-9}	IC_{50} 4 × 10^{-6}
Guinea-pig pulmonary artery	IC_{50} 5.4 × 10^{-9}	IC_{50} 6.4 × 10^{-6}
BK-induced EDRF release	IC_{50} 10^{-8}	not determined
BK-induced Ca^{2+} response	IC_{50} 10^{-9}	not determined

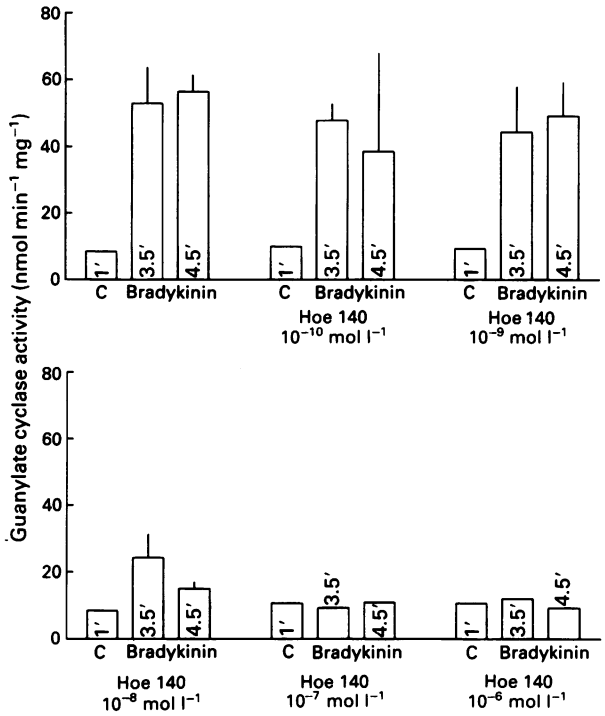

Figure 2 Effect of Hoe 140 on bradykinin (BK, 3×10^{-8} mol l^{-1})-induced EDRF release in aortic endothelial cells of the pig measured as the formation of cyclic GMP in the guanylate cyclase assay. The figures in the columns indicate the time (min) of incubation. The level of significance ($P < 0.05$) was reached with bradykinin (3.5 min and 4.5 min), Hoe 140 10^{-10} mol l^{-1} (3.5 min and 4.5 min), Hoe 140 10^{-9} mol l^{-1} (3.5 min and 4.5 min) and Hoe 140 10^{-8} mol l^{-1} (3.5 min) vs control (C).

BK_1-receptor agonist Des-Arg^9-BK in this isolated vessel preparation.

Bradykinin-induced EDRF release and bradykinin-induced Ca^{2+} response in cultured endothelial cells from pig aorta

Endothelium-derived relaxing factor (EDRF) released by BK (3×10^{-8} mol l^{-1}) from endothelial cells stimulated cyclic GMP formation by activation of guanylate cyclase from 9 (controls) to 53 and 57 nmol min^{-1} mg^{-1} protein, respectively. At 10^{-8} mol l^{-1} of Hoe 140, BK-induced cyclic GMP formation was reduced by about 50% and totally suppressed at

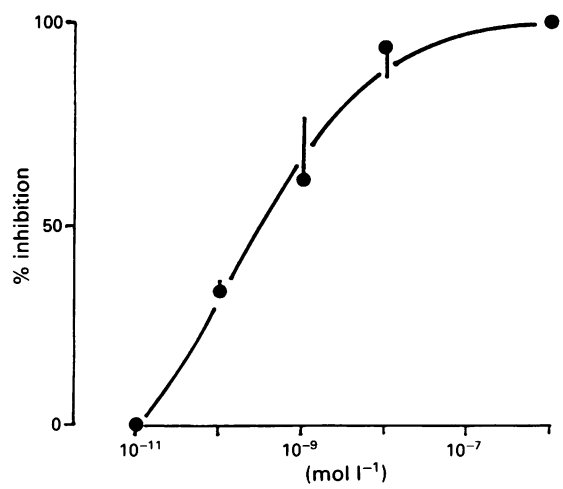

Figure 3 Inhibitory effect of Hoe 140 on the increase in the intracellular free calcium concentration induced by bradykinin (1 nmol l^{-1}) in aortic endothelial cells of the pig.

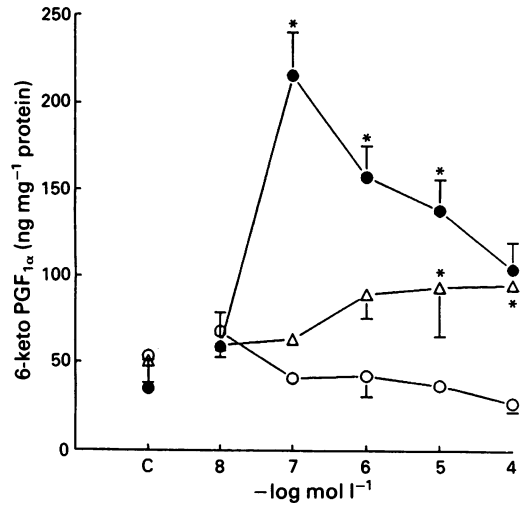

Figure 4 Influence of Hoe 140 and D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK on bradykinin (BK)-induced prostacyclin (PGI_2) release. The first three points designated as C indicate baseline PGI_2 production in the absence of BK with 10^{-7} mol l^{-1} of Hoe 140 (○) and D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK (△) and without antagonist (●). Then both antagonists were tested against increasing concentrations of BK (● BK alone). Incubation with BK (1 min) was preceded by a 5 min incubation period with the antagonists. * Indicates significance ($P < 0.05$) vs the corresponding baseline value.

10^{-7} and 10^{-6} mol l^{-1}. Hoe 140 did not influence basal EDRF release (Figure 2).

BK (10^{-9} mol l^{-1}) induced increase in cytosolic free calcium was inhibited with an IC_{50} of about 10^{-9} mol l^{-1} (Figure 3).

Bradykinin-induced prostacyclin release in cultured endothelial cells from bovine aorta

The incubation of endothelial cells for 1 min with BK (10^{-8} to 10^{-4} mol l^{-1}) dose-dependently stimulated PGI_2 release. Hoe 140 (10^{-7} mol l^{-1}; preincubated for 5 min) totally suppressed the BK-induced PGI_2 release. In comparison to Hoe 140, D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK (10^{-7} mol l^{-1}) preincubated for 5 min showed a weaker antagonism of the BK-induced PGI_2 release (Figure 4). Neither Hoe 140 nor D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK affected basal (control) PGI_2.

Discussion

Hoe 140 is a highly active antagonist of BK_2-receptors *in vitro*. Hoe 140 and D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK showed saturable and high affinity binding in crude membrane preparations of the guinea-pig ileum. Compared with D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK Hoe 140 appeared to be about 70 times more potent. This potency difference was even better in smooth muscle preparations where Hoe 140 was shown to be about three orders of magnitude more potent. A comparison of the *in vitro* activities of both antagonists is given in Table 1. The selectivity of Hoe 140 for BK_2 receptors was established by showing that it was virtually inactive in the isolated rabbit aorta which contains the BK_1-receptor type (Regoli, 1984). The discrepancies in residual agonistic activity of Hoe 140 in the different isolated organ preparations might be explained by differences in species and site. Since the agonist activity of Hoe 140 was lower than that of D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK but Hoe 140 was two to three orders of magnitude more potent as an antagonist, better toleration can be expected.

BK is a potent stimulator of autacoid formation in endothelial cells. One of these autacoids is EDRF (d'Orléans-Juste *et al.*, 1989) which stimulates soluble guanylate cyclase. Hoe 140 markedly reduced BK-induced EDRF production. Basal

EDRF production was not altered by Hoe 140 indicating a lack of agonistic activity at the concentrations tested. Endothelial cell activation by BK involves binding to membrane receptors of the BK_2 type which are likely to be coupled to a phospholipase C via pertussis toxin-insensitive G-proteins (Busse, personal communication). Activation of this enzyme by BK evokes an enhanced production of inositol-1,4,5-trisphosphate, which increases cytosolic free calcium concentration by mobilization of calcium from intracellular stores. BK-induced calcium increase was inhibited by Hoe 140. BK has been shown to be a potent stimulator of prostaglandin synthesis in various tissues (Terragno *et al.*, 1975; Uglesity *et al.*, 1983; Zusmann, 1987). The stimulatory effect of BK on vascular PGI_2 synthesis could be explained by an increase in kinin activity. The smaller increase in prostaglandin formation by higher concentrations of BK could be explained by a possible densesitization of BK receptors. In the rat isolated aorta preparation Hoffmann *et al.* (1990) showed that the BK receptor antagonist D-Arg-[Hyp^2, $Thi^{5,8}$, D-Phe^7]BK completely inhibited BK-induced PGI_2 release. Hoe 140 was even more potent in cultured endothelial cells from bovine aorta than D-Arg-[Hyp^2, $Thi^{5,8}$, D-Phe^7]BK. Hoe 140 is highly stable against enzymatic degradation. It is not a substrate for kininase II and carboxypeptidases and is only slowly degraded in human plasma (König; unpublished data). It differs from the known BK-antagonists by replacement of two residues by more rigid and lipophilic amino acids.

The potent BK-antagonist Hoe 140 is likely to contribute to the investigation of the pathophysiological role of BK. It is to be hoped that the reversal of BK-mediated effects will constitute a new approach to the treatment of certain diseases.

References

COSTELLO, A.H. & HARGREAVES, K.M. (1989). Suppression of carrageenan-induced hyperalgesia, hyperthermia and edema by a bradykinin antagonist. *Eur. J. Pharmacol.*, **171**, 259–263.

D'ORLÉANS-JUSTE, P., DE NUCCI, G. & VANE, J.R. (1989). Kinins act on B_1 or B_2 receptors to release conjointly endothelium-derived relaxing factors and prostacyclin from bovine aortic cells. *Br. J. Pharmacol.*, **96**, 920–926.

ERDÖS, E. (ed.) (1979). *Bradykinin, Kallidin and Kallikrein.* Handbook of Experimental Pharmacology Supplement to Vol. 25, p. 817. Berlin: Springer Verlag.

GRIESBACHER, T. & LEMBECK, F. (1987). Actions of bradykinin antagonists on bradykinin-induced plasma extravasation, prostaglandin E_2 release, nociceptor stimulation and contraction of the iris sphincter muscle of the rabbit. *Br. J. Pharmacol.*, **92**, 333–340.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

HENKE, St., ANAGNOSTOPOULOS, H., BREIPOHL, G., GERHARDS, H., KNOLLE, J., SCHÖLKENS, B. & LEMBECK, F. (1990). Novel highly potent bradykinin antagonists and their impact on allergic diseases. *Pure Appl. Chem.*, (in press).

HOFFMAN, G., PIETSCH, R., GÖBEL, B.O., WEISSER, B., BÖNNER, G., VETTER, H. & DÜSING, R. (1990). Converting enzyme inhibition and vascular prostacyclin synthesis: effect of kinin receptor antagonism. *Eur. J. Pharmacol.*, **178**, 79–83.

JUAN, H. & LEMBECK, F. (1974). Action of peptides and other algesic agents on paravascular pain receptors of the isolated perfused rabbit ear. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **283**, 151–164.

LINZ, W., ALBUS, U., WIEMER, G., SCHÖLKENS, B.A. & KÖNIG, W. (1986). Atriopeptin III induces endothelium-independent relaxation and increases cGMP levels in rabbit aorta. *Klin. Wochenschr.*, **64**, (Suppl. VI), 27–30.

MANNING, D.C., VAVREK, R., STEWART, J.M. & SNYDER, S.H. (1986). Two bradykinin binding sites with picomolar affinities. *J. Pharmacol. Exp. Ther.*, **237**, 504–512.

McPHERSON, G.A. (1986). *Kinetik, EBDA, Ligand, Lowry.* Cambridge: Elsevier-BIOSOFT.

MÜLSCH, A., BÖHME, E. & BUSSE, R. (1987). Stimulation of soluble guanylate cyclase by endothelium-derived relaxing factor from cultured endothelial cells. *Eur. J. Pharmacol.*, **135**, 247–250.

REGOLI, D. (1984). Neurohumoral regulation of precapillary vessels: The kallikrein-kinin system. *J. Cardiovasc. Pharmacol.*, **6**, S401–S412.

REGOLI, D. & BARABÉ, J. (1980). Pharmacology of bradykinin and related peptides. *Pharmacol. Rev.*, **32**, 1–46.

REGOLI, D., RHALEB, N.-E., DION, St. & DRAPEAU, G. (1990). New selective bradykinin receptor antagonists and bradykinin B_2 receptor characterization. *Trends Pharmacol. Sci.*, **11**, 156–161.

ROSSUM, J.M., VAN HURKMANS, J.A.Th.M. & WOLTERS, C.J.J. (1963). Cumulative dose-response curves. II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. *Arch. Int. Pharmacodyn.*, **143**, 299–330.

SACHS, L. (1984). *Angewandte Statistik*, p. 552. Berlin: Springer Verlag.

SCHULTZ, G. & BÖHME, E. (1984). Guanylate cyclase. In *Methods of Enzymatic Analysis*, Vol. 4. pp. 379–389. ed. Bergmeyer, H.U. Weinheim: Verlag Chemie.

STERANKA, L.R., DeHAAS, Ch.J., VAVREK, R.J., STEWART, J.M., ENNA, S.J. & SNYDER, S.H. (1987). Antinociceptive effects of bradykinin antagonists. *Eur. J. Pharmacol.*, **136**, 261–262.

STERANKA, L.R., FARMER, S.E. & BURCH, R.M. (1989). Antagonists of B_2 bradykinin receptors. *FASEB J.*, **3**, 2019–2025.

STERANKA, L.R., MANNING, D.C., DeHAAS, Ch.J., FERKANY, J.W., BOROSKY, S.A., CONNOR, J.R., VAVREK, J.R., STEWART, J.M. & SNYDER, S.H. (1988). Bradykinin as a pain mediator: Receptors are localized to sensory neurons, and antagonists have analgesic actions. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 3245–3249.

TAYLOR, J.E., DeFEUDIS, F.V. & MOREAU, J.P. (1989). Bradykinin-antagonists: Therapeutic perspectives. *Drug Develop. Res.*, **16**, 1–11.

TERRAGNO, D.A., CROWSHAW, K., TERRAGNO, N.A. & McGIFF, J.C. (1975). Prostaglandin synthesis by bovine mesenteric arteries and veins. *Circ. Res.*, **36**, (Suppl. 1), I76–I80.

TSIEN, R.Y., POZZAN, T. & RINK, T.J. (1982). Calcium homeostasis in intact lymphocytes: Cytoplasmic free calcium monitored with a new intracellularly trapped fluorescent indicator. *J. Cell. Biol.*, **94**, 325–334.

UGLESITY, A., KREISBERG, J.I. & LEVINE, L. (1983). Stimulation of arachidonic acid metabolism in rat kidney mesangial cells by bradykinin, antidiuretic hormone and their analogues. *Prostaglandin Leukotriene Med.*, **10**, 83–93.

VAVREK, R.J. & STEWART, J.M. (1985a). Development and modification of competitive antagonists of bradykinin. In *Peptides, Structure and Function.* Proc. 9th Amer. Peptide Symp. ed. Deber, C.M., Hruby, V.L. & Kopple, K.D. pp. 655–658. Rockford, IL: Pierce Chem. Comp.

VAVREK, R.J. & STEWART, J.M. (1985b). Competitive antagonists of bradykinin. *Peptides*, **6**, 161–164.

WILHELM, D.L. (1973). Chemical mediators. In *The Inflammatory Process.* ed. Zweifach, B.W., Grant, L. & McCluskey, R.T., pp. 251–301. New York: Academic Press.

ZUSMANN, R.M. (1987). Effects of converting enzyme inhibitors on the renin-angiotensin-aldosterone, bradykinin, and arachidonic acid-prostaglandin system: correlation on chemical structure and biologic activity. *Am. J. Kidney Dis.*, **10**, (Suppl. 1), 13–23.

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Hoe 140 a new potent and long acting bradykinin-antagonist: *in vivo* studies

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1 The potency, duration of action and tolerability of Hoe 140, a novel and highly potent bradykinin (BK) antagonist *in vitro*, has been tested in different *in vivo* models and compared with the well-known BK antagonist D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK.

2 Hoe 140 is highly potent and long acting in inhibiting BK-induced hypotensive responses in the rat. Four hours after s.c. administration of 20 nmol kg^{-1}, inhibition still amounted to 60% whereas the effect of 200 nmol kg^{-1} of D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK was not significant.

3 BK-induced bronchoconstriction in guinea-pigs was strongly inhibited by Hoe 140. The magnitude and duration of inhibition confirmed the findings obtained in the blood pressure experiments in the rat.

4 Carrageenin-induced inflammatory oedema of the rat paw was considerably inhibited at i.v. doses between 0.1 and 1 mg kg^{-1}.

5 In conscious dogs, intravenous doses of 0.01 and 0.1 mg kg^{-1} of Hoe 140 and D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK were well tolerated. At doses of 1 mg kg^{-1} adverse effects occurred that were attributed to the residual BK agonistic activity of both compounds.

6 Hoe 140 has been shown to be a highly potent and long acting BK antagonist *in vivo* in different animal species and models. This makes it appropriate to investigate further the physiological and pathophysiological role of BK.

Introduction

Hoe 140 is a highly potent, novel and original BK_2-receptor antagonist of bradykinin (BK) as shown *in vitro* in receptor binding assay, in isolated smooth muscle preparations, endothelium-derived relaxing factor (EDRF)-release and prostacyclin (PGI_2)-release (Hock *et al.*, 1991). It exceeds the potency of the hitherto known antagonists by at least two orders of magnitude.

The aim of the present study was to test the efficacy of Hoe 140 *in vivo* in models where BK served as an agonist. For this purpose, models of BK-induced hypotensive reaction and BK-induced bronchoconstriction were used to demonstrate its high potency and long duration of action. For comparison, the BK antagonist D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK which is representative of the class of antagonists synthesized by Stewart (Vavrek & Stewart 1985) was used in blood pressure experiments. To show the efficacy and therapeutic potential of Hoe 140 in a standard model of inflammation the carrageenin-induced rat paw oedema model was selected which has been shown to be highly predictive of anti-inflammatory drug activity in human inflammatory disease. Furthermore, tolerability of Hoe 140 was tested in dogs.

Methods

Duration of action of Hoe 140 assessed by inhibition of bradykinin-induced hypotensive responses in anaesthetized rats

Intra-arterial infusion of bradykinin-antagonists The method of Griesbacher *et al.* (1989) was used; 26 male Sprague Dawley rats (Moellegard, Skensved, DK) weighing between 320 and 350 g were anaesthetized with sodium thiopentone (Trapanal, 100 mg kg^{-1}, i.p.). Systemic blood pressure was measured directly in the left femoral artery by use of a Statham pressure transducer (P23 Db), connected to an 8-channel recorder (Hellige GmbH, Freiburg, F.R.G.).

BK was dissolved in saline with 0.1% bovine serum albumin (BSA) and given as a bolus injection (100 pmol in 0.1 ml i.a.) into the aorta via a cannula in the left carotid artery.

Hoe 140 and D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK were freshly dissolved with 0.1% BSA and doses of 0.015 and 0.15 nmol min^{-1} were infused i.a. at an infusion rate of 0.15 ml min^{-1} with a Braun-Melsungen perfusor (Unita II). The infusion was started 4 min before the third injection of BK and lasted for 5 min. In control animals the vehicle was given instead of the antagonist.

Subcutaneous administration of bradykinin-antagonist In a further experiment in rats, doses of 20 nmol kg^{-1} Hoe 140 (0.1 ml kg^{-1}) and 200 nmol kg^{-1} D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7] BK were given subcutaneously. BK was injected before and at regular intervals after administration of both compounds.

Effect of Hoe 140 on bradykinin-induced bronchoconstriction in the anaesthetized guinea-pig

Guinea-pigs (Hoechst breeding farm) of either sex weighing 400–600 g were anaesthetized with 70 mg kg^{-1} pentobarbitone i.p. and prepared for catherization of the trachea, oesophagus, jugular vein and carotid artery. Pulmonary function was assessed according to the method described by Chapman *et al.* (1985) with some modifications. The animals were mechanically ventilated with a Starling respiratory pump at a rate of 60 strokes min^{-1}. Inspiratory volume was determined by maintaining the inspiratory tracheal pressure at a constant level of 8 cm water.

Succinylcholine chloride (1 mg kg^{-1}, i.v.) was injected for deep muscle relaxation in order to prevent interference from spontaneous respiration. The animals were placed inside a whole body plethysmograph and the tracheal, oesophageal, venous and arterial catheters were connected to onset ports in the wall of the plethysmograph. The tracheal port was connected to a respirator pump and airflow ($\dot{V}$) rates into and out of the plethysmograph were measured as a pressure difference with a Fleisch tube No. 000 and a differential pressure transducer (PM 97 TC, Hellige GmbH, Freiburg, F.R.G.). $\dot{V}$ was calibrated by passing compressed air through a rotameter.

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The tidal volume (VT) was calculated from the flow signal by computer. A calibrated differental pressure transducer (PM 97 TC, Hellige GmbH, Freiburg, F.R.G.) was used to measure the pressure difference between the tracheal port and the oesophagus-catheter representing the transpulmonary pressure (PTP). Calibration of PTP was with a water manometer. The airflow, tidal volume, and transpulmonary pressure signals were fed into an on-line computer system (PO-NE-MAH, Model PF-1, Storrs) for calculation of pulmonary resistance (R_L) and dynamic lung compliance (C_{Dyn}). Computation of these parameters was performed for each breath with a sampling rate of 100 Hz for each cycle. Flow and pressure signals for computation were obtained from PLUGSYS-measuring system (Hugo Sachs Elektronik, Freiburg, F.R.G.). Systemic arterial blood pressure was measured with a Statham pressure transducer (P23 Db) and heart rate was obtained from pressure pulses.

In preliminary experiments the i.v. dose of BK that increased R_L by about 50% was selected. However, tachyphylaxis occurred after repeated injections. Propranolol (10 mg kg^{-1}, s.c.) was needed in order to obtain consistent BK responses throughout the whole experimental period (Lau *et al.*, 1989) and allowed a reduction of the dose of BK by a factor of ten, which did not lower blood pressure. The doses of BK (between 300 and 600 pmol i.v.) were individually adapted to the animals and the selected dose injected at intervals of 10 min for the i.v. and 30 min for the s.c. dose of Hoe 140 throughout the experiment. In preliminary experiments ($n = 6$) it was established that BK injections at intervals of 10 min induced reproducible changes in R_L and C_{Dyn} for at least 1 h. In a small number of animals tachyphylaxis was observed during the initial control injections. In these animals, each subsequent dose of BK was increased by up to 100% of the previous dose in order to maintain a consistent rise in R_L.

Carrageenin-induced paw oedema in rats

The method described by Winter *et al.* (1962) was employed. Groups of 5 male rats (Sprague-Dawley, Möllegard, Skensved, DK, about 120 g) deprived of food overnight were treated intravenously with the test compound, immediately before carrageenin was injected into the left hind paw. Paw volume was measured by water displacement before and 1, 2 and 3 h after injection of carrageenin. Hoe 140 was dissolved in physiological saline and administered into the tail vein at a volume of 1 ml kg^{-1}. Carrageenin was made up 0.5% in saline. Each rat received 0.1 ml of the irritant.

Effects of i.v. administration on blood pressure and heart rate in conscious dogs

The experiments were carried out in 4 male and 1 female Beagle dogs (Hoechst breeding farm), weighing between 13.5 and 19 kg. An ultrasonic blood pressure monitor (Roche Arteriosonde 1011) was positioned on the dog's tail for indirect measurement of blood pressure and heart rate.

D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK and Hoe 140 were given intravenously over 1 min to the conscious dogs in increasing doses of 0.01, 0.1, and 1 mg kg^{-1}. Blood pressure measurements were done before and 1, 3, 5, 10 and 15 min after application of the substances or vehicle (saline 0.2 ml kg^{-1}, i.v.). After a period of about 1.5 h the next dose was administered.

Drugs

Hoe 140 and D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK (Vavrek & Stewart, 1985) were synthesized in Pharma Sythesis at Hoechst AG and were freshly dissolved for each experiment in physiological saline. BK was taken from a frozen stock solution and diluted in physiological saline. All other compounds were purchased from commercial sources.

Statistics

Paired and unpaired data were analyzed for statistical significance ($P < 0.05$) with the appropriate Student's t test (Sachs, 1984).

Results

Duration of action of Hoe 140 assessed by inhibition of bradykinin-induced hypotensive responses in anaesthetized rats

Intra-arterial infusion of bradykinin-antagonists Intra-arterial injections of 100 pmol BK transiently decreased the mean arterial blood pressure (MAP) of 121 to 130 mmHg by 29 to 39 mmHg (range of the means of the groups). In control animals repeated injections of BK at intervals of 10 min consistently produced a transient drop in blood pressure. No tachyphylaxis occurred.

When D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK (0.75 nmol) was infused i.a. over 5 min it attenuated the BK-induced drop in MAP by 56% when compared with the values before antagonist infusion. A reduction by 30% was still observed 20 min after the start of the infusion. From this time course, a half life of the effect of 10 to 15 min was estimated. Hoe 140 (0.075 nmol) infused i.a. over 5 min attenuated the BK-induced drop in MAP by 58%. This effect was about equipotent to the effect obtained with the infusion of 0.75 nmol D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK for the first injection of BK after starting infusion of the BK-antagonists. A half life for the effect of about 30 min was estimated for this dose of Hoe 140.

At an equimolar dose of 0.75 nmol, infused over 5 min, Hoe 140 completely prevented the BK-induced fall in blood pressure. Sensitivity to BK returned after 10 min, but even 1 h after the start of the infusion the response to BK was still impaired by 71%. It is concluded that at this dose, the half life is longer than 60 min.

Subcutaneous administration of bradykinin-antagonists Injections of BK decreased MAP of 115 to 122 mmHg by 27 to 30 mmHg (range of the means of the groups). BK-antagonism by s.c. injections of 20 nmol kg^{-1} of Hoe 140 lasted for more than 5 h. One hour after subcutaneous administration the inhibition was still complete, whereas at 5 h the BK-induced fall in MAP was 12 mmHg. Thus, the response to BK was impaired by 60%. This means that the biological half life is longer than 5 h for Hoe 140 (Figure 1). In contrast, the effects of D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK were not significant although given at a ten times higher dose than Hoe 140.

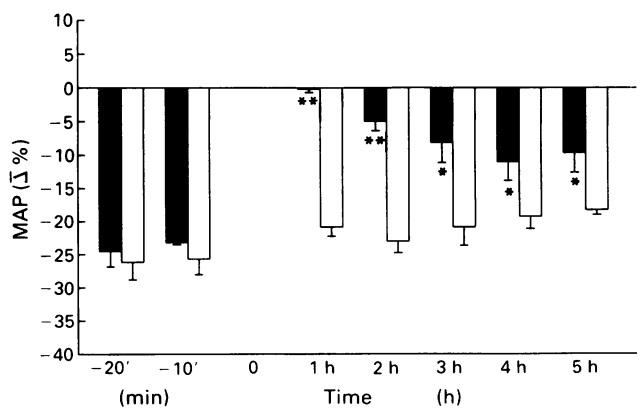

Figure 1 Inhibition of bradykinin (BK)-induced hypotensive responses in rats by 20 nmol kg^{-1} Hoe 140 (solid columns) injected subcutaneously (time zero) in comparison to the vehicle (open columns). BK (100 pmol) was given i.a. Each column represents the mean of % change in mean arterial pressure (MAP) of 6 animals with s.e.mean shown by vertical bars. ** $P < 0.001$ and * $P < 0.05$ compared with the vehicle.

Effect of Hoe 140 on bradykinin-induced bronchoconstriction in the anaesthetized guinea-pig

BK in doses of 300 to 600 pmol i.v. per animal increased R_L by 42–58% compared to baseline levels (0.28 ± 0.01 cmH_2O $ml^{-1}s^{-1}$), while C_{Dyn} decreased by 33 to 39% (0.34 ± 0.03 ml cm^{-1} water). Hoe 140, 1 to 1000 pmol kg^{-1} i.v., caused a dose-dependent inhibition of BK-induced bronchoconstriction with ID_{50} values of 13.4 pmol kg^{-1} for R_L and 31.8 pmol kg^{-1} for C_{Dyn} (Figure 2).

Maximum effects were seen 1 min after administration of Hoe 140. The response to BK was completely re-established within 5 min following 1 pmol kg^{-1}, within 10 min after 10 pmol kg^{-1} and within 10–20 min after 100 pmol kg^{-1} of Hoe 140. Following administration of 1 nmol kg^{-1} however, BK-induced increase in R_L was still inhibited by 60% after 1 h (Figure 3) and the decrease in C_{Dyn} by 46%. BK-induced bronchoconstriction was abolished for 2 h by pretreatment with a s.c. dose of 20 nmol kg^{-1} Hoe 140 administered 30 min before BK. Four hours after pretreatment, the inhibition in terms of R_L-increase was still 50% indicating a long biological half life of at least 4 h for this dose and route of administration.

Seven experiments were performed to determine whether large doses of Hoe 140 showed BK-agonist activity. In animals pretreated with propranolol, a dose of 1 μmol kg^{-1}, i.v. increased R_L by 16% from baseline, while C_{Dyn} was decreased by 14%. When propranolol was omitted ($n = 3$) only slight transitory changes in R_L (+6%) and C_{Dyn} (−5%) were observed.

Carrageenin paw oedema in rats

In the carrageenin-induced inflammation of the paws in rats, intravenous treatment (0.01–1 mg kg^{-1}) inhibited the inflammatory response, the duration of action being longer when the dose was increased (Table 1).

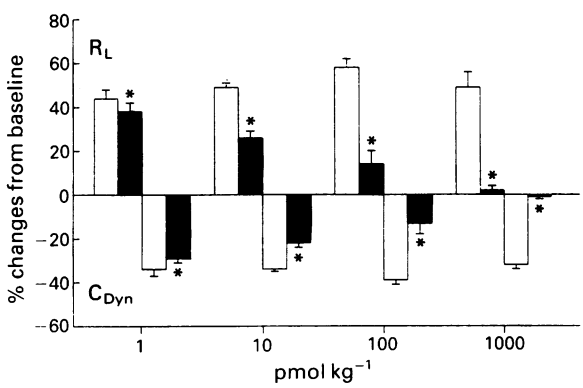

Figure 2 Dose-dependent inhibition of bradykinin (BK)-induced bronchoconstriction in anaesthetized guinea-pigs. Each column represents the mean of 3 to 6 animals before (open columns) and after (solid columns) i.v. administration of Hoe 140 (vertical bars show s.e.mean); * $P < 0.05$.

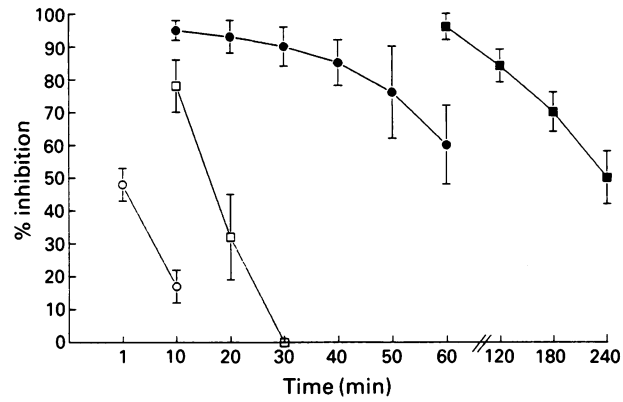

Figure 3 Duration of inhibition of bradykinin (BK)-induced increases in pulmonary resistance (R_L) in anaesthesized guinea-pigs following different doses of Hoe 140 (○, 0.01 nmol kg^{-1}, i.v., □, 0.1 nmol kg^{-1}, i.v.; ●, 1 nmol kg^{-1} i.v. and ■, 20 nmol kg^{-1}, s.c.). Each point represents the mean of 3 to 6 animals with s.e.mean shown by vertical bars.

Effects of i.v. administration on blood pressure and heart rate in conscious dog

In conscious dogs, administration of D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK ($n = 4$) and Hoe 140 ($n = 5$) in doses of 0.01 and 0.1 mg kg^{-1} did not affect systolic, diastolic and mean arterial blood pressure (MAP) or heart rate during a 15 min observation period. At the highest dose of 1 mg kg^{-1} however, Hoe 140 decreased blood pressure drastically in two dogs out of two, for 5 to 10 min with concomitant symptoms like pain reaction, restlessness and hypersalivation. D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK (1 mg kg^{-1}) induced restlessness, hypersalivation, panting and swelling of the mucous membranes in one dog out of four.

Discussion

Our results demonstrate that Hoe 140 is a highly potent BK-antagonist *in vivo* with a long duration of action. In rats, 20 nmol kg^{-1} s.c. Hoe 140 showed a half life of protection against BK-induced hypotensive responses of more than 5 h whereas the effect of 200 nmol kg^{-1} D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK was not significant at any time. The higher potency and longer duration of action of Hoe 140 compared to D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]BK can be explained by a higher receptor affinity and *in vitro* stability (Hock *et al.*, 1991). Hoe 140 is not a substrate for kininase II and is only slowly degraded in human plasma.

In anaesthetized guinea-pigs, Hoe 140 exerted strong protective effects against BK-induced bronchoconstriction. The magnitude and duration of the antagonism were dose-dependent. Even low i.v. doses of 1 to 100 pmol kg^{-1} of Hoe

Table 1 Effects of Hoe 140 on carrageenin-induced paw oedema in rats

Treatment (i.v.)	*Paw volume* (ml) *pre drug* (x ± s.d.)	*Increase in paw volume* (ml) (x ± s.d.) 1 h	2 h	3 h
Control	1.68 ± 0.04	0.36 ± 0.11	0.62 ± 0.26	0.75 ± 0.29
Hoe 140				
0.01 mg kg^{-1}	1.66 ± 0.08	0.06 ± 0.06*	0.45 ± 0.14	0.80 ± 0.18
0.1 mg kg^{-1}	1.66 ± 0.07	0.12 ± 0.06*	0.11 ± 0.09*	0.29 ± 0.15*
1.0 mg kg^{-1}	1.68 ± 0.05	0.14 ± 0.04*	0.14 ± 0.06*	0.27 ± 0.1*

* $P < 0.05$ vs. control (Student's t test)

140 afforded short but considerable protection. At a s.c. dose of 20 nmol kg^{-1}, the half life of the effect was estimated to be at least 4 h. These results are consistent with the results and conclusions obtained in blood pressure experiments in rats concerning the potency and duration of action of Hoe 140. Only weak agonist activity assessed as an increase in pulmonary resistance (+6%) was observed at the very high dose of 1 μmol kg^{-1} of Hoe 140 in the absence of propranolol. Propranolol enhanced this effect to a 16% increase in pulmonary resistance over baseline values. Selectivity against BK was demonstrated by the inability of Hoe 140 to inhibit bronchoconstriction induced by acetylcholine, histamine and 5-hydroxytryptamine in a previous study using the method of Konzett and Rössler.

It has been proposed that BK has an important role in the events leading to allergic asthma and rhinitis (Proud & Kaplan, 1988). It elicits contraction of tracheal smooth muscle in the guinea-pig *in vitro* as well *in vivo*. However, tachyphylaxis occurs *in vivo* in the absence of propranolol, probably due to the release of bronchodilator catecholamines from the adrenal glands by BK (Feldberg & Lewis, 1967). Propranolol potentiates the effect of BK in the guinea-pig (Lau *et al.*, 1989). The assumption of an involvement of BK in human asthma was based on the observation that BK induces bronchoconstriction in asthmatics but not in healthy subjects (Fuller *et al.*, 1987) and further supported by the demonstration of increased BK levels in the bronchoalveolar lavage fluid of asthmatic patients (Christiansen *et al.*, 1987). BK increases neuronal reflex activity (Kaufman *et al.*, 1980) which causes the immediate bronchoconstrictor effect and neurophysiologically corresponds to hyperalgesia. Moreover, it exerts exudative and proinflammatory activity. These actions together could lead to the development of bronchial hyperresponsiveness and chronic inflammation of the airways. The role of inflammatory mediators in asthma is extensively discussed by Barnes *et al.* (1988).

Based on the inflammatory actions of BK, receptor antagonists can be expected to reduce airway inflammation and subsequent destruction of lung tissue. Due to its high potency and long duration of action Hoe 140, given in adequate doses, can be expected to neutralize completely endogenous BK. This makes it important to answer the question as to whether antagonism of excess BK improves clinical asthma.

Hoe 140 markedly reduced carrageenin-induced rat paw oedema. This confirms the results obtained with another BK-antagonist (Costello & Hargreaves, 1989). Carrageenin-induced inflammation has been shown to be highly predictive of anti-inflammatory drug activity in human inflammatory disease. BK is an important mediator in this rat model of inflammation. This was already anticipated by the finding that BK plasma levels rose after carrageenin-induced inflammation in rats (Hargreaves *et al.*, 1988). The assumption of an involvement of BK in inflammation in human disease was strengthened by the observation that BK levels increased in plasma of patients with rheumatoid arthritis and after oral surgery (Hargreaves *et al.*, 1988). Moreover, kallikrein levels are increased in joints of patients with rheumatoid arthritis (Selwyn *et al.*, 1989).

Possible adverse affects of Hoe 140 were tested in dogs by i.v. injection and compared with D-Arg-[Hyp^2, $Thi^{5,8}$, D-Phe^7]BK. Doses of 0.01 and 0.1 mg kg^{-1} of both compounds were well tolerated. The symptoms observed with both antagonists at the very high dose of 1 mg kg^{-1} might be due to their residual BK-agonist activity (Hock *et al.*, 1991).

In summary, our results show that Hoe 140 is a highly potent BK-antagonist with a long duration of action and a good efficacy/tolerability relationship for application in BK-mediated diseases.

References

BARNES, P.J., CHUNG, F. & PAGE, C.P. (1988). Inflammatory mediators and asthma. *Pharmacol. Rev.*, **40**, 49–83.

CHAPMAN, R.W., DANKO, G. & SIEGEL, I.M. (1985). Effect of propranolol on pulmonary function and bronchoconstrictor responsiveness in guinea pigs and rats. *Pharmacol. Res. Commun.*, **17**, 149–163.

CHRISTIANSEN, S.C., PROUD, D. & COCHRANE, C.G. (1987). Detection of tissue kallikrein in the bronchoalveolar lavage fluid of asthmatic subjects. *J. Clin. Invest.*, **79**, 188–197.

COSTELLO, A.H. & HARGREAVES, K.M. (1989). Suppression of carrageenin-induced hyperalgesia, hyperthermia and edema by a bradykinin antagonist. *Eur. J. Pharmacol.*, **171**, 259–263.

FELDBERG, W. & LEWIS, G.P. (1967). The action of peptides on the adrenal medulla: Release of adrenaline by bradykinin and angiotensin. *J. Physiol.*, **171**, 98–108.

FULLER, R.W., DIXON, C.M., CUSS, F.M.C. & BARNES, P.J. (1987). Bradykinin-induced bronchoconstriction in humans. *Am. Rev. Respir. Dis.*, **135**, 176–180.

GRIESBACHER, T., LEMBECK, F. & SARIA, A. (1989). Effects of the bradykinin antagonist B 4310 on smooth muscles and blood pressure in the rat, and its enzymatic degradation. *Br. J. Pharmacol.*, **96**, 531–538.

HARGREAVES, K.M., TROULLOS, E.S., DIONNE, R.A., SCHMIDT, E.A., SCHAFER, S.C. & JORIS, J.L. (1988). Bradykinin is increased during acute and chronic inflammation: Therapeutic implications. *Clin. Pharmacol. Ther.*, **44**, 613–621.

HOCK, F.J., WIRTH, K., ALBUS, U., LINZ, W., GERHARDS, H.J., WIEMER, G., HENKE, St., BREIPOHL, G., KÖNIG, W., KNOLLE, J. & SCHÖLKENS, B.A. (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: *in vitro* studies. *Br. J. Pharmacol.*, **102**, 769–773

KAUFMAN, M.P., COLERIDGE, H.M., COLERIDGE, J.C.G. & BAKER, D.G. (1980). Bradykinin stimulates afferent vagal C-fibres in intrapulmonary airways of dogs. *J. Appl. Physiol.*, **48**, 511–517.

LAU, W.A.K., RECHTMAN, M.P., BOURA, A.L.A. & KING, R.G. (1989). Synergistic potentiation by captopril and propranolol of bradykinin-induced bronchoconstriction in the guinea pig. *Clin. Exp. Pharmacol. Physiol.*, **16**, 849–857.

PROUD, D. & KAPLAN, A. (1988). Kinin formation: mechanisms and role in inflammatory disorders. *Ann. Rev. Immunol.*, **6**, 49–83.

SACHS, L. (1984). *Angewandte Statistik.* p. 552. Berlin: Springer Verlag.

SELWYN, B.M., FIGOUEROU, C.D., FINK, E., SWAN, A., DIEPPE, P.A. & BHOOLA, K.D. (1989). A tissue kallikrein in the synovial fluid of patients with rheumatoid arthritis. *Ann. Rheum. Dis.*, **48**, 128–133.

VAVREK, R.J. & STEWART, J.M. (1985). Development and modification of competitive antagonists of bradykinin. In *Peptides, Structure and Function.* Proc. 9th Amer. Peptide Symp. ed. Deber, C.M., Hruby, V.L. & Kopple, K.D. pp. 655–658. Rockford IL: Pierce Chem. Comp.

WINTER, Ch.A., RISLEY, E.A. & NUSS, G.W. (1962). Carrageenin-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. *Proc. Soc. Exp. Biol.*, **111**, 544–547.

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Deamination of newly-formed dopamine in rat renal tissues

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1 The present study has examined the formation of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) in slices of the rat renal cortex and the renal medulla loaded with exogenous L-β-3,4-dihydroxyphenylalanine (L-DOPA). The effects of pargyline and of two selective inhibitors of monoamine oxidase (MAO) types A and B, respectively Ro 41-1049 and Ro 19-6327, on the deamination of newly-synthesized dopamine in kidney slices incubated with exogenous L-DOPA were also tested. The assay of L-DOPA, dopamine, noradrenaline and DOPAC was performed by means of h.p.l.c. with electrochemical detection.

2 Incubation of renal slices with exogenous L-DOPA resulted in a concentration-dependent accumulation of dopamine and DOPAC; the tissue levels of newly-formed dopamine and DOPAC in slices of the renal medulla were 6–8% of those in cortical slices.

3 Pargyline (0.1 mM) produced a marked decrease (84% reduction) in the formation of DOPAC in kidney slices loaded with 1.0 mM L-DOPA; this effect was accompanied by a 17% increase in the accumulation of dopamine. Similar effects were obtained at higher concentrations of pargyline (0.5 and 1.0 mM). At 5.0 and 10.0 mM pargyline, a marked decrease (46 and 76% reduction) in the accumulation of newly-formed dopamine was observed.

4 The accumulation of dopamine and DOPAC was found to be time-dependent in experiments in which tissues were incubated with 5 and 10 μM L-DOPA for 5, 10, 20 and 30 min. Pargyline (0.1 mM) produced an increase in the accumulation of dopamine at all incubation periods and decreased the formation of DOPAC.

5 Ro 41-1049 (50, 100 and 250 nM) was found to produce a concentration-dependent increase of newly-formed dopamine (16–66% increase) and reduced DOPAC formation (44–89% reduction). Ro 19-6327 (50, 100 and 250 nM), was found not to affect the accumulation of newly-formed dopamine, but significantly reduced the formation of DOPAC.

6 It is concluded that deamination of newly-formed dopamine in kidney slices loaded with L-DOPA constitutes an important mechanism of amine inactivation. The results presented also suggest that most of the MAO, located inside the compartment where the synthesis of dopamine occurs, is of the A type.

Introduction

In kidney, a major source of dopamine derives from the decarboxylation of filtered 3,4-dihydroxyphenylalanine (DOPA) in tubular epithelial cells (Baines & Chan, 1980; Lee, 1982; Suzuki *et al.*, 1984). This is a quantitatively important process as tubular epithelial cells have a high aromatic L-amino acid decarboxylase (AAAD) activity (Adam *et al.*, 1986) and plasma levels of DOPA can attain concentrations up to 15 pmol ml^{-1} (Cuche, 1988). More recently, evidence obtained in studies performed under *in vitro* conditions have confirmed the view that renal tissues are endowed with an enormous capacity to synthesize dopamine from exogenous L-β-3,4-dihydroxyphenylalanine (L-DOPA) and further showed that a substantial amount of newly-formed dopamine in renal tissues is deaminated to 3,4-dihydroxyphenylacetic acid (DOPAC) (Soares-da-Silva & Fernandes, 1990a). The metabolic degradation of dopamine formed in tubular epithelial cells is indeed a matter of considerable importance. Firstly, because there is evidence suggesting that in kidney, dopamine is synthesized in close proximity to the cells endowed with dopamine receptors, the activation of which results in a decrease of tubular reabsorption of sodium (Jose *et al.*, 1986; Siragy *et al.*, 1989). Secondly, there is evidence that renal tissues are endowed with one of the largest monoamine oxidase (MAO) activities in the body (Caramona & Soares-da-Silva, 1990) and deamination represents a major pathway for the inactivation of catecholamines within the kidney (Stöcker & Hempel, 1976; Kopin, 1985).

Most of the AAAD activity in kidney is located in the cortex (Adams *et al.*, 1986) and evidence has also been presented showing that most of the dopamine formed in renal tissues has its origin in cortical elements (Seri *et al.*, 1990; Soares-da-Silva & Fernandes, 1990b), namely in proximal convoluted tubules (Hayashi *et al.*, 1990). The aim of the present work was to compare the processes of deamination of newly-formed dopamine in slices of the rat renal cortex and the renal medulla loaded with exogenous L-DOPA and also to study the effects of non-selective and selective MAO inhibitors on the renal production of dopamine.

Methods

Male Wistar rats (Biotério do Instituto Gulbenkian de Ciência, Oeiras, Portugal) aged 45–60 days and weighing 200–280 g were used in the experiments. Animals were kept two per cage under controlled environmental conditions (12 h light/dark cycle and room temperature 24°C). Food and tap water were allowed *ad libitum*. The experiments were all carried out during day time.

The rats were killed by decapitation under ether anaesthesia and both kidneys removed and rinsed free from blood with saline (0.9% w/v NaCl solution). The kidneys were placed on an ice cold glass plate, the kidney poles removed and slices of renal cortex and renal medulla approximately 1.5 mm thick and weighing 70–80 mg wet weight were prepared with a scalpel. Thereafter, slices of the renal cortex and the renal

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medulla were preincubated individually for 60 min in warm (37°C) and gassed (95% O_2 and 5% CO_2) 10 ml Krebs solution; the pH of the incubation medium was 7.4 for both cortical and medullary slices. After preincubation, renal slices were incubated for 15 min in gassed and warm Krebs solution with added L-DOPA (10–2500 μM); up to a concentration of 5 mM L-DOPA, the pH of the incubation medium remained stable at 7.4. In studies of MAO inhibition, kidney slices containing both the cortex and the medulla were preincubated for 60 min in the presence of the inhibitor, pargyline (0.1–10.0 mM). After preincubation, renal slices were incubated for 15 min in Krebs solution with added L-DOPA (1.0 mM) and containing pargyline, as in the preincubation period. In some experiments, kidney slices (containing both the cortex and the medulla) were incubated for 5, 10, 20 or 30 min with L-DOPA (5 and 10 μM) in the presence of pargyline (0.1 mM). Selective inhibition of type A and B MAO was performed with compounds Ro 41-1049 and Ro 19-6327, respectively (Da Prada *et al.*, 1990). Slices containing both the cortex and the medulla were preincubated for 60 min in the presence of increasing concentrations of the inhibitors (50, 100 and 250 nM); after preincubation, renal tissues were incubated for 15 min with added L-DOPA (10 μM) in the presence of the selective MAO inhibitors. The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, EDTA 0.4, ascorbic acid 0.57 and glucose 11; 1-α-methyl-*p*-tyrosine (50 μM), copper sulphate (10 μM) and tropolone (30 μM) were added to the Krebs solution in order to prevent the synthesis of endogenous catecholamines from tyrosine and to inhibit the endogenous inhibitors of dopamine β-hydroxylase and the enzyme catechol-*O*-methyltransferase (COMT), respectively. The preincubation and incubation were carried out in a shaking water bath at 37°C, in an atmosphere of 95% O_2 and 5% CO_2; renal slices were incubated individually in glass vials containing 10 ml Krebs solution. After the incubation, renal slices were collected, washed for 30 min in ice cold Krebs solution, blotted with filter paper, weighed, minced with fine scissors and placed in 2 ml of 0.2 M perchloric acid at 4°C for the next 24 h, till quantification of tissue catecholamines.

The assay of L-DOPA, dopamine, noradrenaline (NA) and DOPAC in renal tissues was performed by means of high performance liquid chromatography with electrochemical detection (h.p.l.c.-e.c.d.). Aliquots of 1.5 ml perchloric acid in which tissues have been kept were placed in 5 ml conical-based glass vials with 50 mg alumina and the pH of the sample immediately adjusted to pH 8.6 by the addition of Tris buffer. Mechanical shaking for 10 min was followed by centrifugation and the supernatant discarded. The adsorbed catecholamines were then eluted from the alumina with 200 μl of 0.2 M perchloric acid on Millipore microfilters (MF1); 50 μl of the eluate was injected into the h.p.l.c.-e.c.d. system. The h.p.l.c.-e.c.d. system consisted of a pump (Gilson model 302; Gilson Medical Electronics, Villiers le Bel, France) connected to a manometric module (Gilson model 802 C) and a stainless steel 5 μm ODS column (Biophase; Bioanalytical Systems, West Lafayette, Indiana, U.S.A.) 25 cm in length. Samples were injected into the column by means of an automatic sample injector (Gilson model 231) connected to a Gilson dilutor (model 401). The detection was carried out electrochemically by means of a thin-layer cell with a glassy carbon working electrode, an Ag/AgCl reference electrode, and an amperometric detector (model 304 LC-4A; Bioanalytical Systems). The detector cell was operated at 0.75 V. The current produced was monitored with a Spectra-Physics integrator (model SP4270; Spectra-Physics, Darmstadt-Kranichstein, F.R.G.). The mobile phase was a degassed solution of citric acid (0.1 mM), sodium octylsulphate (0.5 mM), sodium acetate (0.1 M), EDTA (0.17 mM), dibutylamine (1 mM) and methanol (10% v/v), pH 3.5 and pumped at a rate of 1.0 ml min^{-1}. Standard solutions of L-DOPA, NA, dopamine, DOPAC and dihydroxybenzylamine (internal standard) were injected at different concentrations and peak height increased linearly. The interassay coefficient of variation was less than 5%. The lower limits for detection of L-DOPA, NA, dopamine and DOPAC were 1.0, 0.9, 1.4 and 2.5 pmol g^{-1}, respectively.

Mean values ± s.e.mean of *n* experiments are given. Significance of differences between two means was estimated by Student's *t* test for unpaired data. Significance of difference between one control and several experimental groups was evaluated by Tuckey-Kramer method (Sokal & Rohlf, 1981). A P value < 0.05 was taken to denote a significant difference.

1-α-Methyl-*p*-tyrosine, dopamine hydrochloride, (−)-noradrenaline bitartrate, L-β-3,4-dihydroxyphenylalanine (L-DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), pargyline hydrochloride and tropolone hydrochloride were purchased at Sigma Chemical Company (St Louis, MO, U.S.A.) and copper sulphate at May and Baker (Dagenham, England). Ro 41-1049 (N-(2-aminoethyl)-5-(*m*-fluoro-phenyl)-4-thiazole carboxamide hydrochloride) and Ro 19-627 (N-(2-aminoethyl)-5-chloro-2-pyridine carboxamide hydrochloride) were kindly donated by Prof. Mosé Da Prada from Hoffman-La Roche, Basle, Switzerland.

Results

As shown in Figure 1a, the accumulation of L-DOPA in kidney slices was found to be dependent on the concentration of the amino acid added to the medium; this was much more evident in cortical slices than in slices containing the renal medulla, as evidenced by the tendency for saturation in the

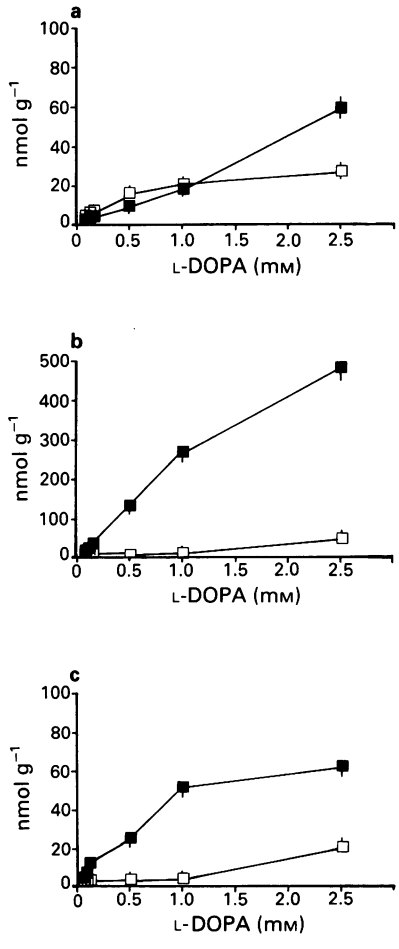

Figure 1 Tissue levels (in nmol g^{-1}) of (a) L-β-3,4-dihydroxyphenylamine (L-DOPA), (b) dopamine and (c) 3,4-dihydroxytryphenlacetic acid (DOPAC) in slices of the (■) rat renal cortex and the (□) renal medulla incubated with increasing concentrations of exogenous L-DOPA (10–2500 μM) for 15 min. Each point represents the mean of four experiments per group; vertical lines show s.e.mean.

accumulation of L-DOPA in this renal area. Incubation of cortical slices with increasing concentrations of L-DOPA for 15 min also resulted in a concentration-dependent accumulation of newly-formed dopamine; the amount of the amine accumulated in the tissue reached values as high as 480 nmol g^{-1} when 2.5 mM L-DOPA was added to the medium (Figure 1b). The production of dopamine from added L-DOPA in slices containing the renal medulla was about 6–8% of that occurring in the renal cortex at 2.5 mM L-DOPA (Figure 1b). The accumulation of DOPAC, the deaminated metabolite of dopamine, in cortical slices was also found to be dependent on the concentration of L-DOPA used and reached values as high as 62 nmol g^{-1} when 2.5 mM L-DOPA was used (Figure 1c). As has been found for dopamine, the formation of DOPAC in the renal medulla was approximately 8% of that occurring in cortex. However, although in the renal cortex there was a trend for saturation in the formation of DOPAC, in the renal medulla such a tendency was not observed. In kidney slices incubated in the absence of exogenous L-DOPA the tissue levels of NA were 1.3 ± 0.14 and 0.40 ± 0.06 nmol g^{-1} ($n = 5$), respectively in the cortex and the medulla; DOPAC was not detectable in these experimental conditions. The tissue levels of NA in kidney slices did not change significantly in the course of these experiments even when 2.5 mM L-DOPA was used (data not shown).

Incubation of slices containing both the renal cortex and the medulla with 1.0 mM L-DOPA resulted in the accumulation of newly-formed dopamine and DOPAC. The addition of 0.1 mM pargyline, the non-selective MAO inhibitor, to the incubation medium resulted in a marked decrease (84% reduction) in the formation of DOPAC; this effect was accompanied by a non-significant rise (17% increase) in the accumulation of newly-formed dopamine in kidney slices (Figure 2). The addition of increasing concentrations (0.5, 1.0, 5.0 and 10.0 mM) of pargyline similarly reduced the formation of DOPAC (85–95% reduction). This effect was accompanied by a decrease in the accumulation of newly-formed dopamine in the presence of 5.0 and 10.0 mM pargyline when tissue levels of dopamine in kidney slices were respectively 54% and 22% of those in control conditions (Figure 2).

Assuming that the compartment where renal synthesis of dopamine takes place might be of limited storage capacity, it was decided to perform additional experiments with a lower concentration of L-DOPA (10 μM) and different periods of incubation (5, 10, 20 and 30 min) were also tested. The accumulation of newly-formed dopamine and DOPAC was shown to be time-dependent (Figure 3). In the presence of 0.1 mM pargyline, the increase in the accumulation of dopamine (20–32% increase; Figure 3a) in kidney slices loaded with 10 μM L-DOPA was less pronounced than the reduction of DOPAC tissue levels (54–55% reduction; Figure 3b). However, when tissues have been incubated for 30 min, the decreasing effect of pargyline upon DOPAC levels (62% decrease) was similar to its increasing effect upon dopamine tissue levels (56% increase) (Figure 3). When kidney slices were incubated with 5 μM L-DOPA (data not shown), the magnitude of the effect of pargyline (0.1 mM) in increasing dopamine tissue levels (20–32% increase) was similar to the magnitude of its effect in decreasing DOPAC tissue levels (33–60% reduction).

The addition of increasing concentrations (50, 100 and 250 nM) of the selective MAO-A inhibitor, Ro 41-1049, resulted in a concentration-dependent increase of newly-formed dopamine tissue levels (16–66% increase) in kidney slices incubated with 10 μM L-DOPA (Figure 4a). This effect was accompanied by a concentration-dependent decrease

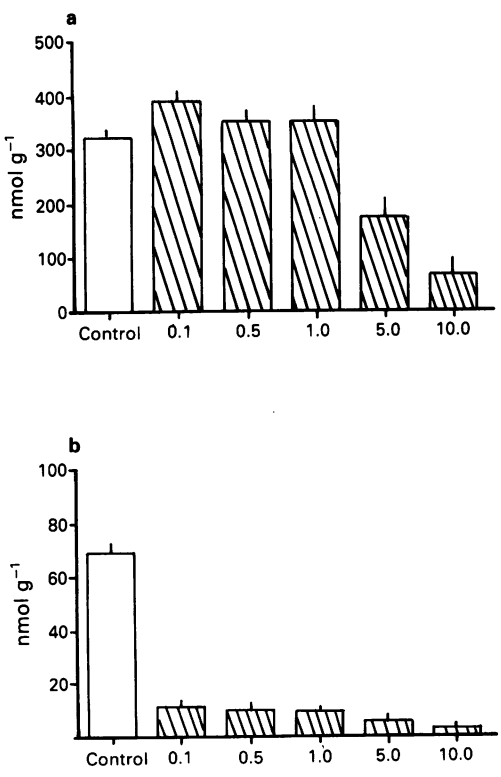

Figure 2 Effect of increasing concentrations of pargyline (0.1, 0.5, 1.0, 5.0 and 10.0 mM) on the tissue levels (in nmol g^{-1}) of (a) dopamine and (b) 3,4-dihydroxyphenylacetic acid (DOPAC) in slices containing both the renal cortex and renal medulla incubated with exogenous L-β-3,4-dihydroxyphenylanine (L-DOPA) (1.0 mM) for 15 min. Each column represents the mean of three experiments per group; vertical lines show s.e.mean. Significantly different from corresponding values of control by the Tuckey-Kramer method (* $P < 0.01$). Control, open columns; pargyline, hatched columns.

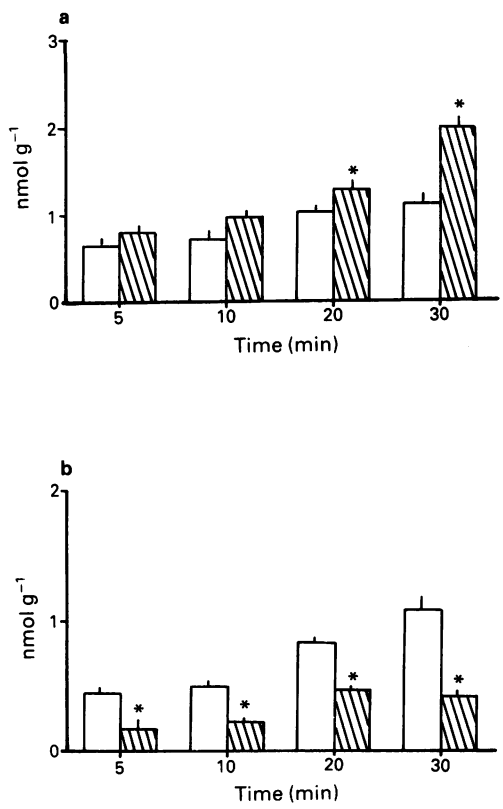

Figure 3 Effect of pargyline (0.1 mM) on the time-dependent accumulation of (a) dopamine and (b) 3,4-dihydroxyphenylacetic acid (DOPAC) (in nmol g^{-1}) in slices containing both the renal cortex and renal medulla incubated with exogenous L-β-3,4-dihydroxyphenylalanine (L-DOPA, 10 μM) for 5, 10, 20 and 30 min. Each column represents the mean of four experiments per group; vertical lines show s.e.mean. Significantly different from corresponding values of control by Student's t test (* $P < 0.01$). Control, open columns; parglyine, hatched columns.

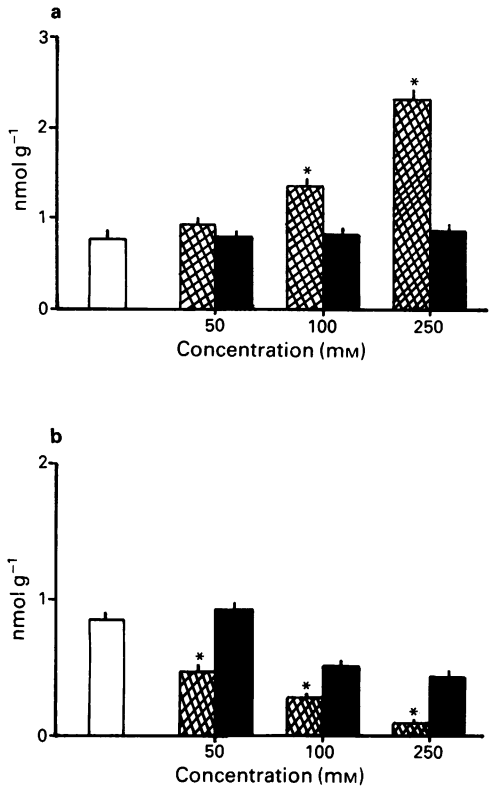

Figure 4 Tissue levels (in nmol g^{-1}) of (a) dopamine and (b) 3,4-dihydroxyphenylacetic acid (DOPAC) in slices containing both the renal cortex and renal medulla incubated with exogenous L-β-3,4-dihydroxyphenylalanine (L-DOPA, 10 μM) for 10 min in control conditions and in the presence of Ro 41-1049 (50, 100 and 250 nM) and Ro 19-6327 (50, 100 and 250 nM). Each column represents the mean of five to six experiments per group; vertical lines show s.e.mean. Significantly different from corresponding values of control by the Tuckey-Kramer method (* $P < 0.01$). Control, open columns; Ro 41-1049, cross hatched columns, Ro 19-6327, solid columns.

(44–89% reduction) of DOPAC tissue content (Figure 4b). Incubation of kidney slices in the presence of the MAO-B selective inhibitor, Ro 19-6327 (50, 100 and 250 nM), was found not to affect the accumulation of newly-formed dopamine, but reduced DOPAC formation (Figure 4). In fact, at 50 nM, Ro 19-6327 did not affect DOPAC formation, but at higher concentrations (100 and 250 nM) it decreased (40–49% reduction) DOPAC accumulation. The tissue contents of both NA and L-DOPA in kidney slices at the end of incubation were unaffected by the presence of either Ro 41-1049 or Ro 19-6327 (data not shown).

Discussion

The results described here are in agreement with previous observations showing that most AAAD activity in renal tissues is located in cortical elements. The finding that a considerable amount of newly-formed dopamine in kidney slices is deaminated to DOPAC gives further support to the view that MAO plays a major role in the disposition of the amine in renal tissues (Stöcker & Hempel, 1976; Kopin, 1985). The present results suggest that both types of MAO may be involved in the deamination of newly-formed dopamine but that most of the MAO located inside the compartment where the synthesis of dopamine takes place appears to be of the A type.

The synthesis of dopamine, in renal tissues, has been suggested to occur either at neuronal and non-neuronal elements: in noradrenergic neurones, dopamine is the percursor for NA; in independent dopaminergic neurones, dopamine may serve as the transmitter substance (Bell, 1987) and there is evidence to suggest that in tubular epithelial cells, dopamine may act as a paracrine substance (Lee, 1982; Siragy *et al.*, 1989). Thus, it might be suggested that decarboxylation of L-DOPA in renal tissues loaded with the amino acid can take place in each of these three cellular elements. However, Baines & Chan (1980) showed that kidney denervation does not affect the formation of dopamine from exogenous L-DOPA; this strongly suggests that most of the decarboxylation of exogenous L-DOPA does occur in non-neuronal structures, namely in tubular epithelial cells (Hayashi *et al.*, 1990). The finding that tissue levels of NA in kidney slices did not change even when 2.5 mM L-DOPA was added to the incubation medium, strongly supports the view that L-DOPA is not decarboxylated inside noradrenergic neurones. Moreover, this further suggests that newly-formed dopamine is not taken up into sympathetic nerve terminals (see Soares-da-Silva, 1988). The possibility that a major proportion of exogenous L-DOPA is decarboxylated inside dopaminergic neurones is also rather unlikely, given the limited size of this hypothetical compartment and the large amounts of L-DOPA which are decarboxylated into dopamine.

Deamination represents a major pathway in the inactivation of catecholamines within the kidney (Stöcker & Hempel, 1976; Kopin, 1985) and renal tissues are endowed with one of the largest MAO activities in the body (Caramona & Soares-da-Silva, 1990). The results presented here show that a substantial amount of newly-formed dopamine is deaminated to DOPAC; this occurs largely in the renal cortex where saturation of deamination of dopamine could be observed, but also to some extent in the medulla and this difference may be related to the limited production of dopamine in the rat renal medulla. Alternatively, it could be hypothesized that in the rat renal medulla the specific activity of MAO may exceed that in the renal cortex, as has been found to occur in the kidney of the cat (Caramona & Soares-da-Silva, 1990). Furthermore, the results indicate a limited ability of MAO to deaminate newly-formed dopamine in renal tissues. In fact, the accumulation of dopamine obtained in the presence of 1.0 or 2.5 mM L-DOPA was far greater than that of DOPAC, whereas with concentrations of 5 and 10 μM L-DOPA the amount of DOPAC accumulated in the tissue was about 80% of that of dopamine.

When kidney slices were loaded with 10 μM L-DOPA the effect (20–32% increase) of 0.1 mM pargyline on the accumulation of dopamine was considerable lower than its decreasing effects on the formation of DOPAC (54–55% reduction). Since pargyline is a non-selective MAO inhibitor, the different effects on the accumulation of dopamine and DOPAC could be the result of the interference with the two different types of MAO, A and B, located in different cellular compartments. For this reason, it was decided to test the effects of selective inhibitors of type A and B MAO, Ro 41-1049 and Ro 19-6327 respectively, on the deamination of newly-formed dopamine. Ro 41-1049 was found to produce a concentration-dependent increase in the accumulation of newly-formed dopamine; this effect was accompanied by a parallel reduction of DOPAC tissue levels. By contrast, incubation of kidney slices in the presence of the MAO-B selective inhibitor, Ro 19-6327, did not affect the accumulation of dopamine in kidney slices, but decreased the formation of DOPAC, at concentrations of 100 and 250 nM. These results appear to suggest that type A and B MAO do not occur in the same cellular compartments. It is possible that most of type A MAO is located in the compartment where the synthesis of dopamine takes place, whereas type B MAO is mainly located outside this compartment and would be responsible for the deamination of dopamine leaking from the storage compartment since MAO-B inhibition does not increase dopamine accumulation but does reduce DOPAC formation.

In recent years, several lines of evidence have suggested that dopamine may exert considerable effects within the kidney and contribute actively in the maintenance of blood pressure at levels considered as acceptable (Lee, 1982; Yoshimura & Takahashi, 1988; Bertorello *et al.*, 1988). The activation of

type D_1 dopamine receptors in the renal vasculature produced vasodilatation resulting in increased renal blood flow and glomerular filtration rate (Felder *et al.*, 1988). Tubular epithelial cells are also endowed with type D_1 dopamine receptors (Hedge *et al.*, 1989) and it has been suggested that dopamine of tubular origin would activate these receptors resulting in an increased sodium excretion (Siragy *et al.*, 1989). Therefore, it might be suggested that inhibition of an important mechanism responsible for the inactivation of tubular dopamine would result in a potentiation of diuretic and natriuretic effects of the amine. Pargyline may not be the most effective MAO inhibitor for this purpose, because of its non-selective effects, but the use a selective MAO-A inhibitor would be preferred if MAO-A predominates in the compartment where the synthesis of dopamine takes place.

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References

ADAM, W.R., CULVENOR, A.J., HALL, J., JARROT, B. & WELLARD, M.R. (1986). Aromatic L-amino acid decarboxylase: Histochemical localization in rat kidney and lack of effect of dietary potassium or sodium loading on enzyme distribution. *Clin. Exp. Pharmacol. Physiol.*, **13**, 47–53.

BAINES, A.D. & CHAN, W. (1980). Production of urine free dopamine from dopa: a micropuncture study. *Life Sci.*, **26**, 253–259.

BELL, C. (1987). Dopamine: precursor or transmitter in sympathetically-innervated tissues. *Blood Vessels*, **24**, 234–239.

BERTORELLO, A., HÖKFELT, T., GOLDSTEIN, M. & APERIA, A. (1988). Proximal tubule Na^+-K^+-ATPase activity is inhibited during high-salt diet: evidence for dopamine-mediated effect. *Am. J. Physiol.*, **254**, F795–F801.

CARAMONA, M.M. & SOARES-DA-SILVA, P. (1990). Evidence for an extraneuronal location of monoamine oxidase in renal tissues. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, 411–413.

CUCHE, J.L. (1988). Sources of circulating dopamine: a personal view. In *Peripheral Actions of Dopamine*, ed. Bell, C. & MacGrath, B.P. pp. 1–23. London: Macmillan Press.

DA PRADA, M., KETLER, R., KELLER, H.H., CESURA, A.M., RICHARDS, J.G., MARTI, J.S., MUGGLI-MANIGLIO, D., KYBURZ, E. & IMHOF, R. (1990). From moclobemide to Ro 19-6327 and Ro 41-1049: The development of a new class of reversible, selective MAO-A and MAO-B inhibitors. *J. Neural. Transm.* [Suppl]., **29**, 279–292.

FELDER, R.A., FELDER, C.C., EISNER, G.M. & JOSE, P.A. (1988) Renal dopamine receptors. In *Peripheral Actions of Dopamine*. ed. Bell, C. & MacGrath, B.P. pp. 124–140. London: Macmillan Press.

HAYASHI, M., YAMAJI, Y., KITAJIMA, W. & SARUTA, T. (1990). Aromatic L-amino acid decarboxylase activity along the rat nephron. *Am. J. Physiol.*, **258**, F28–F33.

HEDGE, S.S., RICCI, A., AMENTA, F. & LOKHANDWALA, M.F. (1989). Evidence from functional and autoradiographic studies for the presence of tubular dopamine-1 receptors and their involvement in the renal effects of fenoldopam. *J. Pharmacol. Exp. Ther.*, **251**, 1237–1245.

JOSE, P.A., FELDER, R.A., HOLLOWAY, R.R. & EISNER, G.M. (1986). Dopamine receptors modulate sodium excretion in denervated kidney. *Am. J. Physiol.*, **250**, F1033–F1038.

KOPIN, I.J. (1985). Catecholamine metabolism: Basic aspects and clinical significance. *Pharmacol. Rev.*, **37**, 333–364.

LEE, M.R. (1982). Dopamine and the kidney. *Clin. Sci.*, **62**, 439–448.

SERI, I., KONE, B.C., GULLANS, S.R., APERIA, A., BRENNER, B.A. & BALLERMANN, B.J. (1990). Influence of Na^+ intake on dopamine-induced inhibition of renal cortical Na^+-K^+-ATPase. *Am. J. Physiol.*, **258**, F52–F60.

SIRAGY, H.M., FELDER, R.A., HOWELL, N.L., CHEVALIER, R.L., PEACH, M.J. & CAREY, R.M. (1989). Evidence that intrarenal dopamine acts as a paracrine substance at the renal tubule. *Am. J. Physiol.*, **257**, F469–F477.

SOARES-DA-SILVA, P. (1988). Dopamine storage in sympathetic nerves. In *Peripheral Actions of Dopamine*. ed. Bell, C. & McGrath, B.P. pp. 24–40. London: Macmillan Press.

SOARES-DA-SILVA, P. & FERNANDES, M.H. (1990a). Synthesis and metabolism of dopamine in the kidney: Effects of sodium chloride, monoamine oxidase inhibitors and alpha-human atrial natriuretic peptide. *Am. J. Hypertens.*, **3**, 7S–10S.

SOARES-DA-SILVA, P. & FERNANDES, M.H. (1990b). Regulation of dopamine synthesis in the rat kidney. *J. Auton. Pharmacol.*, **H10**, Suppl. 1, S25–S30

SOKAL, R.R. & ROHLF, F.J. (1981). *Biometry. The Principles and Practice of Statistics in Biological Research.* New York: Freeman and Company.

STÖCKER, W. & HEMPEL, K. (1976). Inactivation and excretion of dopamine by the cat kidney in vitro. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **295**, 123–126.

SUZUKI, H., NAKANE, H., KAWAMURA, M., YOSHIZAWA, E., TAKESHITA, E. & SARURA, T. (1984). Excretion and metabolism of dopa and dopamine by isolated perfused rat kidney. *Am. J. Physiol.*, **247**, E285–E290.

YOSHIMURA, M. & TAKAHASHI, H. (1988). Dopamine and hypertension. In *Peripheral Actions of Dopamine*. ed. Bell, C. & MacGrath, B.P. pp. 94–107. London: Macmillan Press.

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Contributions that have already been published, or accepted or are under consideration for publication, with essentially the same content will not be considered. This restriction does not apply to results published as abstracts of communications, letters to editors, or as contributions to symposia, provided that the submission adds significantly to the information available in the previously published contribution.

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The Journal will not consider papers which describe experiments on animals which do not fall clearly within the current laws governing animal experimentation in the United Kingdom. Authors must make it clear that the procedures they use were as humane as possible and the doses (initial and subsequent) of anaesthetics and analgesics should be clearly stated; the method of assessing anaesthesia, particularly after the administration of skeletal muscle relaxants (neuro-muscular blocking drugs), must be well defined. The Society has an Ethics Committee which can be consulted by authors through the Secretaries to the Editorial Board.

When investigations on normal human subjects are reported, evidence of approval by a local Ethics Committee must be given. Papers concerned with clinical trials or investigations of the effects of drugs on patients are not appropriate for this Journal.

Authors are strongly urged to keep their manuscripts as short as they reasonably can. An effective way is to reduce the Discussion and the number of figures to a minimum and to avoid repetition of information that has already been published. Authors should remember that a reader may be influenced by literary style and will appreciate simple but accurate prose.

It is important to note that failure to comply with 'Instructions to Authors' may lead to considerable editorial delays.

FULL PAPERS

Manuscripts must be typed on one side of A4 paper. Words at the end of lines should not be divided because they may become incorrectly hyphenated. Handwritten characters or symbols (e.g. Greek letters) should be spelled out in full in the margin. Papers in recent issues of the *British Journal of Pharmacology* should be consulted for the general layout of the paper and also for details. The following subsections are used:

1. Title page
2. Summary
3. Introduction
4. Methods
5. Results
6. Discussion and conclusions
7. Acknowledgements
8. List of references
9. Tables
10. Figures and captions

The type must not be smaller than 12 pitch or 10 point. Each section must be typed in double spacing with margins of not less than 2.5 cm all round and each page should be numbered. **The original and one copy of the typescript should be supplied.**

Title page

The title should normally contain no more than 150 characters and should not consist of a sentence (statement or conclusion) or be interrogative. A short running title containing not more than 50 characters and spaces is also required. The title page should include the names of authors and their appropriate addresses. It should be made clear which address relates to which author. Authors' present addresses differing from those at which the work was carried out should be given as footnotes on the title page and referenced at the appropriate place in the author list by superscript numbers. A footnote may also be used to indicate the author to whom correspondence should be sent. The use of footnotes for any other reason is not allowed. If the address to which proofs should be sent is not that of the first mentioned author, clear instructions should be given in a covering note and not on the title page. The title page should be paginated as page 1 of the paper.

Summary

The summary will be printed at the beginning of the paper. It should not exceed 5% of the length of the paper and should contain a brief account of the problem, the methods, results and the conclusions. It should be arranged in numbered and concise paragraphs. Up to ten **key words** or phrases of two to three words (including names and terms used in the title) should be displayed at the end of the summary. These may be selected from 'Medical Subject Headings' issued by *Index Medicus*. Key words will be used to compile the annual index. The quality of the index will thus be determined by the appropriatness of the key words. Avoid unhelpful or unqualified terms such as 'rat', 'drug' etc.

Introduction

The introduction should give a short and clear account of the background of the problem and the rationale of the investigation. Only previous work that has a direct bearing on the present problem should be cited.

Methods

The methods must be described in sufficient detail to allow the experiment to be interpreted and repeated by the reader. However, detailed repetition of methods which have been adequately described previously should be avoided and references given, although a brief outline is often helpful.

Drugs should be listed in a separate paragraph. Their names should be 'approved names' as published previously in British Approved Names, 1990 (HMSO). If a drug has no 'approved name' its chemical name must be used and the rules set out in the current *Handbook for Chemical Society Authors*

(London, Chemical Society) observed, or its structural formula given. Cumbersome chemical names should be suitably abbreviated for later reference in the paper.

The doses of drugs should be given as unit weight per body weight, e.g. mmol kg^{-1} or mg kg^{-1}; concentrations should be given in terms of molarity, e.g. nM or μM.

Reference should be made to any statistical analyses that have been performed on the results in order, for example, to determine the significance of differences between results obtained under different experimental conditions.

Results

The description of the experimental results should be succinct but, nevertheless, in sufficient detail to allow the experiments to be repeated by others. Typical single experiments may be presented with a clear statement that *n* number of similar experiments had similar results. Where appropriate, however, the mean results with confidence limits or with standard errors of the means and the number of observations should be given. Statistical tests of significance should be performed where appropriate. The results of such tests should be stated as the numerical value of the probability (*P*) that is calculated, with any necessary clarification (e.g. one-tail or two-tail test).

Every effort should be made to avoid unnecessary repetition of data in the text, tables and figures. Conclusions and theoretical considerations should not be elaborated in this section.

Discussion

The purpose of the discussion is to present a brief and pertinent interpretation of the results against the background of existing knowledge. Any assumptions on which conclusions are based must be stated clearly. A mere recapitulation of the results is not acceptable. A review-like treatment, which reduces the impact on the reader, should also be avoided. The main conclusion should be conveyed in a final paragraph.

Acknowledgements

Acknowledgements should be brief but should include reference to sources of support. Sources of drugs not widely available commercially should be acknowledged.

References

In the text, references to other work should take the form: (Bolton & Kitamura, 1983) or, 'Bolton & Kitamura (1983) showed that ...'. If there are more than two authors, the first author's name should be given followed by *et al.* (Bülbring *et al.*, 1981).

References to 'unpublished observations' or 'personal communications' should be mentioned in the text only, and not included in the list of references. Papers which have been **submitted and accepted** for publication, should be included in the list of references with the names of the periodicals and 'in press'. **A photocopy should normally be submitted with the manuscript.** If this is not possible, authors should indicate whether the work cited is an abstract or a full paper. **Papers in preparation or which have been submitted but not yet finally accepted for publication must not be included in the list of references.**

The reference list at the end of the manuscript must be arranged alphabetically according to the surname of the first author. When the surnames of authors are identical, the alphabetical order of their initials takes precedence over the year of publication. The AUTHORS' names are followed by the year of pubication in brackets. If more than one paper by the same authors in one year are cited, a, b, c, etc. are placed after the year of publication, both in the text and in the list of references. The title of the article is given in full, followed by the *abbreviated title of the periodical*, volume **number** and first and last page numbers. The abbreviations used for periodicals are those of the most recent edition of the International List of Periodical Title Word Abbreviations. References to articles in books should consist of names of authors, year of publication, title of article followed by the *title of the book*, the editors, volume number, if any, and page numbers, the place of publication and the names of the publishers. For example:

BOLTON, T.B. & KITAMURA, K. (1983). Evidence that ionic channels associated with the muscarinic receptor of smooth muscle may admit calcium. *Br. J. Pharmacol.*, **78**, 405–416.

BRADING, A.F. (1981). Ionic distribution and mechanisms of transmembrane ion movements in smooth muscle. In *Smooth Muscle: An Assessment of Current Knowledge.* ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. pp. 65–92. London: Edward Arnold.

Tables

Each table should be given on a separate page, paginated as part of the paper. Tables should be numbered consecutively with arabic numericals and the number should be followed by a brief descriptive caption, occupying not more than two lines, at the head of the table. The proportions of the text area should be borne in mind when designing the layout of tables. For the sake of clarity, tables should not have more than 120 characters to a line, with spaces between columns counted as four characters. The absolute maximum is 180 characters to a line. Each column should have a heading and the units of measurement should be given in parentheses in the heading. Except in special circumstances, tables should be self-explanatory; the necessary descriptions should be at the bottom of the table.

Figures

To avoid unnecessary Figures, particularly those requiring half-tone reproduction, only critical points of the text should be illustrated. If coloured Figures are desired, the Authors should discuss their requirements with the Secretaries, preferable before submission.

Please note that unsatisfactory Figures will be returned to the Author for revision. The Journal reserves the right to reject a manuscript if the Figures are unacceptable.

Submission Requirements

(a) The Authors' names and the Figure number must be indicated lightly *in pencil* on the back of each Figure; if necessary, use an adhesive label to avoid damage to the Figure.
(b) *Each* copy of the manuscript must be accompanied by one set of labelled Figures (i.e. complete with lettering and numbering, arrows, etc.). An original set and one high quality photocopy will suffice.
(c) Another original set of Figures identical in size but *without any letters or numbers* must also be supplied for the use of the Publisher. Arrows and event marks on experimental records may be retained, provided they are larger than 3 mm wide. The Publisher will choose the correct style of typeface of an appropriate size to suit the final size of the Figure on the printed page.
(d) No submitted Figure should exceed 210 × 297 mm (A4).
(e) Each Figure must be accompanied by a legend; each legend should be typed on a *separate* sheet of paper and paginated as part of the manuscript. Legends should explain the Figures in sufficient detail that, whenever possible, they can be understood without reference to the text.

Line Figures

It is best to submit an original drawing (black ink on heavy white paper or faint blue graph paper) which has been prepared to conform with the style and convention of the

Line width (axes)	Line width (graphs)	Symbol size	Figure will reduce to this percentage of the original size
———	———	△□○	100 (No reduction)
———	———	△□○	80
———	———	△□○	70
———	———	△□○	60
———	———	△□○	50
———	———	△□○	40

Journal, because redrawing is expensive. The original drawing should be lettered in pencil and should be larger (up to two times as large) than the intended size in the Journal.

It is important that the printed symbols and lines should retain their clarity. To achieve this the symbols and lines in original drawings should be sharply defined and of an even density and breadth. When graphs are generated by computer, lines must not show noticeable stepping. Heavier (broader) lines should be used for curves than for the axes of graphs. The table above illustrates line widths and symbol sizes to be used together on a figure and the appropriate reductions in the final printed form.

Symbols should be chosen from the following set

○ ● □ ■ △ ▲ ▽ ▼ ◇ ◆ + ×

The preferred order to shading of histogram columns is: open (clear), closed (solid), cross-hatched, heavily stippled and other (if required).

The explanation of the symbols and column headings should be given in the Figure legend and not as a key in the Figure itself.

Where the Figure is a composite of more than one graph, experimental record, etc., particular care is needed to minimise the spaces between each part, without over-crowding the entire Figure.

Figure 1 illustrates a simple properly-drawn graph in its original form (a) and in its reduced form (b) as it would appear in the Journal.

Photographs and photomicrographs

These should be submitted, twice as large as their intended published size, as good quality prints of high contrast especially where traces and records are illustrated. The originals must not contain arrows, lettering or numbering; these must be accurately located on a duplicate print (or photocopy). When submitting half-tone illustrations for publication authors should remember that it is not possible to reproduce Figures to a finer quality than the original photographs/photomicrographs provided. Critical areas should be marked on a second copy or on an overlay, so that the Printer can choose the correct exposure. Maximum trim areas should be marked on a second copy of the photograph/photomicrograph or on a tracing overlay, i.e. authors should show any parts of the photographs that could be excluded from the finished half-tone illustration. **A calibration bar must be provided on the photomicrograph** to ensure that, if the Printer reduces the plate, the scale is reduced in the correct proportion.

Proofs

Two sets of page proofs, will be supplied, one of which should be retained by the authors. The other should be corrected immediately and returned to the Publisher. Corrections should be kept to a minimum.

SPECIAL REPORTS

The purpose of *Special Reports*, which have superseded 'Short Communications', is to provide rapid publication for new and important results which the Editorial Board considers are likely to be of special pharmacological significance. *Special Reports* will have publication priority over all other material and so authors are asked to consider carefully the status of their work before submission.

In order to speed publication there is normally no revision allowed beyond very minor typographical or grammatical corrections. If significant revision is required, the Board may either invite rapid re-submission or, more probably, propose that it be re-written as a Full Paper and be re-submitted for consideration. In order to reduce delays, proofs of *Special Reports* will be sent to authors but **essential corrections must reach the Publisher within 48 hours of receipt.** Authors should ensure that their submitted material conforms exactly to the following requirements.

Special Reports should normally occupy no more than two printed pages of the Journal; two illustrations (Figures or Tables, with legends) are permitted. As a guideline, with type face of 12 pitch and double-line spacing, a page of A4 paper could contain about 400 words. The absolute maximum length of the *Special Report* is 1700 words. For each Figure or Table, please deduct 200 words. The manuscript should comprise a Title page, a Summary consisting of a single short paragraph, followed by key words (maximum of 10), Introduction, Methods, Results, Discussion and References (maximum of 10). In all other respects, the requirements are the same as for Full Papers.

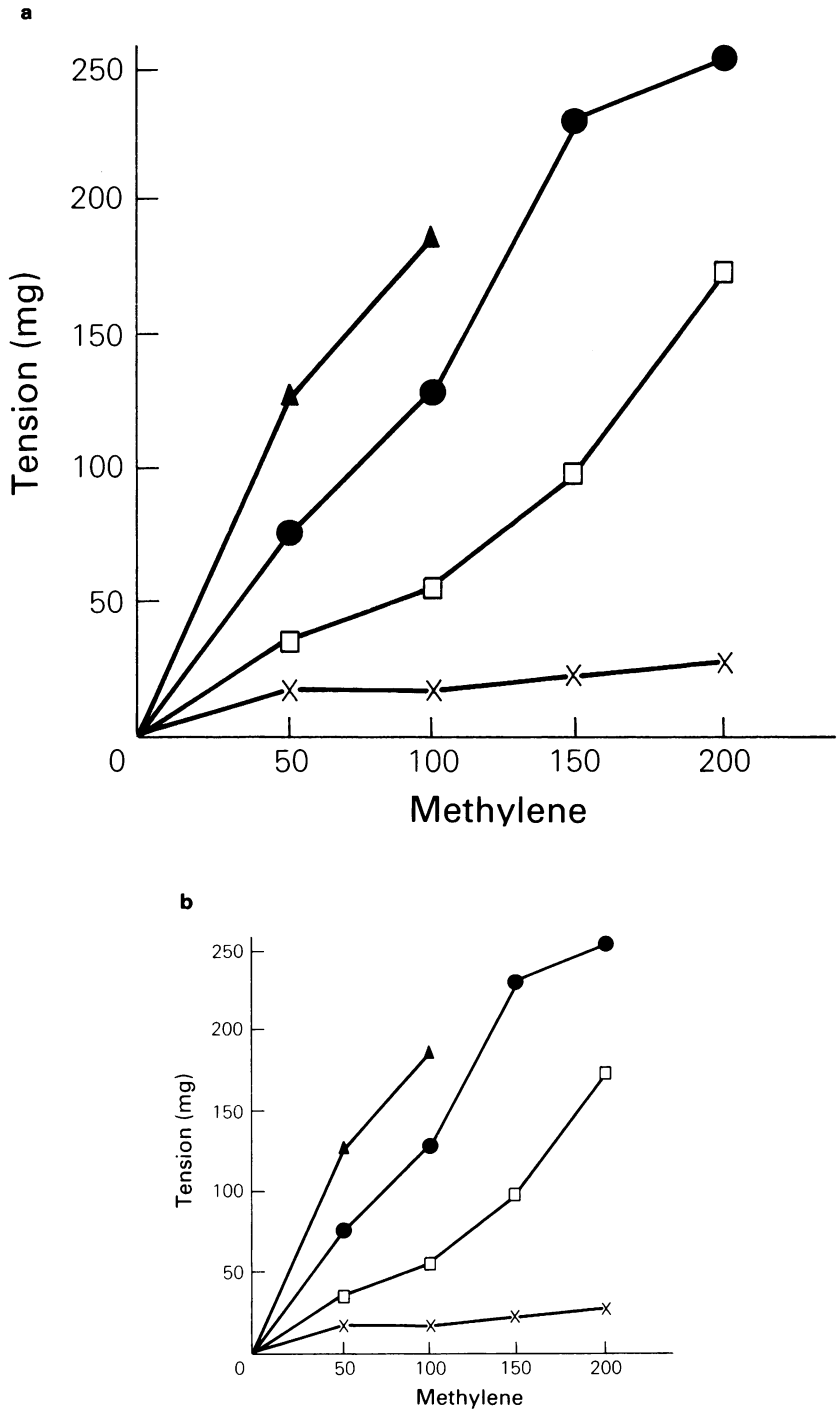

Figure 1. (a) Artwork as drawn. (b) Artwork reduced to 60 per cent of its original size for publication in the Journal.

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The following Statement, Declaration and Copyright Agreement should be read carefully by Authors who should then send a copy of their Declaration with their manuscript following the example given in this section.

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Declaration

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When submitting a manuscript for editorial consideration, Authors should confirm their acceptance of these terms by signing a Declaration to that effect. The recommended wording is given in the example. No paper will be accepted for publication without such a Declaration being signed by each Author (see paragraph 6 above). If the manuscript is not accepted for publication, the assignment will be null and void.

ABBREVIATIONS AND SYMBOLS

Physico-chemical quantities

The *British Journal of Pharmacology* uses the SI symbols for units. The following prefixes for multiples of units should be used:

Multiplier	*Prefix*	*Symbol*
10^{-1}	deci	d
10^{-2}	centi	c
10^{-3}	milli	m
10^{-6}	micro	μ
10^{-9}	nano	n
10^{-12}	pico	p
10^{-15}	femto	f
10^{-18}	atto	a

Multiplier	*Prefix*	*Symbol*
10^{3}	kilo	k
10^{6}	mega	M
10^{9}	giga	G
10^{12}	tera	T

Thus, micron = μm; ångstrom = 0.1 nm. Mixed prefixes are not permissible, thus mμg should be ng. The symbols d (10^{-1}) and c (10^{-2}) should be restricted to those occasions on which there is a strongly felt need for them (e.g. cm).

Use of the solidus

The solidus should be avoided as far as possible and the negative index substituted, e.g. $mg\,kg^{-1}$ rather than mg/kg; $pmol\,mm^{-2}\,min^{-1}$ rather than pmol/mm^2/min.

SYMBOLS

Symbols denoting physical quantities are usually printed as italic capitals (indicated by single underline in typescript). A dash over the symbol indicates a mean value; a dot over the symbol indicates a time derivative. Suffixes may be used to indicate 'where' and 'what'. They are printed as inferiors on the line. Multiple suffixes should be avoided if a simpler symbol adequately defined is unambiguous, but if necessary should be separated by commas e.g. P_{A,CO_2} denotes partial pressure of CO_2 alveolar air.

CHEMICAL AND BIOLOGICAL ABBREVIATIONS

Authors should also consult *Nomenclature Guidelines for Authors* contained in this issue of the Journal. The abbreviations listed may be used without definition *except* those for chemicals, drugs and enzymes which must be written in full at first mention in the title, summary and again in the text. At first mention they should be followed by the abbreviation in brackets. Subsequently, the abbreviation alone may be used.

The list of abbreviations for chemical, drug and enzyme names is clearly not comprehensive and includes only a few commonly used examples.

Use abbreviations sparingly as extensive use can make the text hard to follow.

Physico-chemical quantities

Quantity	*Preferred unit*	*Symbol*
Amount (of substance)	mole	mol
Capacitance	farad	F
Concentration	moles per litre	M or $mol\,l^{-1}$
Current	ampere	A
Electrical conductance	siemens	S
Electromotive force	volt	V
Flow (blood or other liquid)	litres per second (or min)	$1\,s^{-1}$ or $1\,min^{-1}$
Flow (air or other gas)	litres per second (or min)	$1\,s^{-1}$ or $1\,min^{-1}$
Force	newton	N
Frequency of regular event	hertz	Hz
Length	metre	m
Mass	gram	g
Power	watt	W
Pressure (or partial pressure)	pascal*	Pa
Radioactivity	becquerel or curie	Bq (60 d.p.m.) or Ci (3.7×10^{10} Bq)
Resistance (electrical)	ohm	Ω
Temperature	degree celsius	°C
Time	second (preferred)	s
	minute	min
	hour	h
Volume (blood or other liquid)	litre	l
Volume (air or other gas)	litre	l
Work	joule	J

* mm of mercury (mmHg) are allowed if conventional, and if mercury manometer is used for calibration.

Chemical and biological abbreviations

acetylcholine	ACh
acetylcholinesterase	AChE
adenosine 3′ : 5′-cyclic monophosphate	cyclic AMP
adenosine 5′-phosphate	AMP
adenosine triphosphatase	ATPase
γ-aminobutyric acid	GABA
analysis of variants	F
adrenaline	Ad
analytical standard of reagent purity	A.R.
anhydrous	anhyd.
approximate(ly)	approx.
approximately equals	≈
aqueous	aq.
arg-vasopressin	AVP
boiling point	b.p.
bovine serum albumin	BSA
cardiovascular system	CVS
catechol-*O*-methyl transferase	COMT
central nervous system	CNS
cerebrospinal fluid	CSF
chi-squared (statistics)	χ^2
clearance	*c*
coenzyme A	CoA
concentrated	conc.
correlation coefficient	*r*
cubic	cu.
degree of freedom (statistics)	d.f.
deoxyribonucleic acid	DNA
deoxyribonuclease	DNase
dextro-(absolute configuration)	D-
dextro-(optical rotation)	(+)-
diameter	diam.
diameter, inside	i.d.
diameter, outside	o.d.
diffusion coefficient	*D*
3,4-dihydroxyphenylalanine	DOPA
3,4-dihydroxyphenylethylamine	dopamine
direct current	d.c.
disintegration per minute	d.p.m.
dissociation constant	K_D
dissociation constant, negative logarithm of	pK
distilled	dist.
dry ice	solid CO_2
edition	edn
editor(s)	ed.
effective concentration	EC_{50}
effective dose, median	ED_{50}
electrocardiogram	ECG
electrocorticogram	ECoG
electroconvulsive therapy	ECT
electroencephalogram	EEG
electromyogram	EMG
electron spin resonance	e.s.r
endothelial-derived relaxing factor	EDRF
epithelial-derived relaxing factor	EpDRF
equilibrium constant	*K*
equivalent (general use)	equiv.
erythrocyte	r.b.c.
erythrocyte sedimentation rate	ESR
ethylenediaminetetracetic acid	EDTA
excitatory postsynaptic potential	e.p.s.p.
experiment	expt
experimental	exptl

fatty acids, nonesterified	NEFA
figure(s) (with reference number)	Figure(s)
figure (diagram)	figure
gas–liquid chromatography	g.l.c.
glomerular filtration rate	GFR
haemoglobin	Hb
half-life	$t_{1/2}$
high-frequency	h.f.
high performance liquid chromatography	h.p.l.c.
human serum albumin	HSA
hydrogen-ion concentration	$[H^+]$
hydrogen-ion activity, negative logarithm of (hydrogen-ion exponent)	pH
6-hydroxydopamine	6-OHDA
5-hydroxyindoleacetic acid	5-HIAA
5-hydroxytryptamine	5-HT
immunoglobulins	IgA, IgD, IgE, IgG, IgM
inhibitor constant	K_i
inhibitory concentration	IC_{50}
inhibitory postsynaptic potential	i.p.s.p.
insoluble	insol.
international unit	iu
intra-arterial	i.a.
intracellular fluid	ICF
intradermal	i.d.
intramuscular	i.m.
intraperitoneal	i.p.
intracerebroventricular	i.c.v.
intravenous	i.v.
isotope (atomic mass) e.g. iodine-131	^{131}I
isotopically substituted compounds e.g.	$[^{14}C]$-ethanol
laevo-(absolute configuration)	L-
laevo-(optical rotation)	(−)-
lethal dose, median	LD_{50}
leukotriene	LT
logarithm to base e	$\log_e$ or ln
logarithm to base 10	$\log_{10}$
maximum	max.
mean arterial pressure	MAP
mean value of (statistics)	$\bar{x}$
melting point	m.p.
meta	*m*-
Michaelis constant	K_M
minimum	min.
mobility (electrophoresis)	*m*
monoamine oxidase	MAO
noradrenaline	NA
nuclear magnetic resonance	n.m.r.
number	no. or No.
number of observations (statistics)	*n*
ortho	*o*-
packed cell volume	PCV
page/pages	p./pp.
para-	*p*-
paragraph	para. or ¶
parts per million	p.p.m.
per cent	%
platelet activating factor	PAF
posterior	post.
probability (significance level in a statistical test)	P
radioimmunoassay	RIA
rectus (configuration by the sequence rule)	**R**
red blood corpuscle	RBC
relative band speed to front (chromatography)	R_F
relative molecular mass	M_r
relative retention time (gas chromatography)	t_r
renal plasma flow	RPF
resistance (respiratory)	*R*
respiratory conductance	Sgaw
revolutions per minute	r.p.m.
ribonucleic acid	RNA
section	§
sedimentation coefficient (ultracentrifugation)	*s*
sinister (configuration by the sequence rule)	**S**
soluble	sol.
solution	soln.
Spearman rank coefficient	r_s
standard deviation: (of observed sample)	s.d.
standard error (of estimate mean value)	s.e.mean
standard error (of sampling)	s.e.
standard temperature and pressure	STP
subcutaneous	s.c.
sum (statistical): of hypothetical population	Σ
of observed sample	S or Σ
temperature	temp.
thin layer chromatography	t.l.c.
time clock—24 h clock used e.g. 18 h 30 min	*t*
time constant	τ
2-amino-2-hydroxymethyl-propan-1,3-diol	Tris
ultraviolet	u.v.
unit	u
vacuum	vac.
valency	e.g. Fe^{2+}; Fe(II) protoporphyrin
volume by volume	v/v
wavelength	λ
weight	wt.
weight by volume	w/v

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NOMENCLATURE GUIDELINES FOR AUTHORS

With effect from 1 January 1991

The Nomenclature Working Party (NWP) of the Editorial Board of the *British Journal of Pharmacology* has consulted many acknowledged experts in an effort to clarify and standardize receptor and other nomenclature systems for use by Editors until the recommendations of the IUPHAR Commission on Receptor Nomenclature and Classification are made known.

NWP is unanimous in its view that, with rare exceptions, the Journal should use spellings, names and abbreviations that had been chosen by international bodies or specialist groups specially convened for the purpose.

1 *Definition of receptors and subtypes*

In functional studies, pharmacological receptors are to be defined in terms of the relative potencies of agonists and selectivities of antagonists, also by the binding of such ligands, without reference to Second (or other) Messenger Systems.

2 *Format of receptor names**

It was agreed that, until the IUPHAR Commission on Receptor Nomenclature and Classification make their recommendations:

(a) Editors will permit with reluctance new nomenclature systems in papers accepted for publication if and only if there are compelling reasons to introduce a new terminology (or modify an accepted one). The criteria upon which the new receptor type or subtype is defined must be given, together with adequate explanations of the relationship between the previous nomenclature (fully referenced) and the proposed one.

N.B. The new nomenclature should not appear in the Title, Short Title or Key-words, unless qualified by the adjective putative, where appropriate (e.g. ... mediated by the putative β_3-adrenoceptor).

(b) Only well-established and universally accepted subtype names (e.g. muscarinic and nicotinic cholinoceptors; α- and β-adrenoceptors) will be acceptable without any reference to the originator of these terms. In cases of controversy concerning further subdivision of the subtype, full referencing must be given.

(c) Receptor subtypes should be designated by means of a subscript numeral or capital letter. Some double subscripts (i.e. numeral plus letter) have been introduced but, where possible, further introductions should be avoided and must be fully referenced.

* Note on abbreviations: the preferred stype is *capital letters* to designate (a) the first letter of the word (e.g. V, Vasopressin); (b) the first letter of each main syllable or additional word (e.g. NK, neurokinin; GABA, γ-aminobutyric acid; NMDA, N-methyl-D-aspartate). Otherwise, upper and lower case letters should be used (e.g. Enk-IR, enkephalin-like immunoreactivity).

3 *Types of receptor*

(a) *Acetylcholine receptors* (see Cholinoceptors).

(b) *Adrenoceptors* At present, the only adrenoceptor subtypes that should be accepted without a need for very clear definition and full referencing are α_1-, α_2-, β_1- and β_2-adrenoceptors. Reference to either 'atypical' adrenoceptor or the putative β_3-adrenoceptor would be permitted, provided fully referenced.

(c) *Bradykinin receptors* For consistency of style with NK receptors, these should be designated BK_1, BK_2. For the present, possible additional types and subtypes of BK receptors should not be designated, except as discussion points.

(d) *Cholecystokinin (CCK) receptors* The principal subtypes are CCK_A and CCK_B receptors, CCK_B receptors being also known as gastrin receptors.

(e) *Cholinoceptors* The two principal subtypes are muscarinic and nicotinic cholinoceptors (the term acetylcholine receptors is acceptable).

Muscarinic cholinoceptors Until further evidence is forthcoming, the nomenclature should be confined to only three subtypes, namely M_1, M_2 and M_3 cholinoceptors, where M_2 refers to the cardiac subtype and M_3 includes both smooth muscle and glandular subtypes. When the term is used repetitively, muscarinic receptor would be acceptable.

Note that the style m1, m2, etc. refers to nomenclature for molecular structure based on cDNA/genomic cloning.

The abbreviation mAChR and variants are not acceptable.

Nicotinic cholinoceptors The principal subtypes currently accepted are muscle-type and neuronal-type receptors. The abbreviation nAChR and variants are not acceptable.

(f) *Dopamine receptors* Only D_1 and D_2 dopamine receptors are currently recognised. For the present, the possible D_3 subtype must be very clearly defined and fully referenced. There is no need to use DA_1 and DA_2 for peripheral dopamine receptors.

(g) *Excitatory amino acid receptors* At present, no subdivisions of the receptor for N-methyl-D-aspartate (NMDA; see 5 (c) below) are permitted except as discussion points.

Three non-NMDA receptors have been established and are named: (i) Quisqualate- and AMPA-preferring non-NMDA receptors that control cationic channels, to be abbreviated AMPA receptors. (ii) Kainate-preferring non-NMDA receptors, to be abbreviated Kai receptors. (iii) 2-Amino-4-phosphobutyrate receptors (also sometimes known as ABP or AP_4 receptors) to be abbreviated L-AP_4 receptors.

Acceptance of the occurrence of another receptor, described as regulating phosphatidyl inositol pathways, is considered to be premature. The term 'metabotropic receptor' is therefore to be used only as a discussion point.

Otherwise, where appropriate, the term glutamate receptors should be used.

(h) *γ-Aminobutyric acid (GABA) receptors* The principal subtypes are $GABA_A$ and $GABA_B$ receptors. Any other is to be used only as discussion point.

(i) *Histamine receptors* At present, the only histamine receptor subtypes that are acceptable without a need for very clear definition and full referencing are H_1-, H_2 and H_3- although in the latter case, a definition and references are desirable.

(j) *Receptors for 5-hydroxytryptamine* The name 5-hydroxytryptamine (5-HT) is preferred to serotonin (see 4 (b) below). The principal subtypes recognised are 5-$HT_{1A,1B,1C,1D}$, 5-HT_1-like, 5-HT_2 and 5-HT_3. All three should be defined and referenced.

Further putative subtypes may be debated in the Discussion section but, until there is international agreement on the nomenclature, names, such as 5-HT_{1E}, 5-HT_4, are not acceptable, except as discussion points.

(k) *Leukotriene receptors* When first mentioned, the style leukotriene (LT) receptor should be used, thereafter LT receptor. Receptors should be designated according to the leukotriene that selectively or preferentially binds to them.

Editors should be aware that the interpretation of [^{3}H]-LTC_4-binding is hampered by the presence of a binding site for LTC_4 on glutathione S-transferase.

(l) *Neuropeptide Y receptors* Despite some attempts to create subtypes, the view of experts was that no subtypes should be recognised except as discussion points. Full explanations of the basis for proposed subtypes would be required.

(m) *Opioid receptors* The principal subtypes are μ-, δ- and κ-opioid receptors. Other possible subtypes (e.g. ε) are acceptable only as discussion points.

(n) *Oxytocin receptors* (see Vasopressin and oxytocin receptors).

(o) *Prostanoid receptors* The principal types are DP, EP, FP, IP and TP receptors. These should be introduced as prostanoid XP receptors, thereafter simply as XP receptors (where X denotes the type). If subtypes exist, they would be referred to as XP_n, (e.g. EP_1, EP_2, EP_3) receptors.

In the event of possible confusion between a subtype of receptors for prostacyclin IP_3 and a 'receptor' for one of the phosphatidyl inositols (inositol trisphosphate; $InsP_3$), the term prostanoid IP_3 receptor should be used.

(p) *Purinoceptors* The main subtypes permissible are P_1 and P_2. Subdivisions of P_1 into A_1 and A_2 types and of P_2 into P_{2X} and P_{2Y} types are acceptable with appropriate supporting references, provided they are based on agonist potencies and results with antagonists but *not* on activation of particular Second Messenger Systems.

(q) *Tachykinin receptors* Except as discussion points, only the following tachykinin (NK) receptor subtypes are acceptable at present: NK_1, NK_2 and NK_3 and must be fully referenced.

(r) *Vasoactive intestinal peptide (VIP) receptors* Despite some attempts to create subtypes, the view of experts was that no subtypes should be recognised except as discussion points. Full explanations of the basis for proposed subtypes would be required.

(s) *Vasopressin and oxytocin receptors* The principal subtypes are to be designated V_1, V_2, V_3 and OT receptors; V_3 has sometimes been known as V_{1B} but the orignal term V_3 is preferred (see 2(c)).

4 *Naming of nerve fibres*

Many nerve fibres are now known to release more than one transmitter, and future work may show that this is in fact the general rule. In that case, the concept of the same transmitter being released either at different developmental stages or under various experimental conditions would no longer hold, and single adjectives that imply this (e.g. cholinergic, noradrenergic) would become inappropriate when applied to nerve fibres, as distinct from transmitter functions. For the present, those nerve fibres that are known to function by releasing more than one identified transmitter may be described accordingly; for example, noradrenergic-purinergic, cholinergic-peptidergic (in alphabetical order, the order implying no priority of function).

N.B. The suffix 'ergic' should continue to be applied only to nerve fibres and to the transmission event, in accordance with Dale's intentions. For example, 'cholinergic' indicates that the nerve fibre, or the transmission, functions under particular conditions through the release of a choline-like substance. The suffix should not be used loosely to mean 'pertaining to'. Hence, for example, the expression 'cholinergic receptor' (rather than cholinoceptor) is an inappropriate use of the term.

(a) *Catecholamine releasing nerve fibres* The adjective to be applied to nerve fibres that release dopamine as a transmitter is dopaminergic (not DAergic, even in a title).

Nerve fibres that are known to function by releasing noradrenaline are to be described as noradrenergic. The term adrenergic should be reserved for either a nerve fibre that functions by releasing a catecholamine, the identity of which is unknown, or one known to release adrenaline.

(b) *Some other adjectives describing nerve fibre function* NANC is an acceptable abbreviation of non-adrenergic, non-cholinergic for peripheral efferent nerve fibres when the identity of the transmitter(s) is unknown other than the fact that neither (nor)adrenaline nor acetylcholine is involved. It should be defined when introduced. NANCergic, e-NANC (or NANC-e) and i-NANC (or NANC-i) are not acceptable terms.

Glutamatergic, not glutaminergic, should be used to describe nerve fibres releasing glutamate. In referring to peptide-releasing nerve fibres, (e.g. those that may release substance P or vasoactive intestinal peptide), the nomenclature to be used is peptidergic (X), e.g. peptidergic (SP), peptidergic (VIP), not SPergic, VIPergic.

The terms 5-hydroxytryptamine (5-HT) and 5-hydroxytryptaminergic (i.e. nerves releasing 5-hydroxytryptamine) are preferred to those of serotonin and serotoninergic. The term 5-HTergic is not acceptable, except to avoid frequent repetition of 5-hydroxytryptaminergic.

Likewise, the terms purinergic (ATP) and purinergic (adenosine) are preferred.

5 *Other nomenclature requirements*

(a) *Racemates* Authors must state unambiguously in the Methods section of papers which isomers were used, e.g. (+)- or (−)-propranolol, and must bring to the attention of the reader the composite character of drugs that are mixtures of stereo-isomers. Furthermore, the implications of the composite nature of such drugs studied for the interpretation of the data measured and the conclusions drawn must be made explicit. Note that the terms d- or l- for dextro- and laevo-rotatory are now obsolete, and the prefixes (+)- or (−)- respectively should be used. Capital D and L refer to the absolute configurations and of course remain acceptable when appropriate.

(b) *Platelet activating factor (acetyl-glyceryl-ether-phosphorylcholine)* The acronym to be used is PAF (not AGEPC, Paf, Paf-acether or other variant).

(c) *Ligands for NMDA receptors* N-methyl-D-aspartate (NMDA) and N-methyl-DL-aspartate (NMDLA) are to be given in full when introduced in the text.

(d) *Purines* This term should not be used as a synonym for purine nucleotides or nucleosides.

PREPARATION OF MANUSCRIPTS

Authors are strongly recommended to read the full *Instructions to Authors* and *Nomenclature Guidelines for Authors* (*Br. J. Pharmacol.* 1991, **102,** 553–561) before submitting a manuscript for publication in the *British Journal of Pharmacology*. The manuscript and cover letter should be checked against the following list before mailing.

The original and one copy of the manuscript must be supplied. Manuscripts must be typed in double-line spacing on one side of A4 paper, in type not smaller than 12 characters per inch or 10 point. Both copies to include Tables and a set of labelled Figures. One set of Figures without numbers or letters is also to be included. The text to be arranged in the following subsections:

1. **Title**—To have no more than 150 characters on a separate page, which should also include a Short Title (50 characters maximum) and the name and address of the author for correspondence.
2. **Summary**—To be arranged in numbered paragraphs (Full Papers) or a single paragraph (Special Reports).
   —to include aims, principal results and conclusions.
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3. **Introduction**—To contain concise statements of the problem and the aims of the investigation.
4. **Methods**—To have brief but adequate account of the procedures; *full names of drugs (including those referred to by manufacturer's code)*, sources of drugs and statistical tests to be stated.
5. **Results**—To have no repetition of data in Figures, Tables and text.
6. **Discussion**—Findings and conclusions to be placed in context of other relevant work.
   NB Simple repetition of results and unwarranted speculation are not acceptable.
7. **Acknowledgments**—Sources of support. Sources of drugs not widely available commercially.
8. **References**—All references in the text to be included in the Reference List and *vice versa*. References in alphabetical order with complete citations; Journals publishing 'in press' papers identified.
   References to manuscripts submitted to other journals but not yet accepted are not allowed.
9. **Tables**—Each on a separate page and prepared in accordance with current requirements of the Journal.
10. **Figures**—Both labelled and non-labelled Figures to be prepared in accordance with current requirements of the Journal (see *Instructions to Authors*, 1991, vol. **102,** 553–559) and provided with Figure Number and Authors' names on back (*in pencil*)
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11. **Manuscripts**—To be accompanied by a declaration signed by each author that
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