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Dr S J Coker
Department of Pharmacology
University of Liverpool
P.O. Box 147
LIVERPOOL L69 3BX

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SPECIAL REPORT

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Differential effects of hydroxocobalamin on NO-mediated relaxations in rat aorta and anococcygeus muscle

M.A.S. Rajanayagam, C.G. Li & [1]M.J. Rand

Department of Pharmacology, University of Melbourne, Victoria 3052, Australia

In rat aortic rings, hydroxocobalamin (10–30 μM) produced concentration-dependent reductions of the relaxant action of nitric oxide (NO) and the endothelium-dependent, NO-mediated, relaxant action of acetylcholine. In anococcygeus muscles, hydroxocobalamin (10–30 μM) reduced but also prolonged, NO-induced relaxations, but had no effect on non-adrenergic, non-cholinergic-mediated relaxations. Hydroxocobalamin had no effect on the NO-independent relaxant action of papaverine in either tissue. It is suggested that hydroxocobalamin sequesters NO by forming nitrosocobalamin. Nitrosocobalamin did not relax aortic rings, but produced a slowly developing and prolonged relaxation of anococcygeus muscles.

Keywords: Aortic rings (rat); anococcygeus muscle; endothelium-dependent relaxation; hydroxocobalamin; nitrergic transmission; nitric oxide; nitrosocobalamin

Introduction Hydroxocobalamin (vitamin B_{12a}) can be readily converted to nitrosocobalamin (vitamin B_{12c}) in a mild nitrosylating reaction, by use of sodium nitrite and acetic acid (Kaczka *et al.*, 1951). It occurred to us that hydroxocobalamin might sequester nitric oxide (NO) in a biological milieu and thereby inhibit the smooth muscle relaxant action of NO. We tested this hypothesis by studying the effects of hydroxocobalamin on responses to NO and the NO-mediated relaxations produced by acetylcholine (ACh) in endothelium-intact rat aortic rings and non-adrenergic, non-cholinergic (NANC) nerve stimulation in rat anococcygeus muscles, which are thought to be NO-mediated (see Rand, 1992).

Methods Male Sprague-Dawley rats (250–400 g) were killed by decapitation and the thoracic aorta and anococcygeus muscles were removed. Aortic rings of approximately 5 mm in length were mounted under a tension of 2 g in a 10 ml organ bath for isometric recording. In some experiments the endothelium was removed by rubbing the intimal surface of the aortic ring. Relaxations were elicited, during sustained submaximal contractile responses to phenylephrine (1 μM) by the addition of ACh (in rings with intact endothelium), NO or papaverine before or 15 min after exposure to hydroxocobalamin.

The preparation of anococcygeus muscles was as described previously (Li & Rand, 1989). Guanethidine (10–30 μM) was used to raise the tone and relaxations were elicited by field stimulation (1 ms pulses at 1–5 Hz for 10 s), NO or papaverine before and after exposure to hydroxocobalamin. The physiological salt solution (PSS) used for both preparations had the following composition (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 0.45, KH_2PO_4 1.03, $CaCl_2$ 2.5, D-(+)-glucose 11.1, disodium edetate 0.067 and ascorbic acid 0.14. The PSS was gassed with 5% CO_2 in O_2 and maintained at 37°C.

Drugs acetylcholine perchloride (BDH, UK), guanethidine sulphate (Ciba, Australia), hydroxocobalamin acetate (Sigma), nitric oxide gas (Commonwealth Industrial Gases, Melbourne), phenylephrine hydrochloride (Sigma, St. Louis,

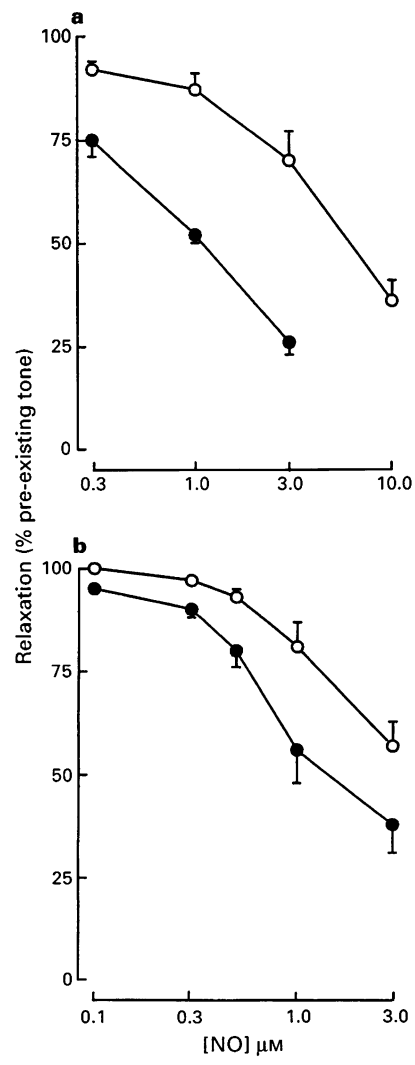


Figure 1 Log concentration-response curves for nitric oxide (NO) in rat aorta (a) and anococcygeus (b) before (●) and after (○) hydroxocobalamin (30 μM). Vertical axis: decreases in tension expressed as a percentage of pre-existing tone. Horizontal axis: concentration of NO in μM. Symbols are means ± s.e.mean (vertical bars).

[1] Author for correspondence.

Mo.) and papaverine hydrochloride (Sigma) were used. NO solutions (2 mM) were made by passing NO gas through de-oxygenated distilled water (Di Iorio, 1981). Nitrosocobalamin was prepared as described by Kaczka *et al.* (1951).

Results *Responses to NO* In rat aortic rings with or without endothelium, and in anococcygeus muscles, hydroxocobalamin produced shifts to the right of the concentration-response curves for NO (Figure 1). In addition, in anococcygeus muscles, the rate of development of relaxation was decreased and the duration was prolonged (Figure 2b).

Responses to papaverine Hydroxocobalamin (30 μM) had no effect on sub-maximal relaxations elicited by 30 and 100 μM papaverine in aortic rings and 100 μM hydroxocobalamin had no effect on relaxations elicited by 0.3 and 1 μM papaverine in anococcygeus muscles (results not shown).

Effects of nitrosocobalamin The presence of a nitroso group in nitrosocobalamin suggests that it might act like a NO-donating nitrovasodilator. It was devoid of relaxant activity in aortic rings in concentrations up to 300 μM, but it produced a slowly developing and prolonged relaxation of anococcygeus muscles (Figure 2c).

Endothelium-dependent relaxant action of ACh Hydroxocobalamin (10 μM) produced a concentration-dependent enhancement of phenylephrine-induced contractions in endothelium-intact but not endothelium-denuded rings. The concentration-response curve for the relaxant action of ACh was shifted to the right. A concentration of 30 μM hydroxocobalamin abolished the responses to ACh (Figure 2a).

NANC nerve stimulation-induced relaxations Hydroxocobalamin (10–30 μM) had no effect on the tone or on the relaxations of the anococcygeus muscle elicited by NANC stimulation at 1–5 Hz (Figure 2b). However, in some experiments with 100 μM hydroxocobalamin, there was a

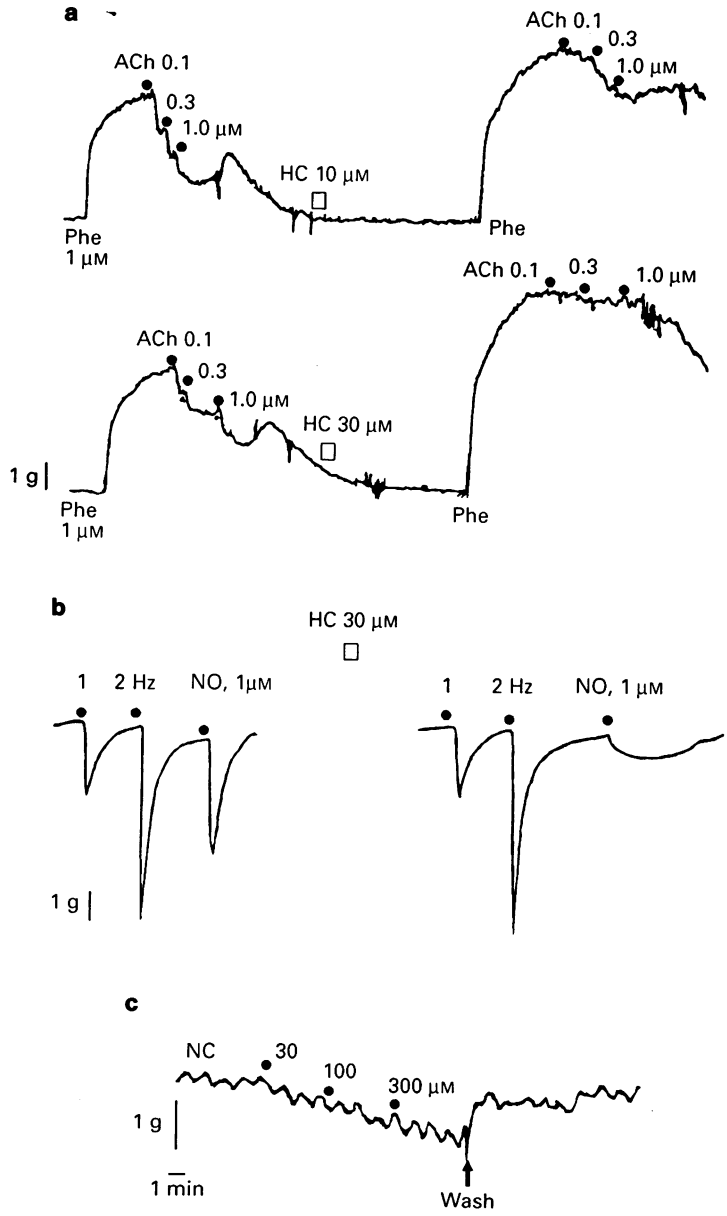


Figure 2 Tracings of responses in (a) endothelium-intact aorta rings: phenylephrine (Phe) and acetylcholine (ACh) before and after hydroxocobalamin (HC, 10 and 30 μM); (b) anococcygeus: non-adrenergic, non-cholinergic nerve stimulation and NO before and after hydroxocobalamin (HC, 30 μM) and (c) anococcygeus: nitrosocobalamin (NC).

slight reduction of the relaxations at the lower frequencies of stimulation.

Discussion Since hydroxocobalamin readily combines with NO to form nitrosocobalamin (Kaczka *et al.*, 1951), we suggest that hydroxocobalamin reduces responses to NO in rat aortic rings by sequestering NO so that the concentration of NO available for activating soluble guanylate cyclase and relaxing the smooth muscle is reduced. The postulated mechanisms of action of hydroxocobalamin is, therefore, like that of haemoglobin which binds NO and thereby reduces the effects of NO and nitrovasodilators in activating guanylate cyclase (Murad *et al.*, 1978) and relaxing vascular smooth muscle (Martin *et al.*, 1985). Since nitrosocobalamin did not produce relaxation of aortic rings, the NO bound in it is not available for activation of the soluble guanylate cyclase in the smooth muscle of this tissue.

Haemoglobin inhibits endothelium-dependent relaxations in vascular preparations (Martin *et al.*, 1985; Ignarro *et al.*, 1987). Likewise, hydroxocobalamin inhibited the endothelium-dependent relaxant action of ACh in aortic rings and, as was reported for haemoglobin (Martin *et al.*, 1985), hydroxocobalamin was more effective in reducing endothelium-dependent than NO-induced relaxations. A possible explanation for this difference is that the total amount of NO generated from ACh-activated endothelial cells is likely to be considerably less than the amount of exogenous NO producing an equivalent relaxation; hence, a given amount of hydroxocobalamin or haemoglobin in the organ bath would more completely mop up the endothelium-derived NO.

Phenylephrine-induced contractions were enhanced by hydroxocobalamin, and this effect has also been reported with haemoglobin (Martin *et al.*, 1985; Khan *et al.*, 1992).

In anococcygeus muscles, hydroxocobalamin reduced the relaxant action of NO but also changed the nature of the relaxation, in that it developed more slowly and was more prolonged. Nitrosocobalamin produced slowly developing and prolonged relaxations in anococcygeus muscles. Therefore it appears that nitrosocobalamin can donate NO to guanylate cyclase in the smooth muscle cells of the anococcygeus.

The concentration of hydroxocobalamin (30 μM) that abolished the endothelium-dependent, NO-mediated, relaxant action of ACh in aortic rings had no effect on NANC nerve stimulation-induced relaxations of the anococcygeus muscle, although there is compelling evidence that the transmitter is NO or a closely related compound (see Rand, 1992). However, haemoglobin blocks NANC transmission in the anococcygeus muscle (Gillespie & Sheng, 1989): the reason for the difference from hydroxocobalamin in this respect is not entirely clear, but one possibility is that nitrosohaemoglobin does not donate NO to guanylate cyclase.

In conclusion, hydroxocobalamin may serve as an additional tool for investigating NO-mediated physiological processes. If it can be established as a general rule that it has a differential action in blocking responses to endothelium-derived but not neurogenic NO, it may help in distinguishing the source of NO in nerve stimulation-induced NO-mediated vasodilatation in such tissues as the salivary gland and corpus cavernosum.

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Protein kinase C inhibitors enhance endothelin-1 and attenuate vasopressin and angiotensin II evoked $[Ca^{2+}]_i$ elevation in the rat cardiomyocyte

Yanjun Xu, L. Sandirasegarane & [1]Venkat Gopalakrishnan

Cardiovascular Risk Factor Reduction Unit (CRFRU), Department of Pharmacology, College of Medicine, University of Saskatchewan, Saskatoon, SK., S7N OWO, Canada

Primary cultures of neonatal rat cardiomyocytes were pretreated for 16 h with either nonselective (staurosporine, 100 nM) or selective (NPC15437, 20 μM) protein kinase C (PKC) inhibitors. These inhibitors did not affect the basal cytosolic free calcium, $[Ca^{2+}]_i$, level (106 ± 12 nM) as determined by fura-2 fluorescence methodology. Both agents significantly enhanced the maximal $[Ca^{2+}]_i$ responses to endothelin-1 (ET-1) and attenuated the peak $[Ca^{2+}]_i$ responses to arginine vasopressin and angiotensin II. They did not alter the EC_{50} values of any of these agonists. Since depletion of $[Ca^{2+}]_o$ led to only partial attenuation of the enhanced response to ET-1 in the treatment groups, it is likely that PKC inhibition results in an exaggerated intracellular mobilization of Ca^{2+} to ET-1. It is concluded that PKC modulates agonist(s)-evoked intracellular Ca^{2+} mobilization and that the nature of regulation is governed by the agonist.

Keywords: Rat cardiomyocyte; fura-2 fluorescence; cytosolic free Ca^{2+}; protein kinase C; staurosporine; vasopressin; angiotensin II; endothelin-1

Introduction Several studies have demonstrated the presence of receptors for vasoactive peptides such as angiotensin II (AII) and endothelin-1 (ET-1) in cardiomyocytes. It was not known if receptors for arginine vasopressin (AVP) were also present on these cells to account for its direct action on the heart. Recently, we have shown that the neonatal rat cardiomyocyte does express V_1 subtype receptors for AVP which are linked to Ca^{2+} mobilization (Xu & Gopalakrishnan, 1991). These receptors are coupled to activation of phosphoinositidase C which stimulates the formation of diacylglycerol, an activator of protein kinase C (PKC). Because PKC could regulate cardiac function, we sought to examine its role in peptide agonist(s)-evoked alterations in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$). Previous studies in non cardiac tissues have utilized phorbol esters which are known to activate PKC (Chardonnens *et al.*, 1990; Simonson & Dunn, 1992; Iijima *et al.*, 1992). However, prolonged incubation with phorbol esters causes downregulation of PKC activity, making studies with these esters difficult to interpret. Therefore, in the present study, we have taken a more direct approach by pretreating the cells with highly potent and selective PKC inhibitors to elucidate the regulatory role of PKC on resting and peptide agonist(s)-evoked alterations $[Ca^{2+}]_i$ using primary cultures of neonatal rat cardiomyocyte.

Methods The methodology for dispersing neonatal rat cardiomyocytes, maintaining these cells in primary culture, fura-2 loading and $[Ca^{2+}]_i$ measurement procedures have been described (Xu & Gopalakrishnan, 1991). In the present study, we added either staurosporine (STS) or 2,6-diamino-N-([1-(1-oxotridecyl)-2piperidinyl]methyl)hexanamide (NPC 15437) to culture flasks so that their final concentrations in the medium were 100 nM and 20 μM respectively. These agents were maintained in culture for 16 h before the experiment. The $[Ca^{2+}]_i$ levels at rest, as well as at the maximal increase evoked by the addition of agonists (AVP, AII and ET-1) were determined. The responses to all the three agonists were tested on the same day with both STS-treated and control groups of cells for comparison. Select experiments for ET-1-evoked increases in $[Ca^{2+}]_i$ were conducted in the absence of extracellular Ca^{2+} with 1 mM EGTA being present in the buffer. Statistical significance of differences between means was estimated by ANOVA.

Materials STS was obtained from Calbiochem (San Diego, CA, U.S.A.). NPC 15437 was a gift from Nova Pharmaceutical Corporation (Baltimore, MD, U.S.A.). Fura-2AM was obtained from Molecular Probes (Eugene, OR, U.S.A.). A stock concentration of STS (1 mM) was prepared in dimethylsulphoxide and subsequent dilutions were made in either culture or incubation medium. AVP, AII and ET-1 were obtained from Peninsula Laboratories (Belmont, CA, U.S.A.).

Results There were no differences in the basal $[Ca^{2+}]_i$ levels between control (106 ± 12 nM) and treated (STS, 113 ± 11 nM; NPC 15437, 103 ± 9 nM) groups. The effect of STS pretreatment on the responses to AVP, AII and ET-1 are shown in Table 1. All three peptides evoked concentration-dependent increases in $[Ca^{2+}]_i$ above the basal levels. The order of potency, as determined by the EC_{50} values, for the evoked maximal $[Ca^{2+}]_i$ values was similar for the three peptides. The EC_{50} values of these agonist(s) were not significantly altered by STS pretreatment. The order of efficacy, as determined by the maximal increase in peak $[Ca^{2+}]_i$ response induced by each agonist, was AII > AVP > ET-1. STS pretreatment led to large reductions ($P < 0.01$) in the evoked maximal $[Ca^{2+}]_i$ responses to both AVP and AII. In contrast, the evoked maximal $[Ca^{2+}]_i$ response to ET-1 was significantly elevated ($P < 0.01$) in the STS-treated cells (Table 1). Removal of $[Ca^{2+}]_o$ led to significant decreases ($P < 0.05$) in the basal $[Ca^{2+}]_i$ values for both control group (from 107 ± 6 to 83 ± 5 nM) and STS (from 115 ± 10 to 87 ± 7 nM) pretreated cells. However, no significant differences were observed in the percentage increases in peak $[Ca^{2+}]_i$ (above the basal) values to ET-1 (25 nM) either in the presence (239 ± 15%) or absence

[1] Author for correspondence.

Table 1 Analyses of the effect of staurosporine (STS) pretreatment (100 nM) on arginine vasopressin (AVP), angiotensin II (AII) and endothelin-1 (ET-1)-evoked cytosolic free $[Ca^{2+}]$ increase in neonatal rat cardiomyocyte (25°C)

Agonists	EC_{50} (nM) Control	STS	$[Ca^{2+}]_i$ – Control	E_{max} (nM) STS
AVP	10 ± 2	12 ± 3	245 ± 30	112 ± 5**
AII	12 ± 4	9 ± 2	960 ± 75	265 ± 16**
ET-1	7 ± 1	11 ± 4	175 ± 11	298 ± 21**

Each value is mean ± s.e.mean of 7 separate determinations. Basal $[Ca^{2+}]_i$ values (in the absence of agonist stimulation) were not significantly different between control (106 ± 12 nM) and STS (113 ± 11 nM) pretreated cells. **$P<0.01$ compared to control values.

(219 ± 11%) of $[Ca^{2+}]_o$. NPC 15437 (20 μM) pretreatment also resulted in a qualitatively similar reduction in responses to AVP and AII and a significant increase in the $[Ca^{2+}]_i$ response to ET-1 (Figure 1).

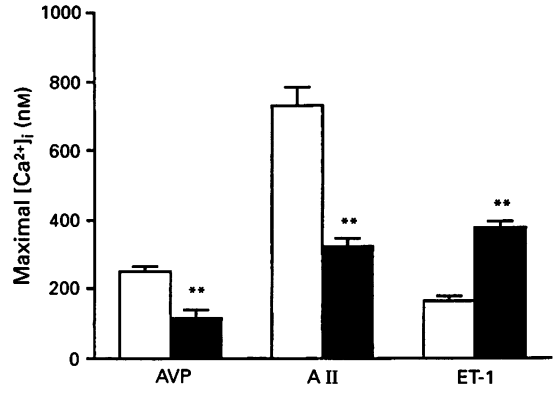


Figure 1 The comparison of peak $[Ca^{2+}]_i$ response to a fixed concentration of 25 nM of arginine vasopressin (AVP), angiotensin II (AII), or endothelin-1 (ET-1) in the dispersed neonatal rat cardiomyocyte in the presence (■) or in the absence (□) of the protein kinase C (PKC) inhibitor, NPC-15437 (20 μM). Each column represents mean ± s.e.mean (vertical bars) of 5 separate experiments performed with different batches of cardiomyocytes. Basal $[Ca^{2+}]_i$ values (in the absence of agonist stimulation) were not significantly different between control group (106 ± 12 nM) and NPC (103 ± 9 nM) pretreated cells. There was a residual response of 20 ± 4 nM increase above the basal $[Ca^{2+}]_i$ values for AVP in the NPC pretreated group.
**Denotes $P<0.01$ compared to control group.

Discussion In addition to comparing the $[Ca^{2+}]_i$ responses to AVP, AII and ET-1 in the neonatal rat cardiomyocytes for the first time, this study demonstrates a qualitatively differential effect of inhibition of PKC on the responses to these peptides: the inhibitors of PKC, STS and NPC 15437, enhanced $[Ca^{2+}]_i$ response to ET-1 and attenuated the responses to AVP and AII. STS is a highly potent but nonselective inhibitor of several kinases and binds to their catalytic moiety. Although several studies in the past have used this agent to elucidate the role of PKC, its selectivity in blocking PKC has recently been questioned (Kageyama *et al.*, 1991). In order to address this issue, we also used a selective inhibitor of PKC, NPC 15437, which blocks the regulatory subunit of PKC, a site at which diacylglycerol binds to activate the enzyme (Sullivan *et al.*, 1992). Neither STS nor NPC 15437 pretreatment *per se* affected fura-2 fluorescence. The observation that similar results were obtained with two inhibitors of PKC that act on different sites of the enzyme adds credibility to the conclusion that the altered responses to the peptide agonists were a consequence of inhibition of PKC.

The $[Ca^{2+}]_i$ response to ET-1 in the presence and absence of inhibition of PKC has not been reported previously for cardiomyocytes. In vascular smooth muscle tissue, ET-1-evoked vasoconstriction was reversed by H-7, a nonselective PKC inhibitor (Sugiura *et al.*, 1989). An increase in the prolonged secondary phase of Ca^{2+} mobilization was demonstrated previously with a fixed high concentration of ET-1 in either STS or phorbol ester pretreated rat mesangial single cells (Iijima *et al.*, 1991; Simonson & Dunn, 1991). Neither of these studies in non-cardiac tissue examined the role of $[Ca^{2+}]_o$. The present study in cardiomyocytes has shown that $[Ca^{2+}]_i$ response to ET-1 in the STS group was attenuated only to a small extent by removal of $[Ca^{2+}]_o$. Therefore, it is likely that the exaggerated $[Ca^{2+}]_i$ response to ET-1 was due primarily to mobilization of Ca^{2+} from intracellular stores. Recently, it has been shown that enhanced PKC activity and reduced receptor density for ET-1 seen in diabetic renal glomerular mesangial cells could be normalized by the inclusion of a PKC inhibitor (Awazu *et al.*, 1991). Thus, on the basis of our results in cardiomyocytes, it seems reasonable to suggest that cell surface ET-1 receptor is tonically downregulated by PKC and that STS or NPC 15437 abolishes this negative feed back modulation by PKC thereby resulting in an exaggerated $[Ca^{2+}]_i$ response to ET-1.

The observation of diminished $[Ca^{2+}]_i$ responses to both AVP and AII in the cells pretreated with PKC inhibitors is consistent with the previous report of decreased $[Ca^{2+}]_i$ responses to these two agonists when PKC is downregulated by prolonged incubation with tumour promoting phorbol ester (Chardonnens *et al.*, 1990). However, the mechanism underlying the diminished response to these agonists needs further study.

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Nitric oxide mediates the inhibition by interleukin-1β of pentagastrin-stimulated rat gastric acid secretion

[1]Juan V. Esplugues, M. Dolores Barrachina, Sara Calatayud, *Josep M. Pique & †Brendan J.R. Whittle

Department of Pharmacology, University of Valencia, Valencia, Spain; *Gastroenterology Unit, Hospital Clínic, Barcelona, Spain and †Wellcome Research Laboratories, Beckenham, Kent

Bolus injection of interleukin-1β (2 μg kg^{-1}, i.v.) inhibited acid secretion induced by intravenous infusion of pentagastrin (8 μg kg^{-1} h^{-1}) in the continuously perfused stomach of the anaesthetized rat. Administration of interleukin-1β did not modify mean systemic arterial blood pressure. Pretreatment with N^{G}-nitro-L-arginine methyl ester (L-NAME, 2–10 mg kg^{-1}, i.v.), but not dexamethasone (5 mg kg^{-1}, s.c. twice over 16 h), restored the acid secretory responses to pentagastrin. The actions of L-NAME were reversed by the prior administration of L-arginine (100 mg kg^{-1}, i.v.), but not by its enantiomer D-arginine (100 mg kg^{-1}, i.v.). L-NAME (5 mg kg^{-1}, i.v.) increased blood pressure but this was not the mechanism by which interleukin-induced acid inhibition was prevented, since similar systemic pressor responses induced by phenylephrine (10 μg kg^{-1} min^{-1}, i.v.), had no such effect. These findings suggest that interleukin-induced inhibition of acid responses to pentagastrin involves synthesis of NO from L-arginine.

Keywords: Gastric acid; interleukin-1β; cytokines; endotoxin; NO; pentagastrin

Introduction Nitric oxide (NO) is formed from L-arginine by a calcium-dependent constitutive NO synthase in a variety of cell types, including the endothelium and neural tissue (Moncada *et al.*, 1991). The induction of a calcium-independent NO synthase is thought to play a key role in the vascular events and cellular injury triggered after several hours by endotoxin (Moncada *et al.*, 1991), while NO can also attenuate the acute vascular damage induced by endotoxin in the gastrointestinal mucosa (Hutcheson *et al.*, 1990). It is well established that endotoxin can stimulate the release of the various cytokines (Dinarello, 1988) which modulate several of the early responses to infection, as well as inducing the expression of a calcium-independent NO synthase in vascular and immune cells (see Moncada *et al.*, 1991). Furthermore, the cytokine interleukin-1β (IL-1β) has been shown to inhibit basal (Uehara *et al.*, 1989; Saperas *et al.*, 1990) and pentagastrin-stimulated (Wallace *et al.*, 1991) acid secretion in the rat. In the present study, we have now investigated by the use of the NO synthase inhibitor, N^{G}-nitro-L-arginine methyl ester (L-NAME, Rees *et al.*, 1990), the role of endogenous NO in the inhibitory effects of IL-1β on pentagastrin-stimulated gastric acid secretion.

Methods Wistar rats of either sex (180–250 g) were fasted for 24 h but allowed drinking water. Under urethane anaesthesia (1.5 g kg^{-1}, i.p.) the trachea was intubated, the jugular vein cannulated and, in some experiments, systemic arterial blood pressure measured from a carotid artery. The gastric lumen was perfused (0.9 ml min^{-1}) with isotonic saline and H^+ output determined at 20 min intervals by automatic titration. IL-1β (2 μg kg^{-1}, i.v.) was administered once acid output stimulated by the i.v. infusion of pentagastrin had remained constant for 120 min, with acid output being determined over the following 120 min and expressed as μEq H^+ per 100 g body weight. Inhibition by IL-1β of acid secretion and the effects of L-NAME, L- and D-arginine were calculated relative to values of acid output stimulated by pentagastrin in each animal before the administration of these compounds or vehicle. In further studies, phenylephrine was co-infused with pentagastrin for the duration of the experiment at a dose (10 μg kg^{-1} min^{-1}) that provided a similar increase in blood pressure to that produced by L-NAME (5 mg kg^{-1}).

Recombinant mouse IL-1β (specific activity of 3.5×10^5 units mg^{-1}) was obtained from Genzyme Corporation (Cambridge, MA, U.S.A.). L-NAME, L-arginine, D-arginine and phenylephrine were from Sigma Chemical (St Louis, MO, U.S.A.). These and pentagastrin (Peptavlon, ICI, UK) and dexamethasone (Fortecortin, Merck, Germany) were dissolved in saline immediately before use and administered in a volume of 1 ml kg^{-1}. Data are expressed as mean ± s.e.mean. Comparisons between groups were made by Student's *t* test for unpaired data, except for blood pressure studies in which Student's *t* test for paired data was used. Differences were considered significant at $P<0.05$.

Results The intravenous infusion of pentagastrin (8 μg kg^{-1} h^{-1}) induced a stable submaximal plateau rate of acid output, which in control rats ($n = 42$) remained between 9 and 11 μEq H^+ 100 g^{-1} 20 min^{-1} for the duration (240 min) of the experiments. As shown in Figure 1, IL-1β administration (2 μg kg^{-1}, i.v., $n = 13$) following 2 h of acid stimulation substantially inhibited ($P<0.001$) the subsequent acid response to pentagastrin. This near-maximal dose of IL-1β, which was chosen from preliminary dose-response studies, did not produce any changes in systemic arterial blood pressure which remained between 95 and 105 mmHg ($n = 4$). Prior administration of dexamethasone (5 mg kg^{-1} s.c., 16 and 4 h before study) did not significantly modify the inhibitory effects of IL-1β on gastric acid secretion (80 ± 6% inhibition by IL-1β, $n = 4$). Pretreatment (i.v.) with 2, 5 and 10 mg kg^{-1} of L-NAME ($n = 4$, 7 and 9 respectively) dose-dependently restored the secretory responses to pentagastrin in IL-treated rats (Figure 1). The reversal by L-NAME of the IL-1β inhibition of acid output was prevented by the administration of L-arginine ($n = 6$, Figure 1), but not by its enantiomer D-arginine (30 ± 6% inhibition by IL-1β, $n = 14$). In control animals, pretreatment with L-NAME (5 mg kg^{-1}) did not significantly modify the secretory response to pentagastrin ($n = 11$), while L-arginine or D-arginine likewise had no significant effect ($n = 4$ for both).

The initial resting blood pressure was significantly

[1] Author for correspondence at Dept. of Pharmacology, Faculty of Medicine, Adv. Blasco Ibañez 15, 46010 Valencia, Spain.

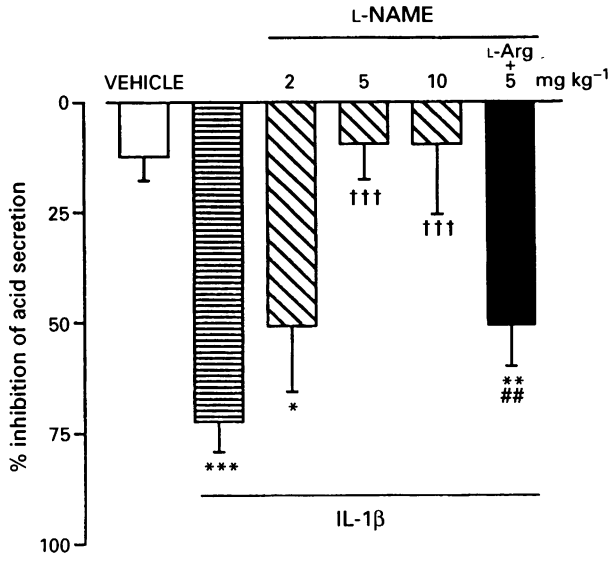


Figure 1 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) (2,5 and 10 mg kg^{-1}, i.v., ▨) on the inhibitory actions of interleukin-1β (IL-1β,2 μg kg^{-1},i.v., ▤) on gastric acid secretion induced by the intravenous infusion of pentagastrin (8 μg kg^{-1} h^{-1}) in the anaesthetized rat. In animals treated with L-NAME (5 mg kg^{-1}) the administration (i.v.) of L-arginine (100 mg kg^{-1}, ■) restored the inhibitory effects of IL-1β on gastric acid secretion. Each column shows mean ± s.e.mean of at least 4 experiments, with % inhibition of acid secretion being calculated relative to values of acid production stimulated by pentagastrin in each animal during an equivalent period of time (120 min) before the administration of IL-1β, or vehicle (□).
Significant difference from the group receiving vehicle is shown as $^*P<0.05$; $^{**}P<0.01$ and $^{***}P<0.001$; from the group receiving IL-1β only as $^{\dagger\dagger\dagger}P<0.001$, and from the group treated with the combination of IL-1β + L-NAME (5 mg kg^{-1}) as $^{\#\#}P<0.01$.

($P<0.01$) increased from 106 ± 11 to 155 ± 7 mmHg by L-NAME (5 mg kg^{-1}, i.v., $n = 4$). Subsequent administration of IL-1β (2 μg kg^{-1}) did not modify the hypertensive effects of L-NAME (143 ± 6 mmHg). In rats treated with IL-1β, the i.v. infusion of phenylephrine (10 μg^{-1} kg^{-1} min^{-1}, $n = 4$) produced a sustained increase in BP, from 104 ± 4 to 133 ± 3 mmHg ($P<0.01$) but did not prevent the inhibition by IL-1β of pentagastrin-stimulated acid output (79 ± 11% inhibition by IL-1β, $n = 4$).

Discussion The present results demonstrate that the NO-synthase inhibitor, L-NAME reverses the suppression by IL-1β of pentagastrin-stimulated gastric acid secretion. These effects of L-NAME were prevented by L-arginine but not by its enantiomer D-arginine, thus indicating the involvement of NO synthesis from L-arginine. However, the observation that administration of L-NAME in control, vehicle-treated animals did not affect the acid secretion stimulated by pentagastrin supports our previous studies using N^G-monomethyl-L-arginine and suggests that NO does not directly modulate H^+ secretion under such conditions (Pique *et al.*, 1992). The restoration of the acid secretory responses by L-NAME is unlikely to be the consequence of its systemic vasopressor actions since phenylephrine, infused at a dose producing a similar vasopressor response as L-NAME, did not modify the acid-inhibitory effects of IL-1β. Furthermore, IL-1β did not produce any significant fall in blood pressure during the infusion of pentagastrin, which suggests an action on gastric acid output independent of any direct vascular changes induced by this cytokine.

It has been demonstrated that the induction of NO-synthase is responsible for the increased vascular synthesis of NO that occurs some 3–6 h after exposure to endotoxin and cytokines, and its expression is inhibited by glucocorticoids (Radomski *et al.*, 1990). It is therefore unlikely that such an inducible enzyme is involved in the inhibitory action of IL-1β on acid secretion since this rapidly occurs within 120 min and, moreover, it is not influenced by pretreatment with dexamethasone in a dose sufficient to prevent such induction (Moncada *et al.*, 1991). Thus, the present results suggest the involvement of an acute release or action of NO in the gastric inhibitory response to IL-1β, but the cellular origin of such NO is not known. If IL-1β inhibits pentagastrin-stimulation by preventing mucosal histamine release (Wallace *et al.*, 1991) this process may thus involve NO. In addition, NO may also influence the release of other endogenous mediators or be involved in the modulation of neuronal mechanisms evoked by IL-1β that could influence gastric acid secretion.

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SPECIAL REPORT

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The non-peptide neurokinin$_1$ receptor antagonist, RP 67580, blocks neurogenic plasma extravasation in the dura mater of rats

Sara L. Shepheard, David J. Williamson, Raymond G. Hill & Richard J. Hargreaves

Department of Pharmacology, Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Harlow, Essex

A non-peptide neurokinin$_1$ (NK_1) receptor antagonist, RP 67580, that is selective for the rodent subtype of the NK_1 receptor, dose-dependently reduced plasma extravasation in the dura mater produced by electrical stimulation of the trigeminal ganglion in rats, with an ID_{50} of 0.6 $\mu g\,kg^{-1}$. Its enantiomer RP 68651 was some 400 fold less active. The results indicate that neurogenic plasma extravasation within the dura mater is NK_1 receptor-mediated and suggest that NK_1 receptor antagonists may have a role as antimigraine agents.

Keywords: Neurokinin$_1$ receptor extravasation; trigeminal ganglion; migraine

Introduction Neurogenic inflammation within cranial tissues has been proposed as a mechanism in the pathogenesis of migraine (Moskowitz, 1992). The dura mater, a pain-producing cranial structure, and its blood vessels are innervated by the trigeminal and upper cervical sensory nerves. Neurogenic plasma extravasation mediated presumably through the release of sensory neuropeptides e.g. substance P and calcitonin gene-related peptide, has been shown to develop within the dura and other receptive fields (lip, conjunctiva, eyelid) of trigeminal sensory nerve fibres upon electrical stimulation of the trigeminal ganglion. It has previously been shown that ergot alkaloids, with clinical effectiveness against migraine, block neurogenic extravasation within the dura evoked by electrical stimulation of the trigeminal ganglion (Saito *et al.*, 1988). Recently sumatriptan, a 5-HT_{1D} receptor agonist with proven anti-migraine activity, has been shown to have similar effects in the trigeminal stimulation model (Buzzi & Moskowitz, 1990) and it was suggested that this was due to inhibition of sensory neuropeptide release. Fardin *et al.* (1992) have described a non-peptide NK_1 receptor antagonist, RP 67580 ((3a**R**, 7a**R**)-7, 7-diphenyl-2(1-imino-2-(2-methoxyphenyl)ethyl)perhydroisindole) which is selective for rodent NK_1 receptors whilst its (3a**S**, 7a**S**) enantiomer, RP 68651, is much less active. These enantiomers, synthesized in our laboratories, had IC_{50}s against [^{125}I]-Tyr^8-substance P on cloned rat NK_1 receptors expressed in CHO cells of 4.5 nM for RP 67580 and >3 μM for RP 68651 (Cascieri; personal communication). Using RP 67580 and RP 68651 we have investigated the role of NK_1 receptors in mediating neurogenic plasma extravasation in the dura and extracranial tissues of rats following trigeminal ganglion stimulation.

Methods Male Sprague-Dawley rats (approx 200 g) were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p.) and a femoral vein and artery were cannulated for intravenous injections and the monitoring of arterial blood pressure respectively. Animals were placed in a stereotaxic frame and burr holes drilled in the skull bilaterally 3.2 mm posterior and 2.5 mm lateral from bregma to allow for placement of stimulating electrodes. After intravenous administration of either RP 67580 (0.1–1000 $\mu g\,kg^{-1}$), RP 68651 (300 $\mu g\,kg^{-1}$) or distilled water vehicle ($n = 8$–10 per dose group), concentric bipolar stimulating electrodes were lowered 9 mm below the surface of the dura bilaterally into the trigeminal ganglia. ^{125}I-radiolabelled bovine serum albumin (70 μCi kg^{-1}) was then given intravenously as a plasma marker. At 10 min after the drug or vehicle injection, the right trigeminal ganglion only was electrically stimulated (5 Hz, 5 ms, 25 V for 5 min, giving a maintained stimulus current in the range of 1 to 2.5 mA measured by voltage drop across a resistance). At the end of the stimulation period the animals were left for 5 min before being exsanguinated prior to dissection of the dura from both sides of the skull. The area of dura immediately surrounding the sites of electrode penetration was discarded. Samples of extracranial tissues (conjunctiva, eyelid and lip) innervated by the trigeminal nerves were also taken. Tissues were rinsed, dried overnight, weighed, then counted for radioactivity. The counts mg^{-1} dry weight of tissue in the stimulated and unstimulated sides were calculated and results are expressed as an extravasation ratio, i.e. the ratio of the stimulated to unstimulated sides. For example, the mean c.p.m. mg^{-1} in the dura of stimulated and unstimulated sides were 455 and 220 respectively giving a ratio of 2.1. Differences were assessed by ANOVA and a Dunnett's t test.

Results Following electrical stimulation of the trigeminal ganglion in vehicle-treated animals, the extravasation ratio in dura, conjunctiva, eyelid and lip was 2.1 ± 0.1, 1.6 ± 0.2, 7.6 ± 1.5 and 7.7 ± 1.2 respectively ($n = 10$, values are mean ± s.e.mean). Pretreatment with RP 67580 produced dose-related reductions in the ratio of extravasation within the dura that were significant ($P < 0.05$) at doses of 10 $\mu g\,kg^{-1}$ and greater when compared to vehicle pretreatment (Figure 1). An inhibition curve fitted to these data using non-linear least squares analysis (RS1) gave an ID_{50} (i.e. dose of RP 67580 producing half maximal inhibition of extravasation) of 0.6 $\mu g\,kg^{-1}$. RP 67580 reduced extravasation in the facial tissues (conjunctiva, eyelid and lip) only at doses of 100 $\mu g\,kg^{-1}$ or more. A comparison of the effects of RP 67580 at a dose of 10 $\mu g\,kg^{-1}$ on the dura and the facial tissues (Figure 1) shows that the extravasation in the dura is almost completely blocked by this dose, but there is little effect on the extravasation in the facial tissues. Thus at low doses (<100 $\mu g\,kg^{-1}$) RP 67580 appears relatively selective in reducing extravasation in the dura over the facial tissues, but at higher doses it loses this apparent tissue selectivity. Pretreatment with RP 68651, the less active (3a**S**, 7a**S**) enantiomer of RP 67850, at a dose of 300 $\mu g\,kg^{-1}$, i.v. gave an extravasation ratio of 1.53 ± 0.1 ($n = 7$) in the dura and had no effect in the facial tissues. In all experiments the mean arterial blood pressure after dosing with vehicle or antagonist was no different at the point of trigeminal ganglion stimulation from that before dosing.

Discussion In the present experiments the NK_1 receptor-selective antagonist, RP 67580, was some 400 fold more active than RP 68651, demonstrating a highly effective (ID_{50} = 0.6 μg kg^{-1}) and enantiomerically selective inhibition of plasma extravasation in the dura of the rat. This indicates that plasma extravasation in the dura after electrical stimulation of trigeminal sensory afferent fibres is predominantly NK_1 receptor-mediated and is likely to be due to substance P release, as this peptide is the endogenous ligand that acts mainly at the NK_1 receptor. The present result contrasts with that of Buzzi and colleagues (cited as unpublished observations, Buzzi *et al.*, 1992) who suggested that NK_1 antagonists have poor activity in this rodent model, although the NK_1 antagonist, CP-96,345 used in their experiments has selectivity for the human NK_1 receptor subtype over the rodent subtype (Beresford *et al.*, 1991; Fardin *et al.*, 1992).

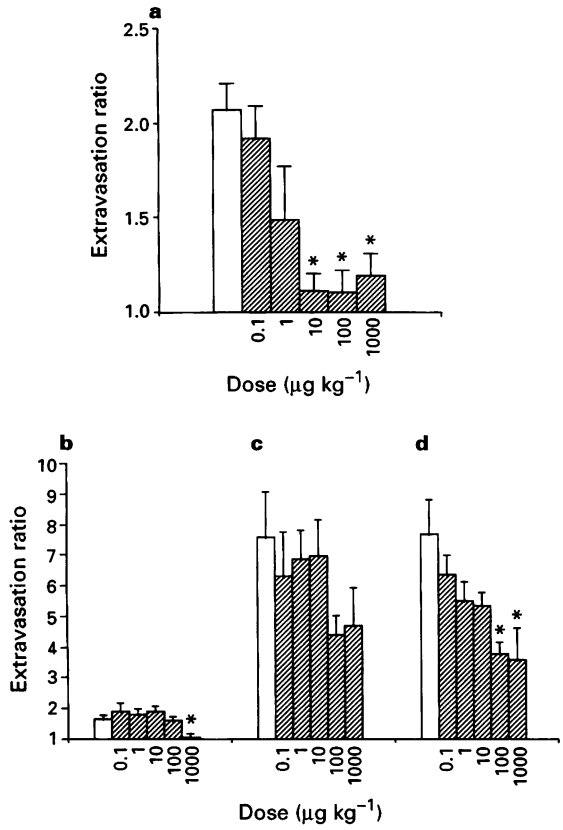


Figure 1 Histograms illustrating the effects of RP 67580 on plasma extravasation produced in dura (a) and facial tissues (conjunctiva (b), eyelid (c), lip (d)) by electrical stimulation of the trigeminal ganglion in the rat. Data are expressed as an extravasation ratio, i.e. ratio of extravasation in stimulated/unstimulated sides. Open column = vehicle (distilled H_2O); hatched column = RP 67580, (0.1, 1.0, 10, 100, or 1000 μg kg^{-1} i.v., $n = 8-10$). Statistical analysis was performed by ANOVA and a Dunnett's test; $*P < 0.05$, vertical bars = s.e.mean.

RP 67580 shows significant inhibition of dural extravasation at doses that have no effect on cardiovascular parameters and thus it is unlikely that the reduction in extravasation seen with this compound is due to any reduction in tissue perfusion pressure. The apparent dural selectivity of RP 67580 over the facial tissues has been observed previously by Buzzi & Moskowitz (1990) with the 5-HT_{1D} agonist, sumatriptan, which markedly inhibited the extravasation in the dura at a dose of 100 μg kg^{-1} but had no effect on extravasation in extracranial tissues at a dose of 300 μg kg^{-1}. The reasons for this selectivity are as yet unknown, but may reflect differences in the physical limits imposed on extravasation in the different tissue types, or differences in the receptor mediated stimulus-response mechanisms.

This study has shown that RP 67580 is more active (maximal inhibition at 10 μg kg^{-1}) at this rodent trigeminal model of migraine than ergotamine tartrate and dihydroergotamine (100 and 50 μg kg^{-1} respectively, Saito *et al.*, 1988) or sumatriptan (100 μg kg^{-1}, Buzzi & Moskowitz, 1990). Clinical studies have shown that the ergot alkaloids and the $5HT_{1D}$ agonist, sumatriptan, are effective anti-migraine agents. The present studies therefore indicate that NK_1 receptor antagonists could also prove effective in the treatment of migraine headache.

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Functional characterization of a 5-HT_3 receptor which modulates the release of 5-HT in the guinea-pig brain

[1]Pierre Blier & Claude Bouchard

Neurobiological Psychiatry Unit, McGill University, 1033 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1

1 The aims of the present study were to confirm the modulation by 5-HT_3 receptors of the electrically evoked release of tritium from slices preloaded with [^{3}H]-5-HT of guinea-pig frontal cortex, hippocampus and hypothalamus, and to assess their functional role in 5-HT release.

2 The selective 5-HT_3 agonist, 2-methyl-5-HT, introduced 8 min before the electrical stimulation, enhanced in a concentration-dependent manner the evoked release of [^{3}H]-5-HT in the three brain regions studied. The 5-HT_3 agonists, phenylbiguanide and *m*-chlorophenyl-biguanide, did not enhance the release of tritium in frontal cortex and hypothalamus slices.

3 In hypothalamus slices, this response was lost when 2-methyl-5-HT was introduced 20 min before the stimulation, thus indicating that these 5-HT_3 receptors desensitize rapidly. When 2-methyl-5-HT was added 20-min before the first stimulation period to desensitize the 5-HT_3 receptors, removed for 24 min, and then re-introduced 8 min before the second stimulation period, the enhancing effect of 2-methyl-5-HT was restored, thus indicating that these 5-HT_3 receptors can rapidly regain normal sensitivity.

4 The enhancing effect of 2-methyl-5-HT was attenuated by the 5-HT_3 receptor antagonists *m*-chlorophenylpiperazine = quipazine = ondansetron $\geq$ ICS 205-930 = BRL 24924 > MDL 72222 = zacopride.

5 The 5-HT reuptake blocker, paroxetine, enhanced the electrically evoked release of tritium when introduced 8 min before stimulation; this effect of paroxetine was blocked by ICS 205-930, thus indicating that these 5-HT_3 receptors can be activated by endogenous 5-HT.

6 In the absence of electrical stimulation, 2-methyl-5-HT (10 μM) produced a marked enhancement of the basal release of [^{3}H]-5-HT which was calcium-dependent and blocked by S-zacopride but not by paroxetine.

7 The enhancing effect of 2-methyl-5-HT was dependent both on the frequency of stimulation, as indicated by the attenuated effect of 120 stimulations delivered at 1 Hz instead of 5 Hz, and on the duration of the stimulation, as indicated by the more pronounced effect of pulses delivered at 5 Hz for 24 s instead of 72 s or 120 s.

Keywords: 5-HT_3 receptors; [^{3}H]-5-HT release; presynaptic modulation; desensitization; 2-methyl-5-HT; ondansetron; ICS 205-930; zacopride; paroxetine; *m*-chloro-phenylpiperazine (*m*CPP)

Introduction

5-Hydroxytryptamine (5-HT) receptors are now divided into four main classes. Each class is defined by several pharmacological criteria. For instance, whereas 5-HT_1 receptors display nanomolar affinity for 5-HT itself, the 5-HT_2, 5-HT_3 and 5-HT_4 receptors have micromolar affinity for their endogenous neurotransmitter. Molecular biology has now established that the multiple 5-HT binding sites do not merely represent pharmacological artifacts since 5-HT_1, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, and 5-HT_{1D}, as well as 5-HT_2 and 5-HT_3 receptors have now been cloned (Maricq *et al.*, 1991; Weinshanck *et al.*, 1992). Most of these receptors have been shown to be coupled to second messenger systems or to ion channels via G proteins, with two possible exceptions: 5-HT_{1B} autoreceptors located on 5-HT terminals in the rat brain, which exert a negative feedback control on 5-HT release, and 5-HT_3 receptors (Blier, 1991; Hoyer & Neijt, 1988). The 5-HT_3 receptors are coupled directly to an ion channel permeable to sodium and potassium (Derkach *et al.*, 1989). Another characteristic of 5-HT_3 receptors is their rapid desensitization following their activation (see Hoyer, 1990 for review).

Activation of 5-HT_3 receptors has been demonstrated to modulate the release of various neurotransmitters in the brain. The selective 5-HT_3 agonist, 2-methyl-5-HT, was shown to increase the potassium-evoked release of [^{3}H]-noradrenaline from preloaded slices of rabbit hippocampus (Feuerstein & Hertting, 1986). This same 5-HT_3 agonist decreases the potassium-evoked release of [^{3}H]-acetylcholine in slices of the rat cerebral cortex (Barnes *et al.*, 1989). Both in *in vitro* and *in vivo* paradigms, the activation of 5-HT_3 receptors results in an increase of the release of dopamine in the rat brain (Blandina *et al.*, 1989; Chen *et al.*, 1991). The activation of 5-HT_3 receptors enhances the release of cholecystokinin in synaptosomes prepared from the rat nucleus accumbens and cerebral cortex (Paundice & Raiteri, 1991). Finally, Galzin *et al.* (1990) reported on the capacity of 5-HT_3 receptors to increase the electrically evoked release of tritium in guinea-pig frontal cortex slices preloaded with [^{3}H]-5-HT_3. The present study was thus undertaken first to confirm the presence of these 5-HT_3 receptors in three regions of the guinea-pig limbic system, namely the frontal cortex, the hippocampus and the hypothalamus, and, second, to assess their physiological relevance for 5-HT release.

Methods

Male guinea-pigs (300–400 g) were killed by decapitation and their brains rapidly removed and dissected on an ice-cold glass plate. Slices from the hypothalamus, hippocampus, or frontal cortex of 0.4 mm thickness were prepared with a McIlwain chopper and incubated for 30 min at 37°C in Krebs

[1] Author for correspondence.

solution containing 0.02 μM [^{3}H]-5-HT creatinine sulphate (specific activity 914 GBq mmol^{-1}; NEN Research Products, Mississauga, Canada) and bubbled with a mixture of 95% O_2:5% CO_2. The composition of the Krebs solution in mmol l^{-1} concentration was: NaCl 118, KCl 4.7, $CaCl_2$ 1.3, $MgCl_2$ 1.2, NaH_2PO_4 1, $NaHCO_3$ 25, glucose 11.1, Na_2EDTA 0.004 and ascorbic acid 0.11. At the end of the incubation period with [^{3}H]-5-HT, one hypothalamic slice or three slices of the hippocampus or two slices of frontal cortex were transferred into each of the glass chambers and superfused continuously at a rate of 0.5 ml min^{-1} with Krebs solution maintained at 37°C and saturated with O_2/CO_2. Nineteen consecutive 4-min fractions were collected starting 52, 60 or 90 min after the beginning of superfusion for the hypothalamus, hippocampus, and frontal cortex slices, respectively. The slices were stimulated twice 8 min (S_1) and 44 (S_2) min after the end of the washing period. The electrical field generated in the chambers between two platinum electrodes (2 cm apart) had a voltage drop of about 5 V cm^{-1}. The following stimulation parameters were generally used to stimulate hypothalamic slices: 20 mA, 2 ms, 3 Hz for 2 min. In some experiments, the pulses were delivered at 1 Hz for 6 min or 5 Hz for 72 s in order to deliver the same number of pulses (360) per stimulation period as in the 3 Hz/2 min condition. In the experiments carried out with hippocampus and frontal cortex slices, the following parameters were used: 30 mA, 2 ms, 3 Hz for 2 min. These frequencies of stimulation were chosen because they are within the range of the firing rate of 5-HT neurones recorded in freely-moving cats (Jacobs, 1985). The first stimulation period (S_1) was always used as control and the putative antagonists were added 20 min before S_1 and remained present thereafter, whereas the other drugs were added 8 or 20 min before S_2 and also remained present until the end of the superfusion. At the end of the experiments, the slices were solubilized in 0.5 ml of Soluene 350 (Packard Instruments, Downers Grove, Il, U.S.A.) and the radioactivity in the slices and superfusate samples was determined by liquid scintillation spectometry. The amount of tritium released per 4-min sample was expressed as a fraction of the total tissue tritium content at the start of the respective collection period. The overflow of tritium produced by the electrical stimulation was calculated as the total increase in radioactivity above the basal outflow of tritium determined in the sample immediately preceding the start of stimulation (Sp_1 or Sp_2). In order to assess the drug-induced changes of electrically-evoked overflow of radioactivity, S_2/S_1 ratios were calculated, and Sp_2/Sp_1 ratios were examined to determine whether the drugs altered the basal outflow of radioactivity. The amount of tritium released by electrical stimulation in rat brain slices under these conditions provides a reliable estimate of the release of tritiated or endogenous 5-HT (Baumann & Waldmeier, 1981; Moret & Briley, 1990; see Results section).

In some experiments, the superfusate was fractionated to determine the ratio of [^{3}H]-5-HT to [^{3}H]-5-hydroxyindoleacetic acid ([^{3}H]-5-HIAA) during the basal outflow and evoked overflow collection periods. One ml of the superfusate was added to 5 ml of *n*-heptane, 1.8 ml of butanol, and 0.1 ml of 2 N HCl. The mixture was vigorously agitated for 10 min and centrifuged at 2,000 *g* for 10 min. The radioactivity was then counted in 4 ml of the organic phase to determine the concentration of [^{3}H]-5-HIAA and in 1 ml of the aqueous phase for [^{3}H]-5-HT (Monroe & Smith, 1985).

The following drugs were used: 2-methyl-5-HT, 5-carboxyamidotryptamine (5-CT), trimethylfluorophenylpiperazine (TFMPP), phenylbiguanide (Research Biochemicals, Natick, MA, U.S.A.), *m*-chloro-phenylbiguanide (Cookson, Southampton, England), ondansetron (Glaxo, Montreal, Canada), ICS 205-930 (3-α-tropanyl)-1H-indole-3-carboxylic ester methiodide; Sandoz, Basel, Switzerland), paroxetine, BRL 24924 ([(±)-(endo)]-4-amino-N-1-azabicylo[3.3.1]-non-4-yl-5-chloro-2-methoxybenzamide), BRL 46470A (endo-N-(8-methyl-8-azabicyclo[3.2.1.]oct-3-yl)2,3-dihydro-3,3-dimethylindole-1-carboxamide HCl) (SmithKline Beecham, Harlow, England), *m*-cholorophenylpiperazine (*m*CPP; Aldrich Milwaukee, WI, U.S.A.), quipazine (Miles, Elkhart, IN, U.S.A.), **R**, **S** and **R/S** zacopride (Delalande, Rueil-Malmaison, France), MDL 72222 (endo-8-methyl-8-azabicyclo[3.2.1.]oct-3-yl-3,5-dichlorobenzoate HCl; Merrell Dow, Cincinnati, OH, U.S.A.) and (+)-tubocurarine (Sigma, St-Louis, MO, U.S.A.).

The *n*'s refer to the number of superfusion chambers studied. All results are expressed as means ± s.e. The means were compared by the two-tailed Student's *t* test, and Dunnett's correction for multiple comparisons with a single control group when necessary. Probability (*P*) values smaller than 0.05 were considered as significant.

Results

Effects of 2-methyl-5-HT on the evoked release of tritium

In the *in vitro* paradigm used in the present study, the electrically evoked release of [^{3}H]-5-HT is entirely calcium-dependent as demonstrated by its complete suppression by the removal of calcium from the superfusion medium (Blier & Bouchard, 1992). The selective 5-HT_3 receptor agonist, 2-methyl-5-HT (0.1–3 μM), introduced 8 min before S_2, produced a concentration-dependent enhancement of the electrically evoked tritium release in hypothalamus, hippocampus, and frontal cortex slices (Figures 1 and 2). As illustrated in Figure 1, 2-methyl-5-HT at a concentration of 1 μM introduced 8 min before S_2 did not induce any enhancement of the

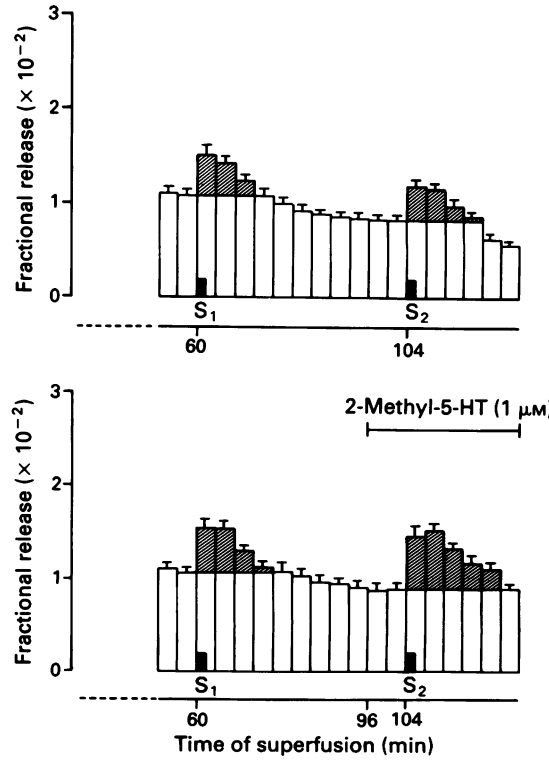


Figure 1 Basal and electrically evoked release of tritium from hypothalamic slices preloaded with [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) in the control condition, and with the introduction of 2-methyl-5-HT 8 min before the second period of electrical stimulation (S_2). Each stimulation period consisted of 360 pulses delivered at an intensity of 20 mA, a duration of 2 ms, and a frequency of 3 Hz. Ordinate scale: amount of tritium released per 4 min sample expressed as a fraction of the total tissue tritium content at the start of the respective collection period. The hatched areas represent the proportion of tritium overflow evoked by the stimulation. Values are expressed as means ± s.e. (vertical bars) of 9 experiments.

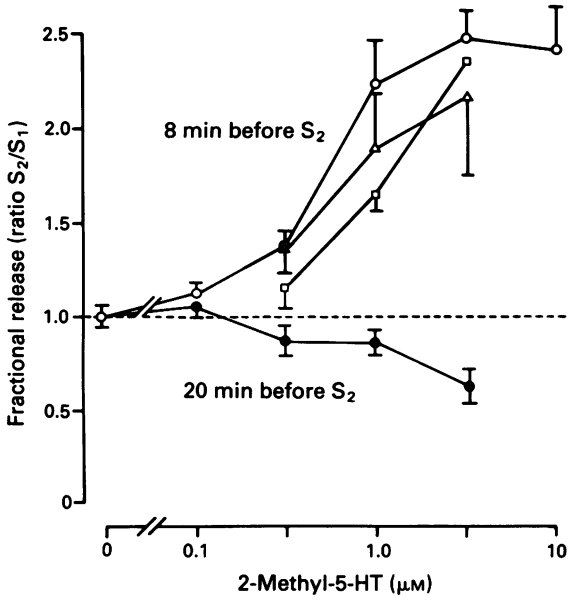


Figure 2 Effect of 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) introduced 8 min before S_2 on the release of tritium elicited by the electrical stimulation of hypothalamus (○), hippocampus (△) and frontal cortex (□) slices preloaded with [^{3}H]-5-HT, and of 2-methyl-5-HT introduced 20 min before S_2 in hypothalamus slices (●). Ordinate scale: fraction of the total tissue radioactivity released by 360 pulses (hypothalamus: 20 mA, 2 ms, 3 Hz; hippocampus and frontal cortex: 30 mA, 2 ms, 3 Hz) expressed as the ratio (S_2/S_1) obtained between the second period of stimulation in the presence of the drug (S_2) and the first period of stimulation carried out in the absence of the drug (S_1). Each point represents the mean ± s.e. (vertical bars) of 5–10 experiments.

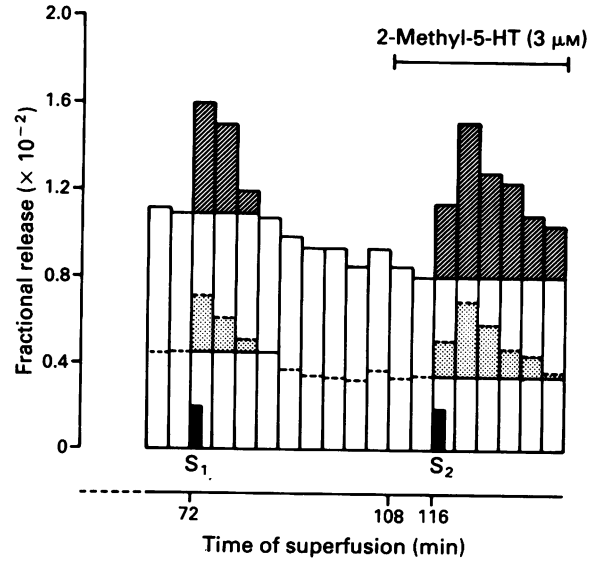


Figure 3 Basal and electrically evoked release of tritium (top line) and [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) (bottom dotted line) from hippocampal slices preloaded with [^{3}H]-5-HT with the introduction of 2-methyl-5-HT 8 min before the second period of electrical stimulation (S_2). Each stimulation period consisted of delivering 360 pulses at an intensity of 30 mA, a duration of 2 ms, and a frequency of 3 Hz. Ordinate scale: amount of tritium released per 4 min sample as a fraction of the total tissue tritium content at the start of the respective collection period. The hatched areas represent the proportion of tritium overflow evoked by the stimulation and the shaded areas represent the proportion of [^{3}H]-5-HT overflow in the corresponding samples. Values represent the mean of two separate experiments, each involving a total of 10 slices.

spontaneous outflow of the radioactivity from hypothalamic slices, thus suggesting that 2-methyl-5-HT did not merely act as a displacing agent of the intraneuronal store of tritium under these conditions. In hypothalamic slices, the Sp_2/Sp_1 ratio was 0.79 ± 0.01 ($n = 29$) in the control condition and it was slightly increased with a 3 μM concentration of 2-methyl-5-HT (0.86 ± 0.04, $n = 5$), but this ratio was only significantly enhanced at a concentration of 10 μM (0.92 ± 0.03, $n = 5$). Similar results for the Sp_2/Sp_1 ratio were also obtained in the hippocampus and cortex (data not shown).

In order to ascertain that the enhanced evoked release of tritium produced by 2-methyl-5-HT was due at least in part to an increased release of [^{3}H]-5-HT, the superfusate was fractionated into [^{3}H]-5-HT and [^{3}H]-5-HIAA. In hypothalmic and hippocampal slices, the proportion of [^{3}H]-5-HT in the superfusate was constant at about 40% with the exception of the two fractions when stimulation was applied in which it increased to 50% (Figure 3).

When 2-methyl-5-HT was introduced 20 min before S_2, its enhancing effect on the electrically evoked release of tritium was totally abolished (Figure 2), thus indicating that the additional 12 min exposure to the agonist was sufficient to desensitize the response. Given this phenomenon, it was deemed crucial to assess whether the response could be restored. To this end, 2-methyl-5-HT (1 μM) was introduced 20 min before S_1 in order to obtain an electrically evoked release of tritium during the desensitized state of the receptor. The slices were then superfused with normal Krebs from the 12th min after the beginning of the first stimulation period (S_1) until 8 min prior to S_2 in order to remove the activation of the 5-HT$_3$ receptors by 2-methyl-5-HT. When 2-methyl-5-HT was again introduced 8 min before S_2, the electrically evoked release was enhanced to approximately the same degree ($S_2/S_1 = 1.82 \pm 0.12$, $n = 9$) as observed when S_1 was carried out in the absence of 2-methyl-5-HT ($S_2/S_1 = 2.25 \pm 0.23$, $n = 8$).

The release of tritium from brain slices preloaded with [^{3}H]-5-HT can also be elicited with fenfluramine which acts by a calcium-independent mechanism (Langer & Moret, 1982). In hypothalamic slices, a 7 μM concentration of fenfluramine superfused for 2 min was required to induce a fractional release of tritium approximately equivalent ($S_1 = 1.77 \pm 0.22\%$, $n = 9$) to that produced by electrical stimulation ($S_1 = 1.48 \pm 0.15\%$, $n = 29$). Under these conditions, 2-methyl-5-HT (1 μM) introduced 8 min before S_2 did not produce a significant alteration of the fenfluramine-evoked release of tritium (control: $S_2/S_1 = 0.97 \pm 0.10$, $n = 4$; 2-methyl-5-HT:$S_2/S_1 = 0.98 \pm 0.07$, $n = 5$).

Effects of phenylbiguanide and m*-chloro-phenylbiguanide on the electrically evoked release of tritium*

Phenylbiguanide and *m*-chloro-phenylbiguanide have been shown to be effective 5-HT$_3$ receptor agonists in rat, but not guinea-pig tissues (see Kilpatrick *et al.*, 1991; Robertson & Bevan 1992; Butler *et al.*, 1990). In frontal cortex slices, phenylbiguanide (0.01 and 0.1 μM), introduced 8 min before S_2, did not significantly alter the electrically evoked release of tritium. At greater concentrations, phenylbiguanide produced a concentration-dependent decrease of the evoked release of tritium (1 μM:S_2/S_1, = 0.61 ± 0.07, $n = 5$; 10 μM:$S_2/S_1 = 0.37 \pm 0.04$, $n = 5$), without altering spontaneous outflow of radioactivity. In hypothalamic slices at concentrations of 0.1 and 1 μM (Figure 4), phenylbiguanide did not alter the electrically evoked release of tritium. In an attempt to reconcile the high affinity of phenylbiguanide for 5-HT$_3$ sites with its lack of effect in the present paradigm, it was then tested as an antagonist in hypothalamic slices. When phenylbiguanide (1 μM) was introduced 20 min before S_1 and remained present until the end of the experiment, it partially blocked the enhancing effect of 1 μM 2-methyl-5-HT introduced 8 min before S_2 (Figure 4). *m*-Chloro-phenylbiguanide, which displays greater affinity for rat 5-HT$_3$ receptors than phenylbiguanide (Kilpatrick *et al.*, 1990), also did not modify the

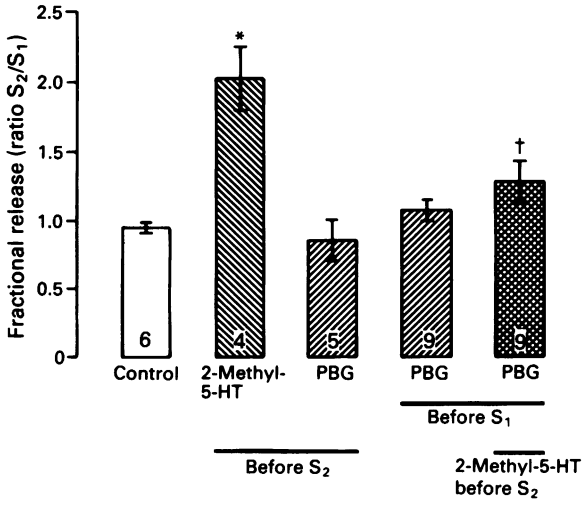


Figure 4 Effect of phenylbiguanide (PBG, 1 μM), introduced 8 min before S_2 or 20 min before S_1 and that of 2-methyl-5-hydroxytryptamine (2-methyl-5-HT, 1 μM) introduced 8 min before S_2 alone or in the presence of phenylbiguanide, on the electrically evoked release of tritium from hypothalamic slices preloaded with [^{3}H]-5-HT. Values are expressed as means ± s.e. (vertical bars) for which the number of experiments per group is given at the bottom of each column.
*$P<0.05$ when compared with the control value; †$P<0.05$ when compared to the value obtained with 2-methyl-5-HT alone.

evoked release of tritium from guinea-pig hypothalamic slices at a concentration of 0.1 μM introduced 8 min before S_2 ($S_2/S_1 = 0.95 \pm 0.13$, $n = 5$); however, a significant attenuation was obtained at a 1 μM concentration of *m*-chlorophenylbiguanide ($S_2/S_1 = 0.51 \pm 0.16$, $n = 5$), but in these conditions the spontaneous outflow was markedly increased.

Effect of 5-HT$_3$ antagonists on the enhancement of the electrically-evoked release of tritium by 2-methyl-5-HT

Few 5-HT$_3$ receptor agonists are available at present while a good number of 5-HT$_3$ antagonists are. Seven compounds with documented 5-HT$_3$ antagonistic properties were tested in hypothalamic slices. These drugs were introduced 20 min before S_1, and 2-methyl-5-HT was introduced 8 min before S_2, both agents remaining present in the superfusion medium from the time of their introduction until the end of the experiment. All 5-HT$_3$ receptor antagonists (1 μM) tested produced a significant attenuation of the enhancing effect of 2-methyl-5-HT (1 μM) with the following order of potency: *m*CPP = quipazine = ondansetron ⩾ ICS 205-930 = BRL 24924 > MDL 72222 = zacopride (Figure 5). The **R**- and **S**-enantiomers of zacopride (1 and 0.1 μM) were also effective in blocking the effect of 2-methyl-5-HT and were equipotent with 1 μM of the racemic compound (data not shown). In contrast, ICS 205-930 was ineffective at 0.1 μM ($S_2/S_1 = 2.18 \pm 0.32$, $n = 5$). It is noteworthy that all these antagonists did not attenuate the electrically-evoked release of tritium when they were superfused 8 min before S_2 in the absence of 2-methyl-5-HT (data not shown), with the exception of 1 μM **S**-zacopride ($S_2/S_1 = 0.68 \pm 0.09$, $n = 5$) and *m*CPP ($S_2/S_1 = 0.65 \pm 0.10$, $n = 5$). The latter drug is a piperazine derivative which is also a 5-HT$_{1D}$ receptor agonist and thus may have directly activated the terminal 5-HT autoreceptor, which is a 5-HT$_{1D}$ receptor in the guinea-pig brain (Hoyer & Middlemiss, 1989).

The capacity of the following drugs to block the enhancement by 2-methyl-5-HT (3 μM) of the electrically-evoked overflow of tritium was also studied in hippocampal slices. The novel 5-HT$_3$ antagonist, BRL 46470A (Newberry *et al.*, 1992), at 1 μM, and **R**- and **S**-zacopride at 0.01 μM blocked the effect of 2-methyl-5-HT (Figure 6). At 0.001 μM, the two stereoisomers of zacopride were ineffective (Figure 6). (+)-Tubocurarine, which effectively blocks 5-HT$_3$ receptors in the nanomolar range in mouse hippocampal preparations (Peters

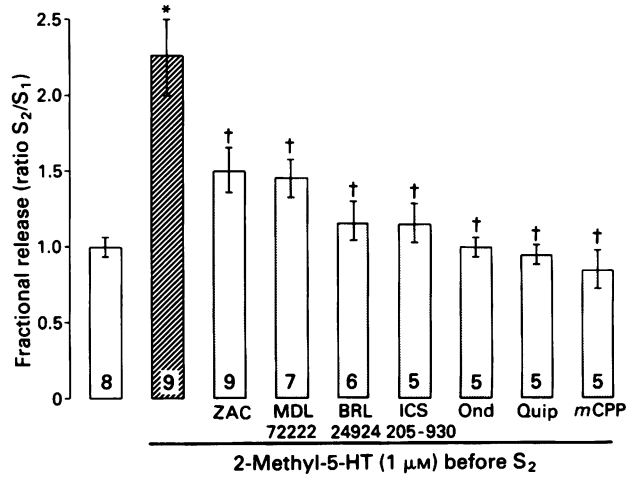


Figure 5 Effect of seven 5-HT$_3$ antagonists on the enhancement of the electrically evoked release of tritium produced by 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) in hypothalamic slices preloaded with [^{3}H]-5-HT. The antagonists (1 μM) were introduced 20 min before the first stimulation period and 2-methyl-5-HT was introduced 8 min before S_2, both agents remaining present in the superfusate until the end of superfusion. Ordinate scale: fraction of the total tissue radioactivity released by 360 pulses expressed as the ratio (S_2/S_1) obtained between the second period of stimulation (S_2) and the first one (S_1). Values are expressed as means ± s.e. (vertical bars) for which the number of experiments per group is given at the bottom of each column.
*$P<0.05$ when compared with the control value; †$P<0.05$ when compared to the value obtained with 2-methyl-5-HT alone.

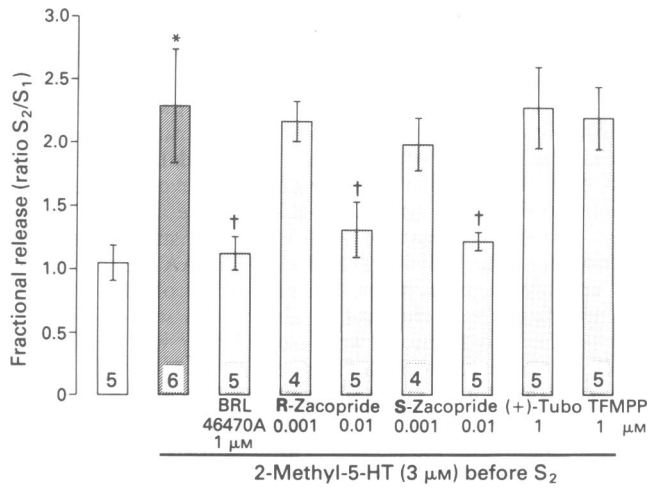


Figure 6 Effect of four compounds with documented 5-HT$_3$ antagonistic properties and of trifluoro-methyl-phenylpiperazine (TFMPP) on the enhancement of the electrically evoked release of tritium produced by 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) in hippocampal slices preloaded with [^{3}H]-5-HT. The compounds tested on the enhancing effect of 2-methyl-5-HT were introduced 20 min before the first stimulation period and remained present in the superfusate until the end of the experiment. Ordinate scale: fraction of the total tissue radioactivity released by 360 pulses expressed as the ratio (S_2/S_1) obtained between the second period of stimulation (S_2) and the first one (S_1). Values are expressed as means ± s.e. (vertical bars) for which the number of experiments per group is given at the bottom of each column.
*$P<0.05$ when compared with the control value; †$P<0.05$ when compared to the value obtained with 2-methyl-5-HT alone. Note that (+)-tubocurarine [(+)-Tubo] was ineffective.

et al., 1990; Yakel & Jackson, 1988) but is virtually inactive at guinea-pig 5-HT$_3$ receptors (Malone *et al.*, 1991; Newberry *et al.*, 1991), did not block the enhancing effect of 2-methyl-5-HT (Figure 6). TFMPP, which like *m*CPP is a piperazine but is devoid of significant affinity for 5-HT$_3$ binding sites (Hoyer, 1991; Robertson *et al.*, 1992), was also ineffective in blocking the effect of 2-methyl-5-HT (Figure 6), despite the fact that by itself it decreased the evoked release by about 40% as was the case with *m*CPP.

Effect of the 5-HT reuptake blocker, paroxetine, on the electrically-evoked release of tritium

In rat hypothalamic slices, selective 5-HT reuptake blockers, introduced 20 min before S_2, do not enhance the electrically evoked overflow of tritium, although increasing the proportion of [^{3}H]-5-HT relative to [^{3}H]-5-HIAA in the superfusate; this is presumably attributable to the potent inhibitory feedback exerted by the enhanced activation of the 5-HT autoreceptor resulting from 5-HT reuptake blockade (Galzin *et al.*, 1985; Blier *et al.*, 1989; Moret & Briley, 1990). In the present experiments carried out under identical conditions in guinea-pig hypothalamic slices, the same lack of effect of the selective 5-HT reuptake blocker, paroxetine, was observed (Figure 7a). However, given the rapid desensitization of the 5-HT$_3$ receptor observed in the present study, it was thus possible that the prolonged 20 min exposure to an enhanced synaptic concentration of 5-HT was sufficient to desensitize the facilitatory 5-HT$_3$ receptors. Paroxetine was then introduced only 8 min before S_2 and it produced a 31% increase of the electrically-evoked overflow of tritium (Figure 7a). In order to ascertain that this moderate, albeit significant, increase was attributable to an enhanced activation of 5-HT$_3$ receptors by endogenous 5-HT, the same experiment was repeated in the presence of the 5-HT$_3$ antagonist, ICS 205-930, introduced 20 min before S_1 and maintained in the superfusion medium until the end of the experiment. Under these conditions, the enhancing effect of paroxetine was blocked (Figure 7a). In the rat hippocampus, paroxetine approximately doubles the evoked overflow of tritium when introduced 20 min before S_2 (Blier, 1991). In the guinea-pig hippocampal slices, paroxetine increased the evoked overflow of tritium by 40% when introduced 20 min before S_2. When introduced 8 min before S_2, paroxetine enhanced the evoked overflow two fold (Figure 7b). This potentiation of the evoked overflow of [^{3}H]-5-HT by paroxetine was completely blocked by ICS 205-930 introduced 20 min before S_1 (Figure 7b). Fractionation of the superfusate from hippocampal slices showed that, in the presence of paroxetine introduced before S_2, the proportion of [^{3}H]-5-HT in the fractions following electrical stimulation was markedly increased (S_2/S_1 for total tritium: 1.45 versus 2.09 for [^{3}H]-5-HT). These results, which are consistent with those observed in hypothalamic slices (Moret & Briley, 1990), indicate that in the absence of a reuptake blocker, an important proportion of the [^{3}H]-5-HT that is released by electrical stimulation is taken back up into 5-HT terminals. In order to provide an additional line of evidence for the desensitization of these 5-HT$_3$ receptors in hypothalamic slices, paroxetine was introduced before S_1 and superfused throughout the experiment in order to increase the tonic activation of these 5-HT$_3$ receptors by 5-HT. Under these conditions, 2-methyl-5-HT (1 μM) did not enhance the electrically evoked release of tritium (paroxetine before S_1 alone: $S_2/S_1 = 0.97 \pm 0.07$, $n = 7$; paroxetine before S_1 and 2-methyl-5-HT before S_2:$S_2/S_1 = 1.04 \pm 0.11$, $n = 8$).

Effects of 2-methyl-5-HT on the spontaneous outflow of tritium

Previous *in vitro* experiments examining the release of tritium in rat spinal cord synaptosomes preloaded with [^{3}H]-5-HT have shown that a 10 μM concentration of 2-methyl-5-HT produced an outflow of [^{3}H]-5-HIAA which was blocked by

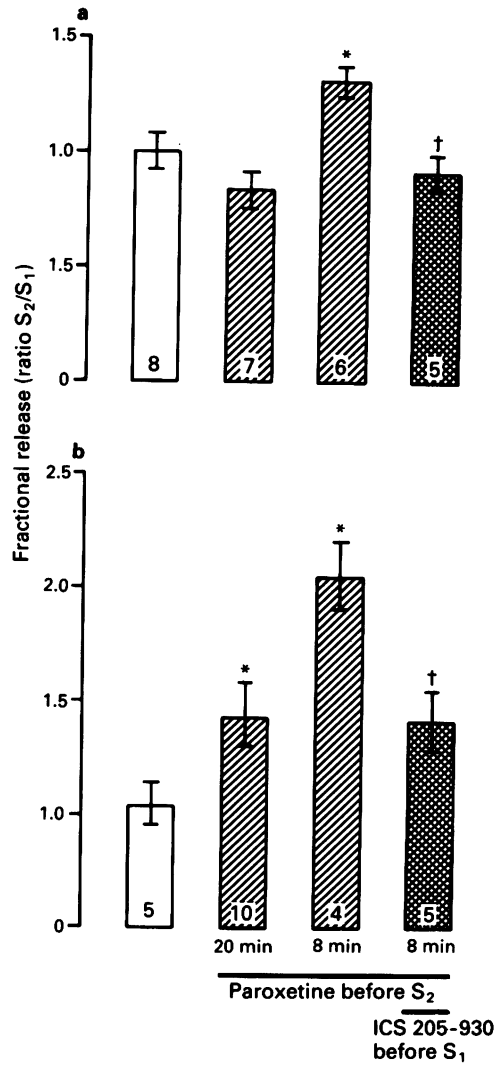


Figure 7 Effect of the selective 5-hydroxytryptamine (5-HT) reuptake blocker paroxetine (1 μM), introduced 20 or 8-min before S_2 and 8 min before S_2 in the presence of ICS 205-930, on the electrically evoked release of tritium from hypothalamic slices preloaded with [^{3}H]-5-HT in (a) and hippocampal slices in (b). Values are expressed as means ± s.e. (vertical bars) for which the number of experiments per group is given at the bottom of each column.
*$P < 0.05$ when compared with the control value; †$P < 0.05$ when compared to the value obtained with paroxetine alone introduced 8 min before S_2.

the selective 5-HT reuptake blocker, fluoxetine, while leaving unaltered the release of [^{3}H]-5-HT (Williams *et al.*, 1992). In order to assess whether the same phenomenon was present in guinea-pig hypothalamic slices, the effect of 2-methyl-5-HT on the outflow of radioactivity was examined by introducing it 8 min before the normal time of the S_2 stimulation period, but without subsequently delivering the stimulations. The amount of radioactivity released in such a sham S_2 period was compared to that released in S_1 produced by delivering 360 pulses at 3 Hz for 2 min. Under these conditions, the 0.3 μM concentration of 2-methyl-5-HT by itself in the absence of electrical stimulations did not produce any effect (Table 1). At 1 μM, however, 2-methyl-5-HT did produce an outflow of tritium which amounted to a fractional release of about 0.3%. Since the spontaneous outflow was not altered in the tube immediately preceding the usual time of the delivery of electrical stimulation (8 min after the introduction of 2-methyl-5-HT, see Figure 1b and Table 1), the 5-HT$_3$ agonist therefore exerted this effect with a delay of at least 12 min, and is thus inconsistent with a simple displacement mechanism of action. When a 10 μM concentration was used,

Table 1 Effect of 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) on the basal release of [^{3}H]-5-HT as compared to the electrically-evoked release of [^{3}H]-5-HT in the same hypothalamic slices[1,2]

	S_1	Sham S_2	Sham S_2/S_1
2-Methyl-5-HT			
0.3 μM	0.89 ± 0.17 (5)	0.08 ± 0.08	0.07 ± 0.06
1.0 μM	0.84 ± 0.10 (5)	0.30 ± 0.06*	0.39 ± 0.10
10 μM	0.85 ± 0.14 (5)	1.00 ± 0.23*	1.14 ± 0.03
2-Methyl-5-HT (10 μM)			
+0 Ca^{2+} [3]	0.75 ± 0.12 (5)	0.16 ± 0.05	0.21 ± 0.07†
+S-Zacopride[4] (0.1 μM)	0.90 ± 0.13 (5)	0.34 ± 0.13	0.37 ± 0.11†
+Paroxetine[4] (1 μM)	1.03 ± 0.18 (4)	0.88 ± 0.14	0.87 ± 0.10

[1]Electrical stimulations (360 pulses: 3 Hz, 0.5 ms, 20 mA) were delivered in S_1 after 60 min of superfusion.
[2]2-Methyl-5-HT was introduced 8 min before the usual time of the S_2 stimulation period, but the electrical pulses were not delivered. The overflow of radioactivity in this sham S_2 period was calculated as the amount of radioactivity released above that released in the sample immediately preceding the introduction of 2-methyl-5-HT.
[3]A Ca^{2+}-free Krebs solution was superfused starting 20 min before the introduction of 2-methyl 5-HT.
[4]S-zacopride and paroxetine were superfused starting 12 min before the introduction of 2-methyl-5-HT.
*$P<0.05$ when compared to the basal outflow of radioactivity; †$P<0.05$ when compared to the sham S_2/S_1 value obtained in the presence of 2-methyl-5-HT (10 μM) alone.

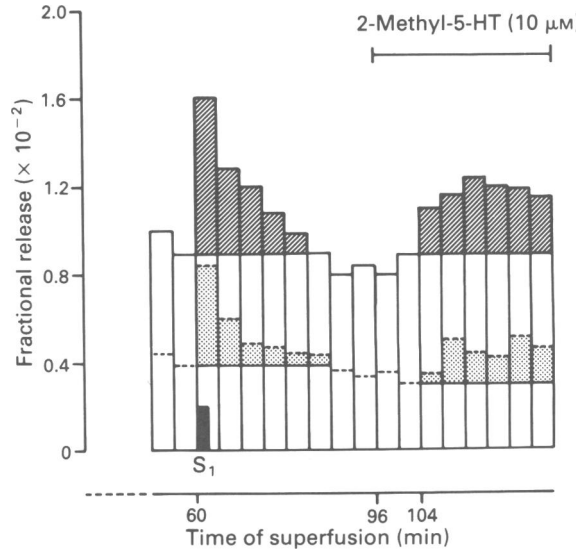


Figure 8 Basal and electrically evoked release of tritium (top line) and [^{3}H]-5-hydroxytrypramine ([^{3}H]-5-HT) (bottom dotted line) from hypothalamic slices preloaded with [^{3}H]-5-HT with the introduction of 2-methyl-5-HT 8 min before the usual time of the second period of stimulation. The stimulation period (S_1) after 60 min of superfusion consisted of delivering 360 pulses at an intensity of 20 mA, a duration of 2 ms, and a frequency of 3 Hz. Note that no electrical stimulation was applied at minute 104. Ordinate scale: amount of tritium released per 4 min sample as a fraction of the total tissue tritium content at the onset of the respective collection period. The hatched areas represent the proportion of tritium released by the electrical stimulations in S_1 and by 2-methyl-5-HT alone. The shaded areas represent the proportion of [^{3}H]-5-HT overflow in the corresponding samples. Values represent the mean of five experiments.

the amount of tritium was about the same as that released in S_1 (Figure 8). This marked enhancement of the spontaneous outflow of tritium by 10 μM 2-methyl-5-HT consisted of about the same proportion of [^{3}H]-5-HT as that released during electrical stimulation (data not shown). Furthermore, it was markedly reduced in slices superfused with a Ca^{2+}-free Krebs solution 20 min before the introduction of 2-methyl-5-HT and also by S-zacopride (0.1 μM) introduced 20 min before S_1 and maintained until the end of the experiment. In contrast with the results obtained in the rat spinal cord synaptosomes (Williams *et al.*, 1992), the selective 5-HT reuptake blocker, paroxetine (1 μM) did not prevent the enhancing effect of 10 μM 2-methyl-5-HT on the spontaneous outflow of tritium (Table 1).

Frequency- and time-dependence of the facilitatory role of the 5-HT$_3$ receptor on the electrically evoked release of tritium

Previous studies have shown that the modulation of 5-HT release by the inhibitory effect of 5-HT autoreceptors located on 5-HT terminals is dependent on the synaptic concentration of 5-HT which varies with the frequency of depolarization (Göthert, 1980; Baumann & Waldmeier, 1981). The effectiveness of an exogenous agonist in suppressing 5-HT release is therefore attenuated when determined at high frequencies of stimulation since it has to compete with a greater amount of endogenous 5-HT resulting from the shortening of the interval between each impulse (Chaput *et al.*, 1986; Blier *et al.*, 1989). In order to test this hypothesis on the function of the 5-HT$_3$ receptors, the effectiveness of the agonist, 2-methyl-5-HT, in enhancing tritium release at 3 Hz for 2 min was compared to that at 5 Hz for 72 s and at 1 Hz for 6 min in order to deliver the same number of pulses (360) during each stimulation period. In keeping with the above-mentioned data, the effectiveness of 2-methyl-5-HT was attenuated when the pulses were delivered at 5 Hz (Figure 9). However, when the pulses were delivered at 1 Hz, a decrease was also obtained instead of the expected enhanced efficacy (Figure 9). Since it was observed in a previous series of experiments that this 5-HT$_3$ receptor can desensitize within a few minutes, it was thus possible that stimulations delivered over a period of 6 min were sufficient to produce a partial desensitization. In order to test this possibility, 1 Hz pulses were delivered but for only 2 min. As illustrated in Figure 10, 2-methyl-5-HT was significantly more effective in enhancing tritium release when the stimulation period lasted 2 instead of 6 min. Since it would be expected that 5 Hz stimulations would lead to a greater degree of activation of 5-HT$_3$ receptors than 3 and 1 Hz stimulations, it was thus possible that a desensitization could also occur over a period of stimulation lasting 72 s. To address this issue, the effectiveness of 2-methyl-5-HT was studied with 5 Hz stimulations delivered for 120 and 24 s. As shown in Figure 11, 2-methyl-5-HT had approximately the same effect when the 5 Hz stimulations were delivered for 72 and 120 s, despite releasing considerably less tritium in the former condition ($S_1 = 1.34 \pm 0.15\%$, $n = 10$) than in the latter ($S_1 = 2.42 \pm 0.15\%$, $n = 8$). In contrast, when the 5 Hz stimulations were delivered for only 24 s, 2 methyl-5-HT (1 μM) actually produced a three fold increase in the release of tritium (Figure 11), despite releasing approximately the same amount of tritium in S_1 ($0.68 \pm 0.13\%$, $n = 15$) as in the 1 Hz/2 min condition ($0.77 \pm 0.11\%$, $n = 13$) when only a two fold increase was observed (Figure 10). It is noteworthy that a two fold increase was obtained with 0.3 μM 2-methyl-5-HT in the 5 Hz/24 s condition ($S_2/S_1 = 1.90 \pm 0.32$, $n = 5$), a concentration of 2-methyl-5-HT that was completely devoid of effect on the basal tritium release in the absence of electrical simulation (Table 1).

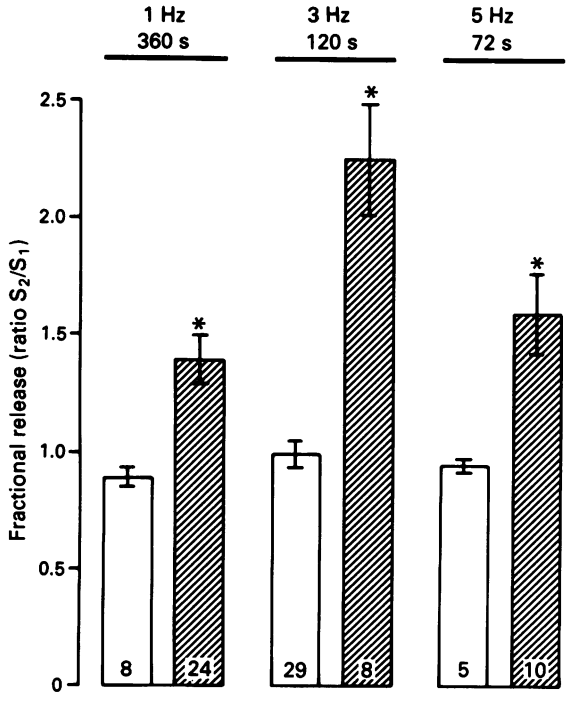


Figure 9 Effect of 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) on the evoked release of tritium from hypothalamic slices, preloaded with [^{3}H]-5-HT, produced by 360 electrical pulses delivered at three frequencies of stimulation. The drug was introduced 8 min before S_2. Ordinate scale: fraction of the total tissue radioactivity expressed as the ratio (S_2/S_1) obtained between the second period of stimulation (S_2) and the first one (S_1) in the absence of the drug. The duration of the stimulation periods is indicated in seconds above the histograms. Values are expressed as means ± s.e. (vertical bars) for which the number of experiments per group is given at the bottom of each column.
*$P<0.05$ when compared with the corresponding control value; open columns, control; hatched columns, 2-methyl-5-HT (1 μM) before S_2.

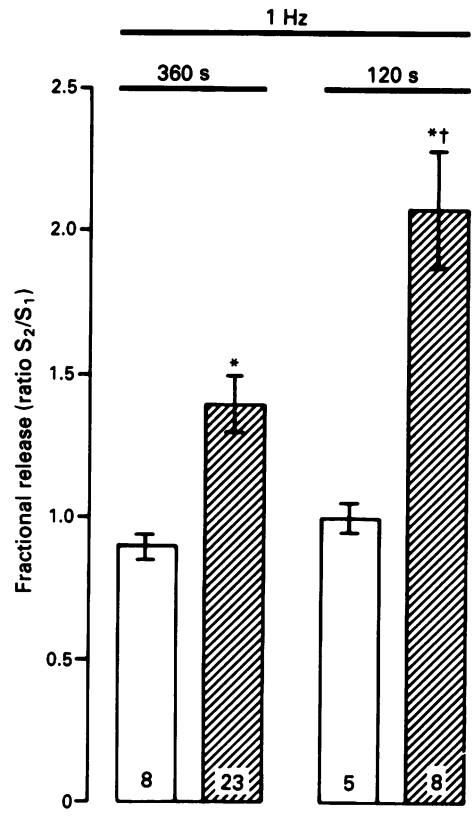


Figure 10 Effect of 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) on the evoked release of tritium from hypothalamic slices, preloaded with [^{3}H]-5-HT, produced by electrical pulses delivered at a 1 Hz frequency of stimulation. The drug was introduced 8 min before S_2. Ordinate scale: fraction of the total tissue radioactivity expressed as the ratio (S_2/S_1) obtained between the second period of stimulation (S_2) and the first one (S_1) in the absence of the drug. The duration of the stimulation periods is indicated in seconds above the histograms. Values are expressed as means ± s.e. (vertical bars) for which the number of experiments per group is given at the bottom of each column.
*$P<0.05$ when compared with the control value, open columns: control; hatched columns: 2-methyl-5-HT (1 μM) before S_2.

Effect of 5-HT$_3$ receptor blockade on the modulation of tritium release by the inhibitory 5-HT autoreceptor

In guinea-pig hypothalamic slices, as in rat hypothalamic slices (Blier *et al.*, 1989), changing the frequency of stimulation from 1 Hz to 3 Hz yielded a smaller fractional release of tritium (1 Hz:S_1 = 1.94 ± 0.11%, $n = 10$; 3 Hz:S_1 = 1.14 ± 0.12%, $n = 10$). Also consistent with the hypothesis mentioned in the previous section, the exogenous 5-HT autoreceptor agonist 5-CT was significantly less effective in decreasing tritium release at 3 Hz for 2 min than at 1 Hz for 6 min (Figure 12). When the 5-HT$_3$ antagonist, ICS 205-930, was superfused starting 20 min before S_1 until the end of the experiment, the effectiveness of 5-CT was not altered at either frequency of stimulation (Figure 12), thus indicating that the facilitatory 5-HT$_3$ receptor does not significantly modulate the frequency-dependence effect of the inhibitory 5-HT$_{1D}$ autoreceptor on tritium release in guinea-pig hypothalamic slices.

Discussion

The results of the present study confirm the capacity of 5-HT$_3$ receptors to enhance the electrically-evoked release of [^{3}H]-5-HT from preloaded slices of the frontal cortex (Galzin *et al.*, 1990) and hypothalamus (Blier & Bouchard, 1992) of the guinea-pig, and demonstrate the presence of such receptors in the hippocampus as well. The selective agonist, 2-methyl-5-HT, did not alter the fenfluramine-induced release of [^{3}H]-5-HT, thus indicating that the activation of these facilitatory 5-HT$_3$ receptors cannot modulate the release of 5-HT by this calcium-independent mechanism. As would be expected from such a positive feedback system, it was also shown to be inactivated by prolonged exposure (20 min) to an exogenous agonist in slices of the frontal cortex (Galzin *et al.*, 1990) and the hypothalamus (Figure 2). It is important to note that this 5-HT$_3$ response had recovered 24 min after superfusion of a medium not containing 2-methyl-5-HT. In *in vitro* electrophysiological experiments carried out in hippocampal neurones (Yakel & Jackson, 1988), however, the desensitization occurred within a few seconds instead of a few minutes as in the present experiments as suggested by the results presented in Figure 2. Nevertheless, the results obtained with the 5 Hz frequency of stimulation suggest that the desensitization of these 5-HT$_3$ receptors may also begin to occur within a matter of seconds, as the effect of 2-methyl-5-HT was markedly attenuated when the stimulations were delivered for 72 s instead of 24 s (Figure 11). The activation of these 5-HT$_3$ receptors by 5-HT itself, as well as their rapid desensitization, was also shown when paroxetine was used to increase the synaptic availability of 5-HT by blocking the 5-HT reuptake carrier (Figure 7). As was the case with the exogenous agonist, 2-methyl-5-HT, blocking the reuptake process for an additional 12 min attenuated the effect of paroxetine on the evoked [^{3}H]-5-HT overflow. Furthermore,

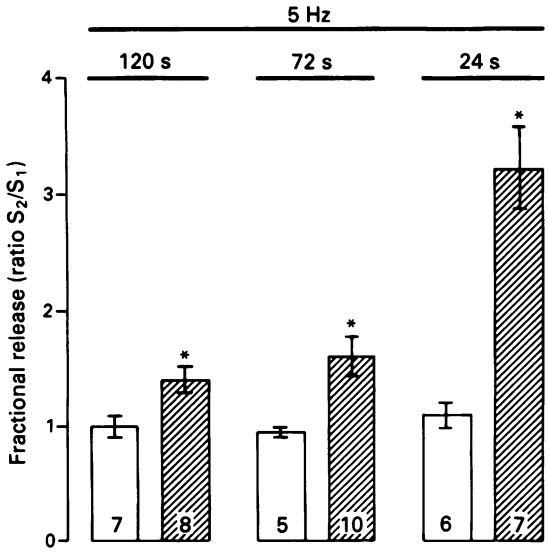


Figure 11 Effect of 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) on the evoked release of tritium from hypothalamic slices, preloaded with [^{3}H]-5-HT, produced by electrical pulses delivered at a 5 Hz frequency of stimulation. The drug was introduced 8 min before S_2. Ordinates scale: fraction of the total tissue radioactivity expressed as the ratio (S_2/S_1) obtained between the second period of stimulation (S_2) and the first one (S_1) in the absence of the drug. The duration of the stimulation periods is indicated in seconds above the histograms. Values are expressed as means ± s.e. (vertical bars) for which the number of experiments per group is given at the bottom of each column.
*$P<0.05$ when compared with the control value; open column: control; hatched columns: 2-methyl-5-HT (1 μM) before S_2.

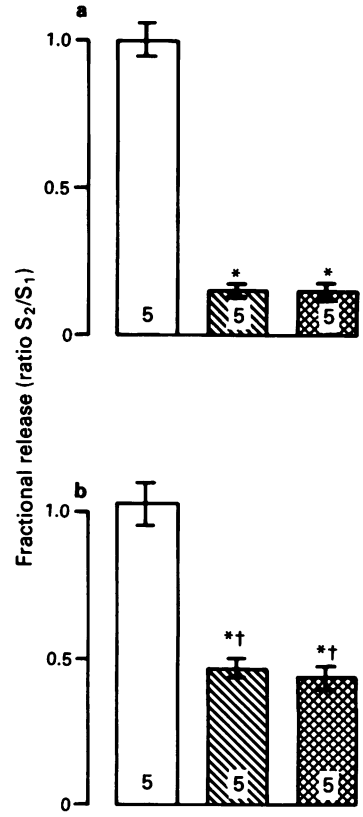


Figure 12 Effect of the 5-hydroxytryptamine (5-HT) autoreceptor agonist 5-carboxyamidotryptamine (5-CT) on the evoked release of tritium from hypothalamic slices, preloaded with [^{3}H]-5-HT, produced by 360 electrical pulses delivered at two frequencies of stimulation (a) at 1 Hz and (b) at 3 Hz. 5-CT (0.003 μM) was introduced 20 min before S_2 and ICS 205-930 (1 μM) was introduced 20 min before S_1 and remained present until the end of the experiment. Ordinate scale: fraction of the total tissue radioactivity expressed as the ratio (S_2/S_1) obtained between the second period of stimulation (S_2) and the first one (S_1). Values are expressed as means ± s.e. (vertical bars) for which the number of experiments per group is given at the bottom of each column.
*$P<0.05$ when compared with the corresponding control value; †$P<0.05$ when compared to the value obtained with the inhibitory effect of 5-CT at the 1 Hz frequency of stimulation. There was no statistically significant difference in the effect of 5-CT in the presence and in the absence of ICS 205-930. Open columns: control; hatched columns: 5-CT before S_2; cross-hatched column: ICS 205-930 before S_1 + 5-CT before S_2.

when paroxetine was present throughout the superfusion experiment, 2-methyl-5-HT no longer enhanced the electrically evoked overflow of [^{3}H]-5-HT. These latter two series of experiments constitute further evidence for the capacity of these 5-HT$_3$ receptors to desensitize.

The purpose of the present study was not to trace a precise pharmacological profile of these 5-HT$_3$ receptors involved in the release of 5-HT. The existence of several subtypes of 5-HT$_3$ receptors is still controversial (Butler *et al.*, 1990; Fozard, 1992; Newberry *et al.*, 1991). Nevertheless, the following points deserve to be emphasized. First, the low micromolar concentrations of the 5-HT$_3$ agonist, 2-methyl-5-HT, required to detect a significant effect on the evoked release of [^{3}H]-5-HT are fully consistent with the micromolar affinity of this drug for 5-HT$_3$ binding sites and with previous results obtained in *in vitro* studies assessing the effect of this 5-HT$_3$ agonist on dopamine and acetylcholine release (Barnes *et al.*, 1989; Blandina *et al.*, 1989; Hoyer, 1990). Furthermore, consistent with previous reports that 2-methyl-5-HT is more potent in rat than in identical guinea-pig preparations (Newberry *et al.*, 1991), preliminary observations from our laboratory indicate that this drug is about 10 fold more potent in enhancing the electrically evoked release of [^{3}H]-5-HT from rat than from guinea-pig frontal cortex slices. Second, the selective 5-HT$_3$ ligand, phenylbiguanide, was ineffective in enhancing the electrically-evoked release of [^{3}H]-5-HT, consistent with previous reports on its lack of agonistic activity in guinea-pig tissues (Butler *et al.*, 1990; Newberry *et al.*, 1991). It is noteworthy that in the present study, phenylbiguanide appeared to act as an antagonist whereas in the study by Butler *et al.* (1990), it did not. This is perhaps attributable to phenylbiguanide having a very weak partial agonist activity at the present 5-HT$_3$ receptors. It is not clear, however, why in the preliminary experiments reported by Galzin *et al.* (1990), phenylbiguanide appeared to be an effective, although weak, 5-HT$_3$ agonist in frontal cortex slices of guinea-pigs. Third, although a 1 μM concentration for the 5-HT$_3$ antagonists is greater than that used in previous studies carried out in the rat, the present results are nevertheless fully congruent with these drugs being one to two log units less potent in guinea-pig preparations than in the rabbit or the rat (Butler *et al.*, 1990; Newberry *et al.*, 1991). Nethertheless, R- and S-zacopride were effective down to a concentration of 10 nM. The relative potency order of the antagonists observed in the present study relates fairly well to the data obtained in the guinea-pig vagus preparation (see Hoyer, 1990). Finally, (+)-tubocurarine was ineffective in the present experiments at a 1 μM concentration. This is also fully consistent with the estimated IC_{50} value of about 10 μM for this drug required to block 5-HT$_3$ responses in guinea-pig ganglionic neurones, as opposed to nanomolar concentrations necessary to block 5-HT$_3$ responses in mouse tissue preparations (Peters *et al.*, 1990; Newberry *et al.*, 1991; Malone *et al.*, 1991).

Since the 5-HT$_3$ antagonist, ICS 205-930, blocked the enhancement of the evoked release of [^{3}H]-5-HT produced by the selective 5-HT reuptake blocker, paroxetine, it thus appears that endogenous 5-HT can activate this facilitatory

5-HT$_3$ receptor. An endogenous activation of this receptor could not be shown in hypothalamic slices by studying the effect of antagonists superfused alone, with the exception of 1 μM S-zacopride which did produce an inhibition of the electrically-evoked release of [^{3}H]-5-HT. This is probably attributable to a property of this drug other than its capacity to block 5-HT$_3$ receptors. Such an endogenous tone also does not seem to be present *in vivo* in the rat frontal cortex and hippocampus, as indicated by the lack of effect of ondansetron and MDL 72222, respectively, on the extracellular level of 5-HT measured by microdialysis (Ge *et al.*, 1991; Martin *et al.*, 1992). It is noteworthy that in the latter report, 2-methyl-5-HT enhanced the extracellular level of 5-HT, and this enhancing effect was prevented by MDL 72222 (Martin *et al.*, 1992). However, given the capacity of this 5-HT$_3$ receptor to desensitize rapidly and the markedly different quantitative effect it can exert over a very narrow range of 5-HT release, it would seem that microdialysis may not be ideally suited to study its influence on the modulation of 5-HT release *in vivo*. A technique which monitors 5-HT release over much shorter time periods would appear more appropriate.

5-HT$_3$ receptors share several characteristics with the nicotinic acetylcholine receptor. For instance, they both belong to the family of ligand-gated ion channels, and a recent molecular biology study has shown that they have an important degree of similarity in their amino acid sequences (Maricq *et al.*, 1991). On a functional basis, the facilitatory 5-HT$_3$ receptors characterized in the present study also present common features with the presynatic nicotinic receptor that was shown to modulate the release of [^{3}H]-acetylcholine in the rat hippocampus and cerebral cortex (Rowell & Winkler, 1984; Aurajo *et al.*, 1988). First, they both subserve the role of enhancing the release of their respective neurotransmitter even in the absence of depolarizing stimuli. Second, they both appear to desensitize rapidly. However, these nicotinic receptors seem to be located on cholinergic terminals, and are thus autoreceptors, whereas the facilitatory 5-HT$_3$ receptors are not. This latter contention was derived from the lack of effect of 2-methyl-5-HT in synaptosomes of the guinea-pig hypothalamus (Blier *et al.*, unpublished). Evidence was recently provided that the 5-HT$_3$ receptors which modulate the release of dopamine are also not located on 5-HT terminals and that the 5-HT$_3$ receptors which modulate the release of cholecystokinin are located on cholecystokinin-containing terminals in the rat nucleus accumbens (Chen *et al.*, 1991; Paundice & Raiteri, 1991). In addition, the 5-HT$_3$ receptors which inhibit the release of [^{3}H]-acetylcholine in the cerebral cortex are located on cholinergic terminals (Barnes *et al.*, 1989; Maura *et al.*, 1992).

Previous studies have documented the capacity of various types of presynaptic receptors to modulate 5-HT release, such as the benzodiazepine type 1 receptor (Lista *et al.*, 1988), and also interactions between receptors demonstrated to be located on the same nerve terminals, such as between the α_2-adrenoceptor and the 5-HT autoreceptor (Blier *et al.*, 1990). In the present study, the blockade of 5-HT$_3$ receptors with ICS 205-930 did not alter the differential effectiveness of an agonist of the terminal 5-HT$_{1D}$ autoreceptor in attenuating the release of [^{3}H]-5-HT at two frequencies of stimulation. Conversely, in the presence of a sustained activation of the terminal 5-HT$_{1D}$ autoreceptor with the piperazine TFMPP, which is devoid of affinity for 5-HT$_3$ binding sites (Hoyer, 1991; Robertson *et al.*, 1992), the effectiveness of 2-methyl-5-HT in enhancing the electrically evoked overflow of [^{3}H]-5-HT was not altered (Figure 6). Taken together, these results indicate that, under the conditions used in the present study, there appeared to be no direct interaction between the facilitatory 5-HT$_3$ receptor and the inhibitory 5-HT$_{1D}$ autoreceptor.

In conclusion, facilitatory 5-HT$_3$ receptors appeared to be present in the frontal cortex, hypothalamus, and hippocampus of the guinea-pig. These receptors can be activated by endogenous 5-HT. Given their capacity to desensitize rapidly and their efficacy in enhancing 5-HT release in conditions when synaptic availability of 5-HT is low, it would appear that these receptors subserve the role of increasing synaptic 5-HT when 5-HT release is at its lowest, even in the absence of impulse flow in 5-HT terminals, as shown by the enhancing effect of a large concentration of 2-methyl-5-HT in the absence of electrical stimulations. Although repeated electroconvulsive shocks did not alter the capacity of these 5-HT$_3$ receptors to modulate the electrically-evoked release of [^{3}H]-5-HT (Blier & Bouchard, 1992), it will be interesting to assess their sensitivity following long-term administration of other antidepressant treatments that have been shown to increase the synaptic availability of 5-HT, such as selective 5-HT reuptake blockers and monoamine oxidase inhibitors.

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Attenuation of tachykinin-induced airflow obstruction and microvascular leakage in immature airways

[1]Kenichi Tokuyama, Tatsuya Yokoyama, Akihiro Morikawa, Hiroyuki Mochizuki, Takayoshi Kuroume & *Peter J. Barnes

Department of Pediatrics, Gunma University School of Medicine, Maebashi, Gunma 371, Japan and *Department of Thoracic Medicine, National Heart and Lung Institute, Royal Brompton Hospital, London SW3

1 To study the effect of maturation on substance P (SP)- and neurokinin A (NKA)-induced airflow obstruction and airway microvascular leakage (MVL), we have measured changes in both lung resistance (R_L) and extravasation of Evans blue dye in anaesthetized immature (aged 14 ± 1 days) and adult guinea-pigs (aged 80 ± 3 days).

2 R_L and its recovery after hyperinflation at 5 min were measured for 6 min after i.v. SP (0.2, 1 and 30 nmol kg^{-1}), NKA (1 and 10 nmol kg^{-1}) or vehicle (0.9% NaCl). After measurement of R_L, MVL in trachea, main bronchi and intrapulmonary airways was also examined.

3 The order of potency in inducing airflow obstruction did not change with age (NKA > SP) but immature animals required a larger dose of SP or NKA than adults to cause a significant increase in R_L.

4 The order of potency in inducing airway microvascular leakage was SP > NKA in both immature and adult animals. The amount of extravasated dye after SP was significantly less in immature airways, especially in central airways.

5 Phosphoramidon (2.5 mg kg^{-1}), a neutral endopeptidase (NEP) inhibitor, significantly increased R_L after 0.2 nmol kg^{-1} SP only in adult airways. Phosphoramidon enhanced the dye extravasation after 0.2 nmol kg^{-1} SP in both immature and adult airways with a significantly greater amount of dye in adult animals, suggesting that mechanisms other than changes in NEP activity may be responsible for this age-related difference.

6 R_L after hyperinflation following SP was not correlated with the degree of extravasation of Evans blue dye in immature animals, whereas it was closely correlated in adult animals.

7 SP and NKA may be less potent in causing both bronchoconstriction and microvascular leakage in immature airways.

8 Airway oedema caused by microvascular leakage may contribute less in immature airways to airflow obstruction after SP or NKA.

Keywords: Aging; airway obstruction; asthma; oedema; neutral endopeptidase; phosphoramidon; plasma exudation; vascular permeability

Introduction

Substance P (SP) and neurokinin A (NKA) are generated from a large precursor preprotachykinin (Nakanishi, 1987) and mediate nonadrenergic noncholinergic (NANC) neural effects in a number of organs including airways (Barnes, 1986). These peptides may mediate neurogenic inflammation in the airways through various pathological effects, including contraction of smooth muscle (Advenier *et al.*, 1987), mucus secretion from submucosal glands (Webber, 1989) and from goblet cells (Tokuyama *et al.*, 1990; Kuo *et al.*, 1990) and increased microvascular leakage from post-capillary venules (Rogers *et al.*, 1988).

The potency of SP or NKA in causing airway inflammation may change with age. Capsaicin, the pungent principle of hot red peppers, is an experimental tool used to deplete primary afferent C-fibres of stored neuropeptides such as SP and NKA (Buck & Burks, 1986). In adult animals, capsaicin treatment causes severe bronchoconstriction and sometimes death, even after pretreatment with bronchodilators (Lundberg *et al.*, 1983). In neonates, by contrast, no mortality is associated with this procedure (App *et al.*, 1991). Maturational changes in the potency of neuropeptides included in the NANC nervous system such as SP and NKA may underlie the different airway responsiveness of neonatal and mature airways following capsaicin treatment. Neutral endopeptidase (NEP) is an enyzme located in the airways and is known to degrade tachykinins to inactive metabolites. A recent observation that NEP activity changes with age (Haver *et al.*, 1992) might also support our speculation. A decrease in tachykinin content in the airways with increased age (Asai *et al.*, 1991) may reflect the presence of maturational changes in the potency of SP or NKA in the airways.

The purpose of this study was to examine the age-related differences in SP- and NKA-induced airflow obstruction and airway microvascular leakage in the same animal, because airway oedema resulting from airway microvascular leakage may be a component of airflow obstruction after aerosolized SP or vagal nerve stimulation in guinea-pigs (Lötvall *et al.*, 1990; 1991). For this purpose, we have measured changes in lung resistance (R_L) and in the amount of extravasated Evans blue dye, a parameter of airway microvascular leakage, following intravenously administered SP and NKA in immature and adult guinea-pigs. Some of the animals were pretreated with phosphoramidon, a NEP inhibitor, in order to study the role of NEP in maturational changes in tachykinin-induced airway responses.

Methods

Experiments were performed on 80 pathogen-free male Dunkin-Hartley guinea-pigs divided into two age groups

[1] Author for correspondence.

(Clerici et al., 1989a,b): immature (197 ± 2 g; 14 ± 1 days) and adult (532 ± 12 g; 80 ± 3 days) animals. Animals were anaesthetized with an initial dose of urethane (6–8 ml kg^{-1}; 25% w/v in 0.9% saline) injected intraperitoneally. Additional urethane was given as required to maintain anaesthesia. A tracheal cannula (10 mm in length and 2.7 mm inner diameter for adults and 8 mm in length and 2.0 mm inner diameter for immature animals) was inserted into the lumen of the cervical trachea through a tracheostomy and secured with a suture. A polyethylene catheter was inserted into the left carotid artery to monitor both blood pressure and heart rate using a pressure transducer. The right external jugular vein was cannulated for the administration of SP, NKA, phosphoramidon or vehicle (0.9% NaCl) and Evans blue dye.

Measurement of airway function

Guinea-pigs were placed in a supine position with the intratracheal cannula connected to a constant volume mechanical ventilator (Model SN-480-7; Shinano Co., Tokyo, Japan). A tidal volume of 10 ml kg^{-1} and a frequency of 60 breaths per min was used. Transpulmonary pressure was measured with a pressure transducer (Model TP-603T; 50 cmH_2O; Nihon Koden Co., Tokyo, Japan), with one side attached to a catheter inserted into the right pleural cavity and the other side attached to a catheter connected to a side port of the intratracheal cannula. The ventilatory circuit had a total volume of 18 ml. Airflow was measured with a pneumotachograph (Model TU-241T; Nihon Koden Co., Tokyo, Japan) connected to a transducer (Model TP-602T; 5 cmH_2O; Nihon Koden Co., Tokyo, Japan). All signals were recorded on a 4-channel recorder (WT-645 GC; Nihon Koden Co., Tokyo, Japan). Lung resistance (R_L) was calculated by the method described by von Neergaard & Wirz (1927).

Protocol

Immature and adult guinea-pigs were divided into six groups each (total 12 groups) in order to compare the effects of serially-diluted SP (0.2, 1 and 30 nmol kg^{-1}; $n = 5$, 5 and 5 in adult and $n = 6$, 6 and 5 in immature animals, respectively), NKA (1, 10 nmol kg^{-1}; $n = 4$ and 5 in adult and $n = 6$ and 5 in immature animals, respectively) or vehicle (0.9% NaCl; $n = 5$ and 5 in adult and immature animals, respectively) on airway microvascular leakage and on lung resistance (R_L) up to 6 minutes after administration. Ten minutes after connection to the ventilator, Evans blue dye (20 mg kg^{-1}) was administered i.v.. One minute later, SP, NKA or the vehicle was administered i.v. R_L, transpulmonary pressure and mean blood pressure were recorded every minute. Five minutes after drug administration, the animals were hyperinflated with twice the tidal volume by manually blocking the outflow of the ventilator, and 1 min later disconnected from the ventilator. In order to examine the role of NEP activity, 4 groups of animals were pretreated with i.v. phosphoramidon (2.5 mg kg^{-1}) 5 min before administration of 0.2 nmol kg^{-1} SP or vehicle. SP (0.2 nmol kg^{-1}) was administered to 5 immature and 5 adult animals; the vehicle was administered to 4 immature and 4 adult animals. We defined R_L at 6 min as 'recovery R_L' (Lötvall et al., 1990; Tokuyama et al., 1991a,b,c), since hyperinflation would be expected to diminish airway smooth muscle contraction and abolish airflow obstruction induced by areas of atelactasis. We calculated 'recovery ratio' as:

$$\text{recovery } R_L/\text{baseline } R_L$$

to compare the degree of recovery in R_L between immature and adult airways, which have significantly different baseline R_L from each other.

Determination of plasma extravasation

After disconnection from the ventilator, the thoracic cavity was opened, and a cannula was inserted into the aorta through the left ventricle. Animals were perfused with 0.9% NaCl at a pressure of 100 mmHg, to remove intravascular dye. The lungs were removed, and the lung parenchyma carefully scraped off. The trachea, main bronchi and intrapulmonary airways (IPA) were separated from each other, and the intrapulmonary airways were divided into two equal portions lengthwise, arbitrarily named proximal (P-IPA) and distal (D-IPA). All tissues were weighed wet. Evans blue dye was extracted in 2 ml of formamide at 40°C for 48 h, and measured in a spectrophotometer (UV-150-02; Shimazu Co., Kyoto, Japan) at 620 nm. The extracted Evans blue dye was quantified by interpolation on a standard curve of dye concentrations in the range of 0.5–10 μg ml^{-1}, and expressed as ng dye mg^{-1} tissue. Evans blue dye extravasation has been previously shown to correlate closely with the extravasation of radiolabelled albumin in guinea-pig airways (Rogers et al., 1989).

Drugs and chemicals

The following drugs and chemicals were used: urethane (Kanto Chemical Co., Tokyo, Japan); Evans blue dye (Aldrich Chemical Co., Milwaukee, U.S.A.); phosphoramidon (Sigma Chemical Co., St. Louis, U.S.A.); 0.9% NaCl (Fuyo Chemical Co., Osaka, Japan); SP and NKA (Peninsula, U.S.A.).

Data analysis

Data are reported as means ± s.e.mean. Non-parametric analysis of variance (Kruskal-Wallis method) was used to determine the significant variance between groups. If a significant variance was found, a Mann-Whitney U-test was performed to assess the significant difference between individual groups. For correlation between variables, Spearman's rank analysis was used. A P value less than 0.05 was considered significant.

Results

Comparison of lung resistance

Baseline R_L in immature animals was significantly higher than that in adult animals in all of the groups studied (Figures 1 and 2). In adults, both 1 and 10 nmol kg^{-1} NKA caused significant increases in R_L compared to the vehicle (0.9% NaCl) with peaks within few seconds (1.31 ± 0.34 and 9.86 ± 2.36 cmH_2O ml^{-1} s^{-1}, respectively). In immature animals, only 10 nmol kg^{-1} NKA induced a significant increase in R_L with a peak at 1 min after the administration (5.85 ± 2.40 cmH_2O ml^{-1} s^{-1}, Figures 1 and 2). As for SP-treated animals, 30 nmol kg^{-1} SP caused a significant increase in R_L in both immature and adult animals with peaks within seconds. In adults receiving 1 nmol kg^{-1} SP, R_L increased gradually to peak at 5 min, while no increase in R_L was observed in immature animals (Figures 1 and 2). In both immature and adult animals receiving 0.2 nmol kg^{-1} SP, no significant increase in R_L was found compared to the control animals (0.9% NaCl). In both immature and adult airways, 10 nmol kg^{-1} NKA induced a significantly higher maximal R_L than 30 nmol kg^{-1} SP (Figure 1). Recovery R_L was still significantly high after 10 nmol kg^{-1} NKA and 1 and 30 nmol kg^{-1} SP in adult animals, while this was the case only after 10 nmol kg^{-1} NKA and 30 nmol kg^{-1} SP in immature animals (Figures 1 and 2). The recovery ratio was significantly less in immature airways after 1 and 30 nmol kg^{-1} SP compared to adult animals receiving corresponding

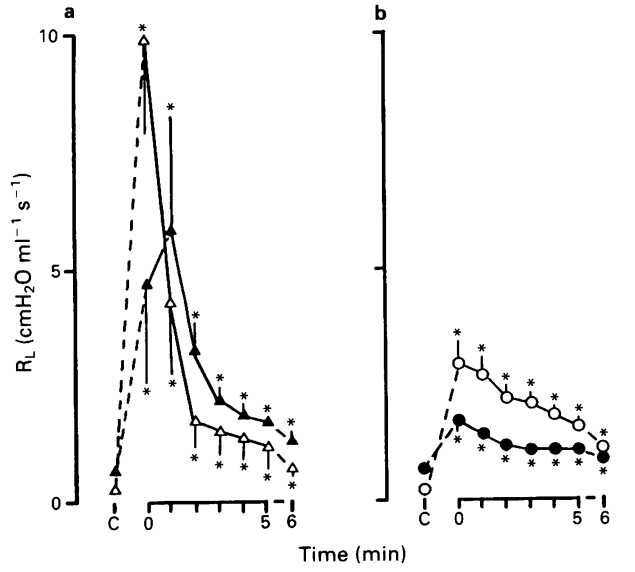


Figure 1 Time-course of changes in lung resistance (R_L) and its recovery after hyperinflation at 5 min after 10 nmol kg^{-1} neurokinin A (NKA) (a) and 30 nmol kg^{-1} substance P (SP) (b). The time-course of R_L after exposures of 10 nmol kg^{-1} NKA and 30 nmol kg^{-1} SP in immature (▲ and ●, respectively) and adult animals (Δ and O, respectively) is shown. Both 10 nmol kg^{-1} NKA and 30 nmol kg^{-1} SP induced a significant increase in R_L within minutes in both age groups. R_L after hyperinflation (recovery R_L) was still significantly higher after either of the tachykinins at these concentrations in both animal groups of different ages. In both immature and adult airways, 10 nmol kg^{-1} NKA induced a significantly higher maximal R_L than 30 nmol kg^{-1} SP. Maximal increase in R_L after either NKA or SP was higher in adult airways than in immature animals receiving corresponding doses of these tachykinins, although it was not significant. The recovery ratio (recovery R_L/baseline R_L) after 30 nmol kg^{-1} SP was significantly less in immature airways than in adult animals receiving a corresponding dose ($P<0.05$).
*$P<0.05$ when compared to controls (0.9% NaCl) at corresponding time points.

doses ($P<0.05$ at both doses). A similar tendency was observed after NKA, although this was not significant.

Comparison of extravasation of Evans blue dye

No significant difference was found between immature and adult animals in the amount of extravasation of Evans blue dye after administration of vehicle (0.9% NaCl) at any airway level (Figure 3). Both SP and NKA produced a greater amount of extravasated dye at trachea and main bronchi than at intrapulmonary airways in either age group. SP induced a greater amount of extravasation than NKA. At 1 nmol kg^{-1}, the amount of extravasated dye was significantly greater after SP than after NKA in both immature and adult airways. A comparison of animals receiving corresponding doses of SP or NKA showed that these tachykinins induced greater amounts of extravasated dye in adult airways, with significant differences after 0.2 nmol kg^{-1} SP in trachea and main bronchi and after 30 nmol kg^{-1} SP in P-IPA. (Figure 3).

Correlation between recovery ratio and extravasated Evans blue dye

In adult animals that were exposed either to SP or to NKA, there was a significant correlation between the degree of extravasation and the recovery ratio in R_L especially in central airways. This was the case in immature airways only after NKA, but not after SP (Table 1).

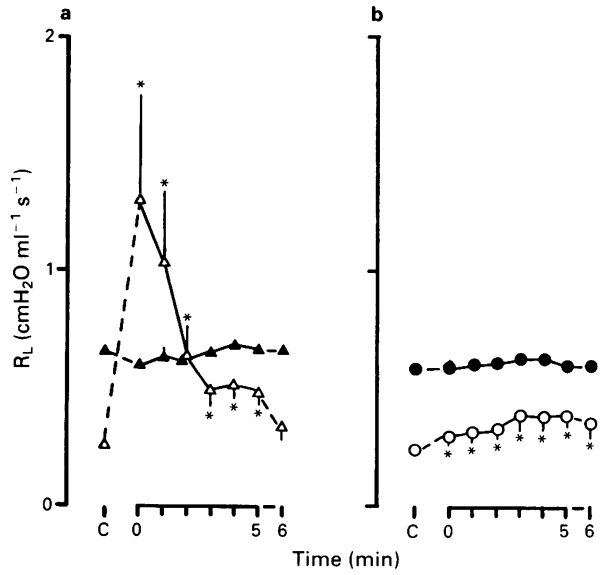


Figure 2 Time-course of lung resistance (R_L) and its recovery after hyperinflation at 5 min after 1 nmol kg^{-1} neurokinin A (NKA) (a) and 1 nmol kg^{-1} substance P (SP) (b). The time-course of R_L after exposures to 1 nmol kg^{-1} NKA and SP in immature (▲ and ●, respectively) and adult animals (Δ and O, respectively) is shown. Both NKA and SP induced significant increases in R_L in adult airways, although this was not the case in immature airways. In adult airways, maximal increase in R_L was found within seconds after i.v. NKA, while i.v. SP induced an increase in R_L with gradual onset, peaking at 5 min after administration with persistent elevation of recovery R_L. *$P<0.05$ when compared with controls (0.9% NaCl) at corresponding time points.

Effect of phosphoramidon pretreatment

In both immature and adult animals receiving the vehicle for SP, the time-course in R_L was not significantly different between groups pretreated with either phosphoramidon or the vehicle. In adults, phosphoramidon caused a significant increase in R_L but not in immature animals. The increase in R_L in adult animals was of gradual onset with a prolonged elevation even after hyperinflation (Figure 4). Phosphoramidon produced no significant difference in extravasation of Evans blue dye after the vehicle for SP between immature and adult animals at all airway levels (Figure 5). Compared to corresponding animals receiving SP after the vehicle pretreatment, phosphoramidon enhanced SP-induced dye extravasation with percentage increases of 73% and 103% at trachea, 65% and 136% at main bronchi, 34% and 167% at proximal intrapulmonary airways and 5% and 75% at distal intrapulmonary airways in immature and adult airways, respectively.

Changes in blood pressure

Both intravenous SP and NKA caused a dose-dependent fall in systemic blood pressure (Table 2). There were no significant differences between immature and adult animals receiving corresponding doses of SP or NKA. Phosphoramidon significantly enhanced the decrease in blood pressure to a similar degree in immature and adult animals (Table 3).

Discussion

We have shown that the order of potency in inducing airflow obstruction is NKA> SP in both immature and adult guinea-pigs. Immature animals required a larger dose of SP or NKA than adults to achieve a similar degree of airflow obstruction.

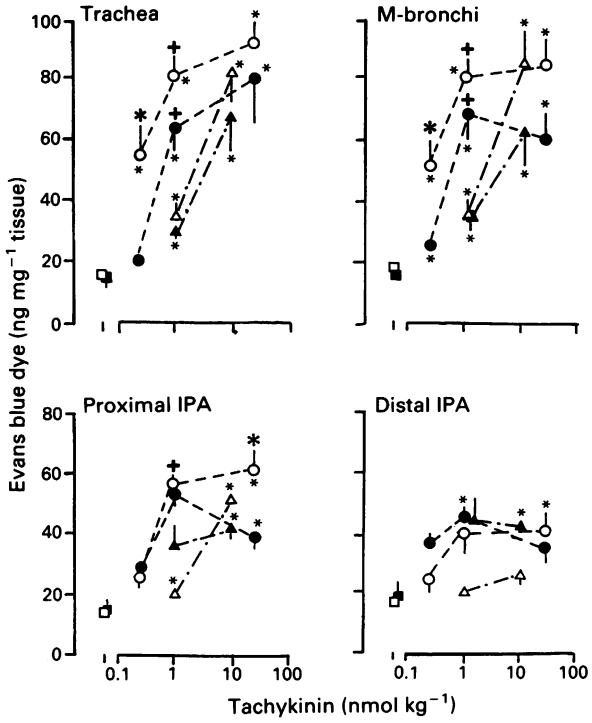


Figure 3 Airway microvascular leakage after i.v. substance P (SP) and neurokinin A (NKA) in immature and adult guinea-pigs. Evans blue dye extravasation at 4 different airway sites is shown. In adult:(○) SP; (△) NKA; (□) vehicle. In immature guinea-pig:(●) SP; (▲) NKA; (■) vehicle. No age-related difference in Evans blue dye extravasation was observed after vehicle (0.9% NaCl). Both SP and NKA produced greater amounts of extravasated dye at trachea and main bronchi (M-bronchi) than at intrapulmonary airways (IPA) in animals of both age groups. SP induced greater amounts of extravasated dye than NKA in both immature and adult airways. $*P<0.05$ when compared with control animals (0.9% NaCl) at corresponding ages. $^{+}P<0.05$ when comparing animals receiving corresponding doses of NKA (1 nmol kg^{-1}) at corresponding ages. $*P<0.05$ when compared with immature animals receiving corresponding doses of SP.

Table 1 Correlation between recovery ratio in R_L and extravasation of Evans blue dye at different airway levels after intravenous substance P (SP) or neurokinin A (NKA)

	n	*Trachea*	*Main bronchi*	*P-IPA*	*D-IPA*
Substance P					
Adult	15	0.67**	0.63*	0.47	0.42
Immature	17	− 0.19	− 0.36	− 0.44	− 0.52*
Neurokinin A					
Adult	9	0.70*	0.78*	0.40	0.05
Immature	11	0.75**	0.70*	− 0.09	− 0.23

Values are Spearman rank correlation coefficients (r_s). P- and D-IPA:proximal and distal intrapulmonary airways. $*P<0.05$ and $**P<0.01$.

By contrast, the order of potency in causing extravasation of Evans blue dye was SP>NKA in both age groups. The degree of extravasation was less in immature airways than in the airways of adult animals. Phosphoramidon did not affect the difference between immature and adult animals.

Exogenous SP and NKA induce both airway smooth muscle contraction and airway microvascular leakage in guinea-pigs (Saria *et al.*, 1983, Rogers *et al.*, 1988). It has been suggested that SP and NKA induce these effects by activating two distinct receptors, called neurokinin-1 (NK_1)

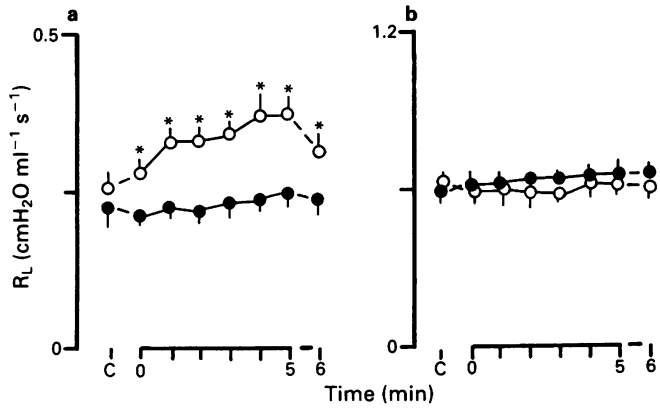


Figure 4 Effect of phophoramidon on time-course of changes in lung resistance (R_L) and its recovery after hyperinflation at 5 min after 0.2 nmol kg^{-1} substance P (SP) in adult (a) and immature airways (b). The time-course of R_L after exposures to 0.2 nmol kg^{-1} SP with and without pretreatment of phosphoramidon (○ and ●, respectively) is shown. A significant increase in R_L was observed only in adult animals receiving phosphoramidon. The increase in R_L was of gradual onset, peaking at 5 min after administration with persistent elevation of recovery R_L. $*P<0.05$ when compared to controls (0.9% NaCl) at corresponding time points.

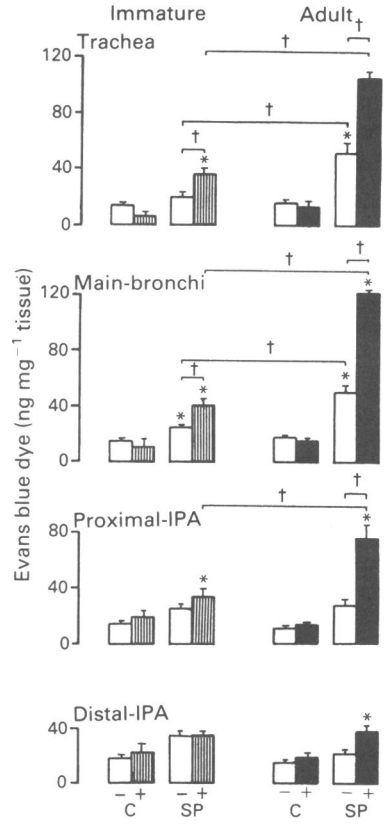


Figure 5 Effect of phosphoramidon on airway microvascular leakage after i.v. substance P (SP) in immature and adult guinea-pigs. Evans blue dye extravasation at 4 different airway sites is shown. + and − represent animals pretreated with phosphoramidon and its vehicle (0.9% NaCl), respectively. SP and C represent animals receiving intravenous substance P and its control (0.9% NaCl), respectively. $*P<0.05$ when compared to control animals of corresponding ages (pretreated with 0.9% NaCl and injected vehicle for SP). $\dagger P<0.05$: phosphoramidon significantly enhanced dye extravasation both in immature and in adult airways, especially at central airways.

Table 2 Percentage change in mean systemic blood pressure after i.v. administration of substance P or neurokinin A in immature and adult guinea-pigs

		Animals	
		Immature	*Adult*
Control (0.9% NaCl)		4.6 ± 7.9	0.5 ± 1.7
Substance P (nmol kg^{-1})	0.2	− 33.0 ± 4.9#	− 32.6 ± 3.0#
	1	− 57.2 ± 3.0#	− 38.6 ± 7.5#
	30	− 70.4 ± 2.0#	− 57.9 ± 5.3#
Neurokinin A (nmol kg^{-1})	1	− 26.5 ± 5.6#	− 21.6 ± 6.3#
	10	− 41.7 ± 8.3#	− 33.9 ± 3.1#

Values are mean ± s.e.;
$P < 0.05$ compared to control animals of corresponding ages. No significant differences were found in the degree of fall in systemic blood pressure between immature and adult animals after corresponding doses of substance P or neurokinin A.

Table 3 Effect of phosphoramidon on substance P-induced percentage change in mean systemic blood pressure in immature and adult guinea-pigs

	Animals	
Pretreatment	*Immature*	*Adult*
0.9% NaCl	− 33.0 ± 4.9	− 32.6 ± 3.0
Phosphoramidon	− 56.0 ± 2.9#	− 46.6 ± 4.0#

Values are mean ± s.e.; % fall in mean systemic blood pressure was significantly greater in all groups given substance P (SP) compared to animals receiving 0.9% NaCl after a pretreatment with 0.9% NaCl with corresponding age.
$P < 0.05$ compared to the age-matched animals given SP following a pretreatment with 0.9% NaCl. No significant difference was found in the degree of fall in systemic blood pressure between immature and adult animals receiving the same pretreatment.

and neurokinin-2 (NK_2) receptors (Iversen, 1985). SP is believed to be more potent in stimulating NK_1 receptors, and NKA more potent in stimulating NK_2 receptors (Iversen, 1985). A third receptor subtype, neurokinin-3 (NK_3) receptor, is more specific for neurokinin B (NKB), but this peptide has not been found in guinea-pig airways (Hua *et al.*, 1985). The NK_1 receptor is believed to be predominant in the vasculature, and mediates vasodilatation and microvascular leakage, whereas the NK_2 receptor mediates airway smooth muscle contraction (Iversen, 1985). In the present study, increases in R_L peaked immediately after exposures to SP and NKA with an exception after 1 nmol kg^{-1} SP in adult animals, suggesting that smooth muscle contraction plays a major role in SP- and NKA-induced airflow obstruction. The maximal increase in R_L was significantly greater after NKA than after SP in both age groups. Therefore, our study may suggest that the predominant tachykinin receptor for airway smooth muscle contraction is NK_2, both in immature and in adult airways. Studies using specific NK_1 and NK_2 receptor agonists will clarify our speculation. SP and NKA caused less increase in R_L in immature airways than in the airways of adult animals. As previously observed (Clerici *et al.*, 1989a,b; Arakawa *et al.*, 1992), baseline R_L was significantly higher in immature animals, presenting smaller airway diameters in resistance airways than in adult animals. Smaller initial airway diameters have been shown to cause a higher increase in airflow resistance to the constrictor stimuli, because resistance is inversely proportional to the fourth power of the airway radius. Our results, therefore, suggest that tachykinins cause less airflow obstruction in immature airways. Our data may confirm previous observations concerning the different responsiveness to capsaicin treatment between adults and neonates in rats. In contrast, Grunstein *et al.* (1984) showed that airway sensitivity to SP diminished with age in rabbits. The discrepancy between their results and ours might result from interspecies variability. We did not determine the mechanism of the age-related difference in response to SP or NKA in the present study. The number of tachykinin receptors may change with age. Several enzymes to degrade SP and NKA such as neutral endopeptidase have been shown to be present in the airways (Johnson *et al.*, 1985). Enzymatic activity may be greater in immature airways. However, this is unlikely, because our results showed that phosphoramidon, a NEP inhibitor, increased both R_L and microvascular leakage in adult rather than in immature airways. A decrease in the quantity of airway smooth muscle in immature airways, as has been described in human airways (Matsuba & Thurlbeck, 1972) may contribute to this age-related difference. Another possibility is that, as discussed later, less microvascular leakage in immature airways may partly result in less airflow obstruction after SP or NKA administration.

In the present study, SP and NKA caused greater amounts of extravasation of dye in trachea and main bronchi than in intrapulmonary airways in both immature and adult airways. These results confirm previous observations that electrical stimulation of the vagus nerve causes a significant increase in plasma extravasation at trachea and main brochi, but not at intrapulmonary airways (Belvisi *et al.*, 1988), and i.v. SP and NKA caused a greater amount of microvascular leakage in central airways than in intrapulmonary airways (Rogers *et al.*, 1988). Airway microvascular leakage was greater after SP than NKA in both immature and adult animals. These results may suggest that the predominant tachykinin receptor mediating airway microvascular leakage is the NK_1 receptor in both age groups. Again, direct studies using specific NK_1 and NK_2-receptor agonists will solve this question. Both SP and NKA caused less microvascular leakage in immature than in adult airways, especially in trachea and main bronchi. We do not know the mechanism for this age-related difference in airway microvascular leakage. The numbers of tachykinin receptors in postcapillary venules, which is the site of plasma exudation, may increase with age. The activity or distribution of enzymes which degrade tachykinins may also change with age. However, this is unlikely because, as mentioned above, phosphoramidon enhanced dye extravasation in adult rather than in immature airways. In an *in vitro* study using rabbit trachea, Haver *et al.* (1992) suggested that NEP activity may increase in adult compared to neonatal animals. Our present *in vivo* results confirm their findings and suggest that NEP is not responsible for the reduced extravasation of dye after SP administration in immature airways. Age-related changes in blood flow, especially in trachea and main bronchi, might also be a reason for this difference, since blood flow is a major determinant of the degree of microvascular leakage in airways (Persson, 1987). However, this is also unlikely because, in a previous study (Arakawa *et al.*, 1992), we have shown that histamine, a potent mediator in inducing airway microvascular leakage, caused a similar degree of extravasation in trachea and main bronchi in both immature and adult guinea-pigs. Furthermore, histamine induced a significant increase in extravasated dye in intrapulmonary airways in immature animals compared to adults. Changes in systemic blood pressure after SP or NKA might result in a different response between immature and adult animals. This is also unlikely, because no difference in systemic blood pressure response to tachykinins was found between the two groups.

In adult airways, we have shown that airway microvascular leakage may partly contribute to airflow obstruction

induced by aerosolised histamine (Tokuyama *et al.*, 1991a), platelet-activating factor (PAF) (Tokuyama *et al.*, 1991b,c) and SP (Lötvall *et al.*, 1990). The present study suggests that microvascular leakage may also contribute to airflow obstruction, at least in part, after intravenous SP or NKA in adult airways and to a greater extent than that in immature animals, since SP and NKA induce a greater amount of leakage in adult airways. Hyperinflation of the lung would be expected to diminish airway smooth muscle constriction (Lötvall *et al.*, 1990) and abolish airflow obstruction induced by areas of atelectasis, whereas airway narrowing induced by thickening of the airway wall caused by airway microvascular leakage would be less readily reversed. In the present study, R_L after hyperinflation (recovery R_L) was significantly higher in adult animals receiving 1 and 30 nmol kg^{-1} SP and 10 nmol kg^{-1} NKA compared to the vehicle controls, and the recovery ratio (recovery R_L/baseline R_L) was greater in adult than in immature airways. We also found a significant correlation between the recovery ratio and the amount of extravasated dye in the airways especially in the trachea and main bronchi. In addition, the increase in R_L after 1 nmol kg^{-1} SP, as well as after 0.2 nmol kg^{-1} SP following phosphoramidon pretreatment, was of gradual onset, peaking at 5 min with recovery R_L significantly higher than that of the vehicle. The time-course is similar to that seen after inhaled PAF which causes small increases in R_L but induces marked degrees of microvascular leakage throughout the tracheobronchial tree in the guinea-pig (Tokuyama *et al.*, 1991b,c). One possibility is that increases in R_L after 1 nmol kg^{-1} SP in adult animals might be secondary to an increase in microvascular leakage, which would lead to oedema formation in the airways. However, more direct evidence is needed to support this hypothesis. In immature airways, the recovery ratio was lower than in adults, with a significant difference after SP, indicating that the response after SP or NKA may be more easily reversible in immature than in adult airways. Furthermore, the recovery ratio in animals receiving SP did not correlate with the amount of extravasation of dye in proximal airways and even correlated inversely in D-IPA. We do not know the reason for this inverse relationship. However, these results suggest that, in immature airways, microvascular leakage may contribute less to airflow obstruction than in adult animals.

We have demonstrated that immature airways show less responsiveness both in airflow obstruction and in airway microvascular leakage caused by SP or NKA. The exact mechanism for this age-related difference in airway responsiveness is unclear and deserves further study.

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Role of extracellular and intracellular sources of Ca^{2+} in sarafotoxin S6b-induced contraction of strips of the rat aorta

Chiaki Watanabe, Katsuya Hirano & [1]Hideo Kanaide

Division of Molecular Cardiology, Research Institute of Angiocardiology, Faculty of Medicine, Kyushu University, Fukuoka, Japan

1 The effect of sarafotoxin S6b (sarafotoxin), a vasoconstrictor peptide, on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and force in rat aortic strips loaded with fura-2 was determined by front-surface fluorometry. The objective was to elucidate the role of extracellular and intracellular Ca^{2+} in the mechanism of action of this peptide.

2 In the presence of extracellular 1.25 mM Ca^{2+}, sarafotoxin induced a biphasic response consisting of an initial rapid increase in $[Ca^{2+}]_i$ followed by a secondary sustained increase. Tension developed slowly but was sustained during the application of sarafotoxin. Diltiazem (10 nM–0.1 mM) partially inhibited both the increases in $[Ca^{2+}]_i$ and tension.

3 In the presence of extracellular Ca^{2+}, the force developed in relation to the increase in $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$-force relationship) observed with sarafotoxin was much greater than that observed upon K^+ depolarization. In the presence of diltiazem the sarafotoxin-induced $[Ca^{2+}]_i$-force relationship was shifted even further to the left.

4 In the absence of extracellular Ca^{2+}, sarafotoxin induced a transient increase in $[Ca^{2+}]_i$ and a sustained contraction. Extending the incubation time in Ca^{2+}-free physiological solution, resulted in smaller responses. However, after 60 min in Ca^{2+}-free solution, sarafotoxin induced a sustained contraction but no change in $[Ca^{2+}]_i$. This residual contraction was inhibited by H-7, which is known to inhibit protein kinase C.

5 After treatment with caffeine to reduce intracellular stored Ca^{2+}, sarafotoxin could still elicit increases in $[Ca^{2+}]_i$ and in tension, showing that the caffeine-sensitive intracellular Ca^{2+} store partially overlaps with the sarafotoxin-sensitive store.

6 We conclude that, in addition to those components of contraction dependent on extracellular- and on intracellularly stored Ca^{2+}, sarafotoxin can also induce contraction without increasing $[Ca^{2+}]_i$. This component may be partially linked to the activation of protein kinase C and may contribute, in part, to the leftward shift of the $[Ca^{2+}]_i$-force relationship in the presence of sarafotoxin.

Keywords: Sarafotoxin; cytosolic free calcium; endothelin; protein kinase C

Introduction

Sarafotoxin S6b (sarafotoxin) is a highly potent vasoconstrictor peptide isolated from the venom of the snake *Atractaspis engaddensis* (Kochva *et al.*, 1982). Its peptide sequence is homologous to that of endothelin-1 and consists of 21 amino acids including four cysteines in identical positions in both peptides (Lee & Chiappinelli, 1988; Takasaki *et al.*, 1988b). Sarafotoxin causes cardiac arrest in mice, probably due to coronary vasoconstriction (Lee *et al.*, 1986; Takasaki *et al.*, 1988a). It has been reported that sarafotoxin specifically inhibits binding of endothelin-1 to membranes prepared from rat brain and atria (Ambar *et al.*, 1989) and cultured rat vascular smooth muscle cells (Hirata *et al.*, 1989). It is known that sarafotoxin-induced contractile responses are associated with increases in phosphatidylinositol phosphate turnover (Kloog *et al.*, 1988; Galron *et al.*, 1990) and in $[Ca^{2+}]_i$ (Hirata *et al.*, 1989). However, the detailed mechanisms of sarafotoxin-induced vasoconstriction, and the relationship between changes in $[Ca^{2+}]_i$ and force during sarafotoxin-induced contractions, required clarification. We have made use of front-surface fluorometry to determine simultaneously changes in $[Ca^{2+}]_i$ and force during sarafotoxin-induced contractions in fura-2-loaded strips of rat aorta.

[1] Author for correspondence.

Methods

Tissue preparation

Male Wistar rats (12–13 weeks old, 350–400 g) were anaesthetized with intraperitoneally administered pentobarbitone (2 ml kg^{-1}) and exsanguinated from the femoral artery. The thoracic aorta was excised and the surrounding tissue was mechanically removed. The aorta was opened longitudinally and the endothelial cells rubbed off with a cotton swab. The preparations thus obtained were cut into transverse strips about 1 mm wide. The tissue density of the strip was assumed to be 1.05 g cm^{-3} (Murphy *et al.*, 1974) and the cross-sectional area of each strip was calculated from the following equation: cross-sectional area (m^2) = wet weight (mg)/(length (mm) × 1.05 (g cm^{-3}) × 10^6). The mean value of cross-sectional area was $1.57 \pm 0.28 \times 10^{-7}$ m^2 ($n = 10$).

Fura-2 loading and force measurement

Aortic strips were incubated in a physiological salt solution (composition in mM: NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25, and D-glucose 11.5), 50 μM fura-2/AM, 0.08% cremophore and 1 mM probenecid (Di Virgilio *et al.*, 1988), and bubbled with a mixture of 95% O_2 and 5% CO_2 for 3 h at 37°C. The strips were then mounted vertically between hooks in a 10 ml quartz organ bath and left in normal physiological salt solution with

1.0 mM probenecid for about 90 min to remove extracellular fura-2. During this period, the strip was repeatedly stimulated at 10 min intervals with 118 mM K^+-containing solution (prepared by equimolar replacement of Na^+ by K^+ in the physiological salt solution) for 5–10 min until a steady force development was obtained (5–6 times) and the basal tension in physiological salt solution was readjusted to 200–300 mg. Maximal force elicited in K^+ solution was taken as 100%. It has been reported that probenecid, a blocker of organic anion transport, prevents both secretion and intracellular sequestration of fura-2 in macrophages without affecting resting or stimulated $[Ca^{2+}]_i$ levels (Di Virgilio *et al.*, 1988). In the present study, exposure to 1 mM probenecid for 30 min had no apparent effect on contractility. Loading with fura-2 did not alter the time course of force development nor the maximum force attained. Contractions were measured with a force transducer (TB-612T, Nihon Koden, Tokyo, Japan) and results were expressed as a percentage of the maximal response elicited in 118 mM K^+-containing solution (Hirano *et al.*, 1990).

Front-surface fluorometry

Fura-2 fluorescence was measured with a front-surface fluorometer specially designed for fura-2 fluorometry (CAM-OF-1), with the collaboration of Japan Spectroscopic Co., Tokyo, Japan (Hirano *et al.*, 1990; Abe *et al.*, 1990). In brief, strips were illuminated by guiding alternating (400 Hz) 340 nm and 380 nm, excitation light from a xenon light source through quartz optic fibres arranged in a circle (3 mm diameter). Surface fluorescence of the strips was collected by glass optic fibres arranged in a circle around the excitation fibres (7 mm diameter) and introduced into a photomultiplier through a 500 nm band-pass filter (10 nm full width at half maximal transmission). The distance between the arterial strip and the end of the optic fibres was kept constant and as short as possible during each measurement. The ratio of the fluorescence at 340 nm excitation (F_{340}) to that at 380 nm excitation (F_{380}) was expressed as a percentage of the maximal response elicited in 118 mM K^+-containing solution. The absolute value of $[Ca^{2+}]_i$ was calculated from the fluorescence ratio by the equation of Grynkiewicz *et al.* (1985) and a dissociation constant of 224 nM, and is shown in parentheses following the fluorescence ratio in the text. The statistical significance of changes in $[Ca^{2+}]_i$ was determined by using values of fluorescence ratio. To examine the release of Ca^{2+} from intracellular store sites, the strip was exposed to Ca^{2+}-free solution (composition similar to physiological salt solution except that it contained 2 nM ethylene glycol-*bis* (β-aminoethyl ester)-N, N, N′, N′-tetraacetic acid (EGTA) instead of 1.25 mM $CaCl_2$) for at least 10 min before the application of stimuli. When the effects of diltiazem, a Ca^{2+} entry blocker, or H-7, a relatively specific inhibitor of protein kinase C (Hidaka *et al.*, 1984) on sarafotoxin-induced contraction were examined, the compounds were applied 15 min before and during the application of sarafotoxin, because 15 min are required for equilibration of binding of diltiazem to the sarcolemma (Miwa *et al.*, 1986). To examine the contribution of Ca^{2+} present in caffeine-sensitive stores on the sarafotoxin-induced contraction, these were practically depleted by 4 different applications of 20 mM caffeine in Ca^{2+}-free solution before the application of sarafotoxin, according to the following protocol. The first application of caffeine was at 5 min in Ca^{2+}-free solution followed by wash out when the increase in $[Ca^{2+}]_i$ reached a peak. After three successive applications of caffeine in Ca^{2+}-free solution, sarafotoxin was finally applied; i.e. after 15 min in Ca^{2+}-free solution. We have already reported that repetitive applications of caffeine in Ca^{2+}-free solution completely depleted Ca^{2+} present in caffeine-sensitive stores in cultured vascular smooth muscle cells (Kobayashi *et al.*, 1986; Kanaide *et al.*, 1987; Matsumoto *et al.*, 1990) and vascular strips (Kodama *et al.*, 1989; Abe *et al.*, 1990). The $[Ca^{2+}]_i$-force relationship for K^+-depolarization-induced contraction was obtained in the following manner: the strips were exposed to Ca^{2+}-free solution containing 2 mM EGTA for 10 min, and then to 118 mM K^+ Ca^{2+}-free solution without EGTA. During exposure to 118 mM K^+ solution, extracellular Ca^{2+} was applied in a step-wise manner, from 0 to 5 mM.

Materials

Sarafotoxin S6b was purchased from Peptide Institute (Osaka, Japan). Caffeine and noradrenaline were purchased from Sigma Co. Ltd, (St Louis, Mo, U.S.A.). H-7 (1-(5-isoquinoline sulphonyl)-2-methylpiperazine dihydrochloride) and fura-2 were purchased from Seikagaku-Kogyo Co. Ltd, (Tokyo, Japan) and Molecular Probes Inc (Eugene, Or, U.S.A.), respectively. Diltiazem, a calcium entry blocker, was kindly donated by Tanabe Seiyaku (Osaka, Japan).

Data analysis

Value are expressed as mean ± s.e.mean Student's *t* test and Duncan's multiple range test were used to determine the statistical significance. Differences were considered significant at a probability of <0.05 using a two tailed test.

Results

Effects of sarafotoxin on $[Ca^{2+}]_i$ and force developed in the presence of extracellular Ca^{2+}

Figure 1 shows representative recordings of changes in the F_{340}: F_{380} ratio and in force induced by exposure to 118 mM K^+-containing solution and then to 0.1 μM sarafotoxin. Exposure to 118 mM K^+-containing solution caused an abrupt increase in F_{340} and a decrease in F_{380} and therefore increased their ratio. There was a slow increase in force, and steady levels of fluorescence ratio and force were reached after about 10 min. In the presence of 0.1 μM sarafotoxin, $[Ca^{2+}]_i$ increased rapidly to its peak, fluorescence ratio of

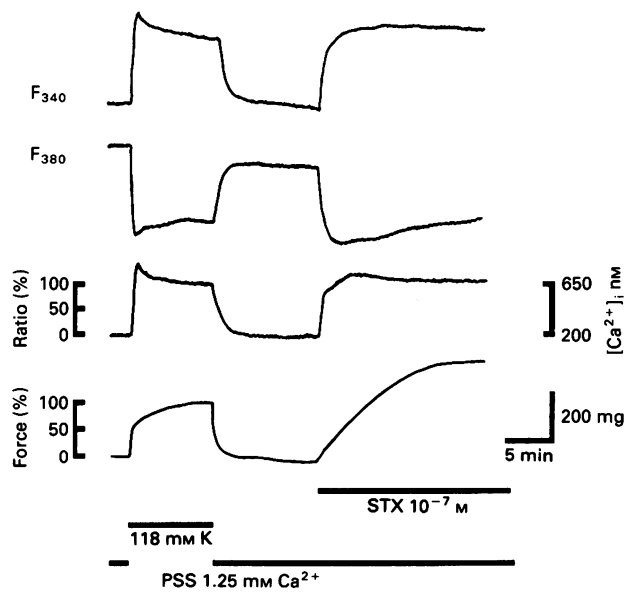


Figure 1 Representative time courses of changes in $[Ca^{2+}]_i$ and force of strips from rat aorta induced by 0.1 μM sarafotoxin (STX) in solution containing 1.25 mM Ca^{2+}. The traces of F_{340} and F_{380} show the changes in 500 nm fluorescence intensities evoked at 340 nm and 380 nm, respectively. Ratio is the F_{340}/F_{380} fluorescence intensity quotient. The bottom trace shows the simultaneously monitored force development. Prior to the application of sarafotoxin, the vascular strip was exposed to 118 mM K^+ solution to determine 100% changes of ratio and force.

111.6 ± 8.6% ($n = 6$), ([Ca^{2+}] = 744.9 nM). The force developed gradually to reach the peak within about 15 min and then remained at this level (170.1 ± 22.7%, $n = 6$) during the application of sarafotoxin. The effects of 1 nM to 1 μM sarafotoxin on both force development and $[Ca^{2+}]_i$ were concentration-dependent (Figure 2). The EC_{50} for force development was approximately 5.2 nM.

The effects of 10 nM to 0.1 mM diltiazem on the time course of changes in $[Ca^{2+}]_i$ and force development induced by 0.1 μM sarafotoxin are shown in Figure 3. At concentrations greater than 1 μM diltiazem significantly decreased the resting $[Ca^{2+}]_i$ level but did not affect the resting force. Between 0.1 μM and 0.1 mM diltiazem concentration-dependently inhibited both the early rapid and the late sustained increase in $[Ca^{2+}]_i$ elicited by sarafotoxin and also reduced maximal force development. A 0.1 mM concentration of diltiazem inhibited increases in $[Ca^{2+}]_i$ and force induced by 0.1 μM sarafotoxin only by about 50%. However, in the case of contractions induced by the presence of 118 mM K^+, 0.1 mM diltiazem almost completely inhibited both the increase in $[Ca^{2+}]_i$ and the force development.

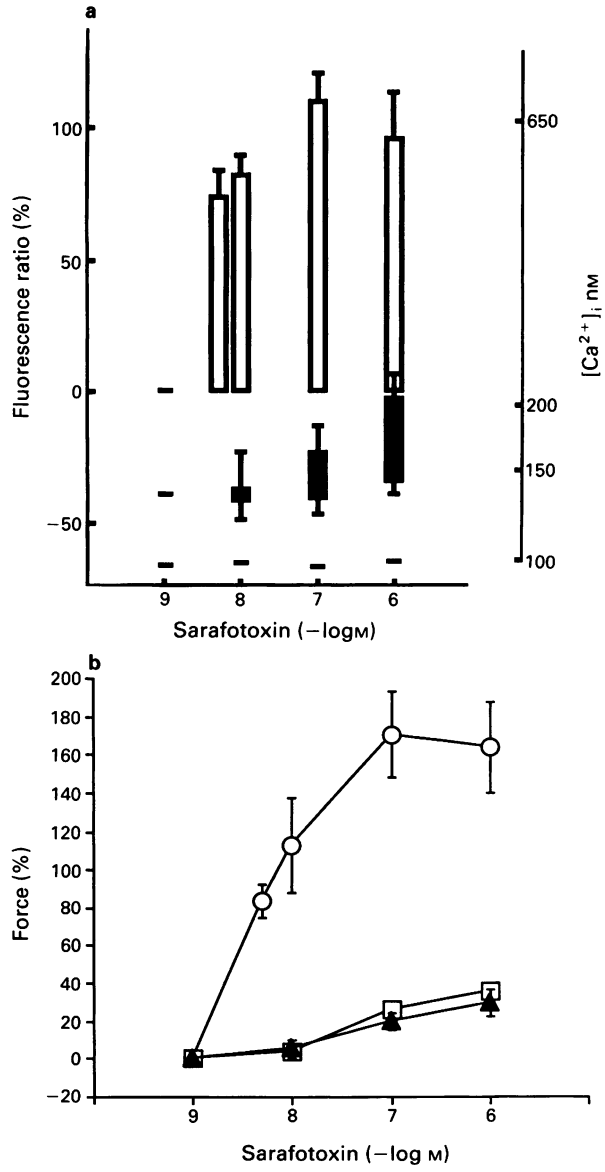


Figure 2 Concentration-dependent effect of sarafotoxin on $[Ca^{2+}]_i$ (a) and force (b). Increases in $[Ca^{2+}]_i$ and force development were measured at their peaks. (a) Open columns, in 1.25 mM Ca^{2+}-containing solution; solid columns after 15 min exposure to Ca^{2+}-free solution; bars, after 60 min exposure to Ca^{2+}-free solution. The bottom and top of each column indicate the peak values of the ratio just before and after application of sarafotoxin, respectively. No change in $[Ca^{2+}]_i$ was observed by sarafotoxin-application following 60 min of exposure to Ca^{2+}-free solution. (b) Responses in 1.25 mM Ca^{2+}-containing solution (○) and after 15 (□) and 60 (▲) min exposure to Ca^{2+}-free solution. The vertical bars indicate s.e. mean.

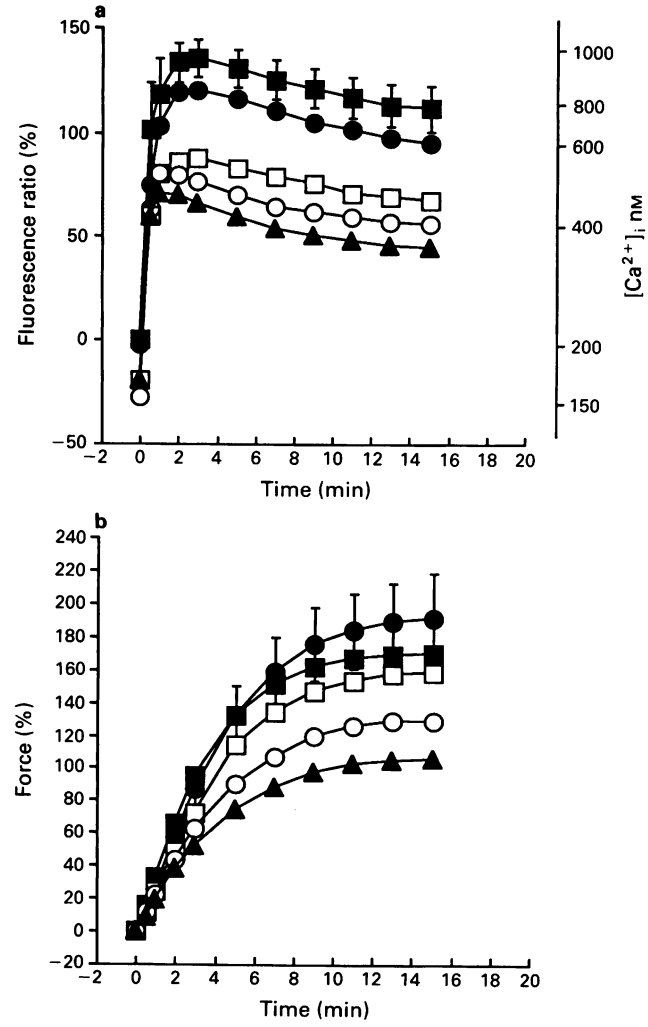


Figure 3 Effects of various concentrations of diltiazem (■, zero; ●, 0.1 μM; □, 1 μM; ○, 10 μM; ▲, 0.1 mM) on sarafotoxin-induced increases in $[Ca^{2+}]_i$ (a) and force development (b). Data are mean ± s.e.mean of 4 observations. Vertical bars on the upper curve represent s.e. mean. Standard errors associated with the other points were of a similar magnitude.

Effects of sarafotoxin on $[Ca^{2+}]_i$ and force in the absence of extracellular Ca^{2+}

Figure 4 shows time courses of $[Ca^{2+}]_i$ and force development in aortic strips induced by 0.1 μM sarafotoxin, after they had been exposed to Ca^{2+}-free solution containing 2 mM EGTA. When these strips were exposed to Ca^{2+}-free solution, resting $[Ca^{2+}]_i$ gradually decreased (fluorescence ratio: −39.2 ± 5.2%, $n = 5$; $[Ca^{2+}]_i$ 138.1 nM) at 15 min without a change in force. Applications of sarafotoxin at this time induced transient concentration-dependent increases in $[Ca^{2+}]_i$ and a maximum was reached at about 40 s. A reversion of pre-sarafotoxin levels occurred within 2 min (Figure 2a). Force developed gradually, reached a maximum after about 10 min (25.9 ± 3.4%, $n = 5$) and remained steady even though $[Ca^{2+}]_i$ returned to pre-sarafotoxin levels (Figure 4a). The maximum increase in force induced by 1 μM sarafotoxin after 15 min in Ca^{2+}-free solution was 35.5 ± 4.0% ($n = 4$) and its EC_{50} was

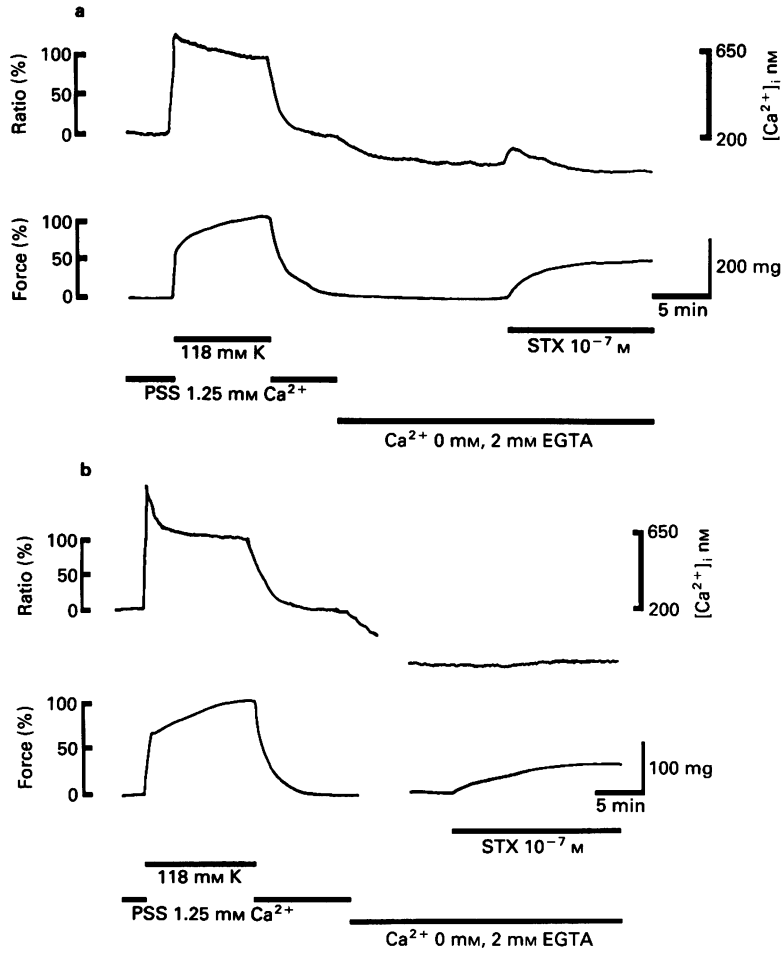


Figure 4 Representative time course of changes in $[Ca^{2+}]_i$ and force induced by 0.1 μM sarafotoxin (STX) after 15 min (a) and 60 min (b) exposure to Ca^{2+}-free solution containing 2 mM EGTA.

about 44 nM. Sarafotoxin (1 μM) induced the development of a peak force of 35.5% (after 10 min), whereas the peak $[Ca^{2+}]_i$ obtained (after 40 s) was only $-0.55 \pm 7.2\%$ (indicating a $[Ca^{2+}]_i$ of 207.3 nM; $n = 4$). Hence the peak $[Ca^{2+}]_i$ was less than that observed in the tissue at rest before exposure to Ca^{2+}-free solution. Thus, under these conditions, more force was developed relative to increases in or absolute $[Ca^{2+}]_i$ levels than was the case for K^+-depolarization.

Increases in $[Ca^{2+}]_i$ and force development induced by 0.1 μM sarafotoxin, were reduced in a time-dependent manner by incubations in Ca^{2+}-free solution (Figure 5). Tissues exposed to Ca^{2+}-free solution for 60 min showed no increase in $[Ca^{2+}]_i$ on exposure to 1 nM to 1 μM sarafotoxin (Figures 2a and 4b). However, force developed gradually under these conditions and a steady state level was reached ($19.7 \pm 3.8\%$, $n = 3$). Thus sarafotoxin was able to induce a sustained, concentration-dependent, extracellular and intracellular Ca^{2+}-independent contraction (Figures 2b, 4b and 5). In strips exposed to Ca^{2+}-free solution for 60 min and then to normal physiological solution for 10 min, a subsequent exposure to a 118 mM K^+-containing solution induced increases in fluroescence ratio and force similar to those observed before exposure to Ca^{2+}-free solution. When tissues were exposed to 10 μM H-7, the Ca^{2+}-independent contraction induced by sarafotoxin after a 60 min exposure to Ca^{2+}-free solution was inhibited by $83.6 \pm 3.7\%$ ($n = 3$).

Effects of depletion of the caffeine-sensitive Ca^{2+} store on sarafotoxin-induced contractions

In the absence of extracellular Ca^{2+}, repeated applications of caffeine induced transient increases in $[Ca^{2+}]_i$ and force with a progressive reduction in their maxima (Figure 6). A fourth application of caffeine produced little or no increase in $[Ca^{2+}]_i$ or force. Hence Ca^{2+} in the caffeine-sensitive store was probably much depleted. When 0.1 μM sarafotoxin was subsequently added to Ca^{2+}-free solution after 10 min, there were transient increases in the fluorescence ratio from $-52.1 \pm 6.6\%$ to $-40.9 \pm 6.8\%$ (119.7 nM to 135.6 nM $[Ca^{2+}]_i$) and force ($27.9 \pm 1.2\%$, $n = 3$). Exposure of tissues incubated in Ca^{2+}-free solution to 0.1 μM sarafotoxin, without repeated application of caffeine, induced a transient increase in fluorescence ratio from $-41.7 \pm 8.4\%$ to $-20.9 \pm 10.8\%$ (134.4 nM to 167.9 nM $[Ca^{2+}]_i$) and force ($27.0 \pm 4.4\%$, $n = 3$). Although $[Ca^{2+}]_i$ elevation in the caffeine-treated groups was lower than that of controls ($P < 0.05$) force development was not changed by exposure to caffeine.

The $[Ca^{2+}]_i$-force relationship of sarafotoxin

The $[Ca^{2+}]_i$-force relationship of tissues observed during exposure to 118 mM K^+-containing solution with various concentrations of extracellular Ca^{2+} (Figure 7) was similar to the pCa-force relationship noted with skinned arterial fibres of various species and organs (Itoh *et al.*, 1982; Saida & Nonomura, 1978; Kuriyama *et al.*, 1982). The $[Ca^{2+}]_i$-force relationship for sarafotoxin was evaluated from the data in Figures 2 and 3 and was found to be to the left of that observed after exposure to 118 mM K^+-containing solution. Thus, for any given $[Ca^{2+}]_i$, sarafotoxin induced a greater force than K^+-containing solution (Figure 7b). Treating the vascular strips with diltiazem inhibited the sarafotoxin-induced increase in $[Ca^{2+}]_i$ more effectively than its induction of force, and hence shifted the $[Ca^{2+}]_i$-force relationship left-

wards relative to tissues that had not been treated with diltiazem. After 15 min incubation in Ca^{2+}-free solution, the $[Ca^{2+}]_i$-force relationship induced by sarafotoxin shifted even further to the left, and after 60 min in Ca^{2+}-free solution it was perpendicular to the abscissa scale since 1 μM sarafotoxin induced a force of approximately 30% without increasing $[Ca^{2+}]_i$.

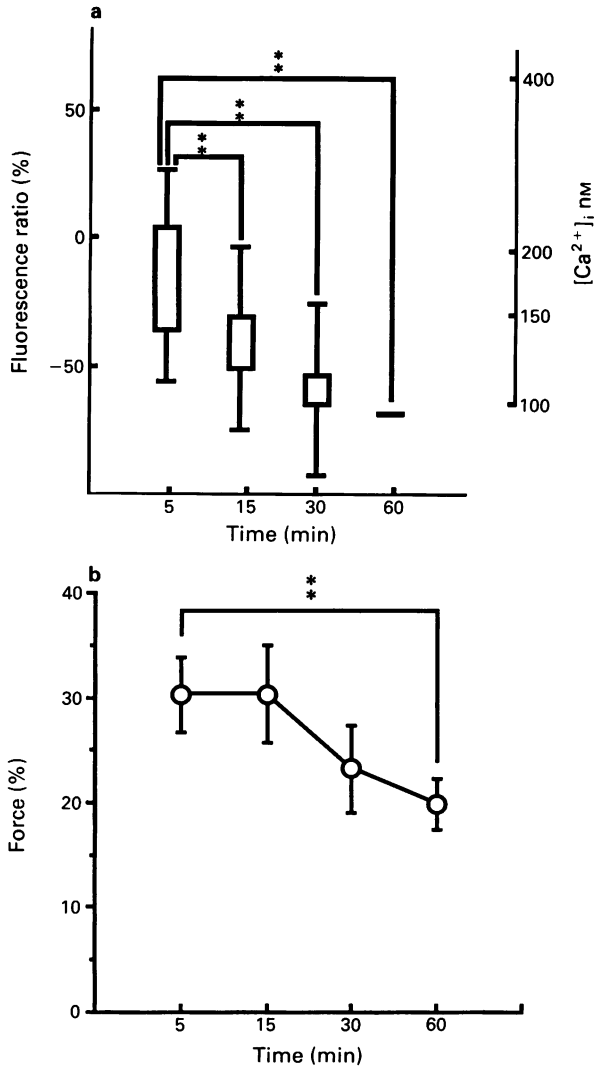


Figure 5 Effects of the duration of exposure to Ca^{2+}-free medium on the peak level of $[Ca^{2+}]_i$ (a) and force development (b) induced by 0.1 μM sarafotoxin. Data are mean ± s.e.mean ($n = 3$). Ordinates: (a) % fluorescence ratio change. The bottom and top of each column indicate the $[Ca^{2+}]_i$ just before and at the peak level obtained by the application of sarafotoxin, respectively. (b) % developed force. Abscissa scale: incubation time (min) in Ca^{2+}-free medium (**$P<0.01$, *$P<0.05$, compared with the value obtained after 5 min incubation in Ca^{2+}-free solution). Vertical bars represent s.e.mean.

Discussion

Changes in $[Ca^{2+}]_i$ play a central role in determining the contractile force of vascular smooth muscle. The precise relationship between $[Ca^{2+}]_i$ and force in intact smooth muscles is not clear, because of the difficulty in directly and simultaneously recording changes in such tissues. It has been reported that the $[Ca^{2+}]_i$-force relationship may vary during contraction (Rasmussen *et al.*, 1987; Murphy, 1989). We have found that the $[Ca^{2+}]_i$-force relationship of contractions induced by K^+-containing solution is similar to that obtained in chemically skinned preparations and that histamine, a receptor stimulating compound, causes a proportionally greater extent of force development for a given change in $[Ca^{2+}]_i$ than does exposure to a K^+-containing solution (Abe *et al.*, 1990; Hirano *et al.*, 1990). In the present study, we used front-surface fluorometry of fura-2 loaded rat aortic strips to examine the roles of extracellular and intracellular Ca^{2+} and of the $[Ca^{2+}]_i$-force relationship in contractions induced by sarafotoxin. Since sarafotoxin and endothelin-1 have homologous amino acid sequences (Lee & Chiappinelli, 1988), share common binding sites (Ambar *et al.*, 1989), and induce marked long-lasting coronary vasospasms (Lee *et al.*, 1986; Takasaki *et al.*, 1988b), it was considered that elucidation of the mechanisms involved in sarafotoxin-induced contraction would shed light on the mechanisms of the vasospastic actions of peptides from the endothelin and sarafotoxin families. In the presence of extracellular Ca^{2+}, sarafotoxin induced a rapid increase in $[Ca^{2+}]_i$ followed by a sustained phase. Force developed slowly but was sustained during the application of sarafotoxin. Both the sarafotoxin-induced increase in $[Ca^{2+}]_i$ and in force in the absence of extracellular Ca^{2+}, were much smaller than those observed in its presence. Also, a maximally effective concentration of the Ca^{2+} entry blocker, diltiazem, inhibited maximal sarafotoxin-induced increases in $[Ca^{2+}]_i$ by about 60% and force by about 38%. Therefore, it is likely that a major part of the sarafotoxin-induced increase in $[Ca^{2+}]_i$ and force depends on extracellular Ca^{2+}. These characteristics are similar to those of endothelin-1 (Kodama *et al.*, 1989; Sakata *et al.*, 1989; Huang *et al.*, 1990).

Fifteen min after removal of extracellular Ca^{2+}, sarafotoxin induced a transient increase in $[Ca^{2+}]_i$, thereby indicating that it can release Ca^{2+} stored intracellularly. When the caffeine-sensitive intracellular Ca^{2+} store was practically depleted by repetitive application of 20 mM caffeine in the absence of extracellular Ca^{2+} (Kodama *et al.*, 1989; Abe *et al.*, 1990; Matsumoto *et al.*, 1990), a subsequent application of sarafotoxin induced a transient increase in $[Ca^{2+}]_i$, which was smaller than that observed without caffeine-treatment.

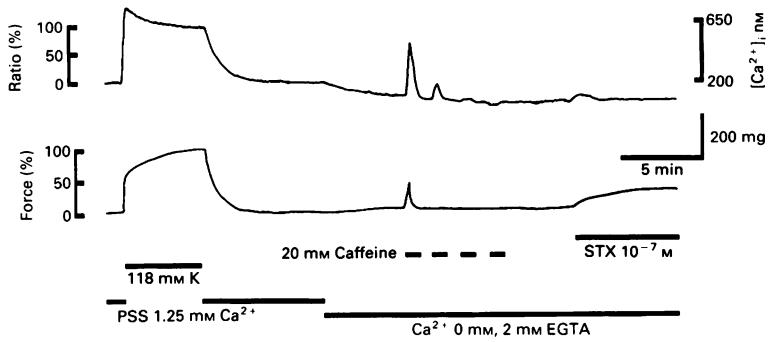


Figure 6 Representative recordings of changes in $[Ca^{2+}]_i$ (Ratio) and force development (Force) induced by 0.1 μM sarafotoxin (STX) after repeated application of 20 mM caffeine in Ca^{2+}-free solution.

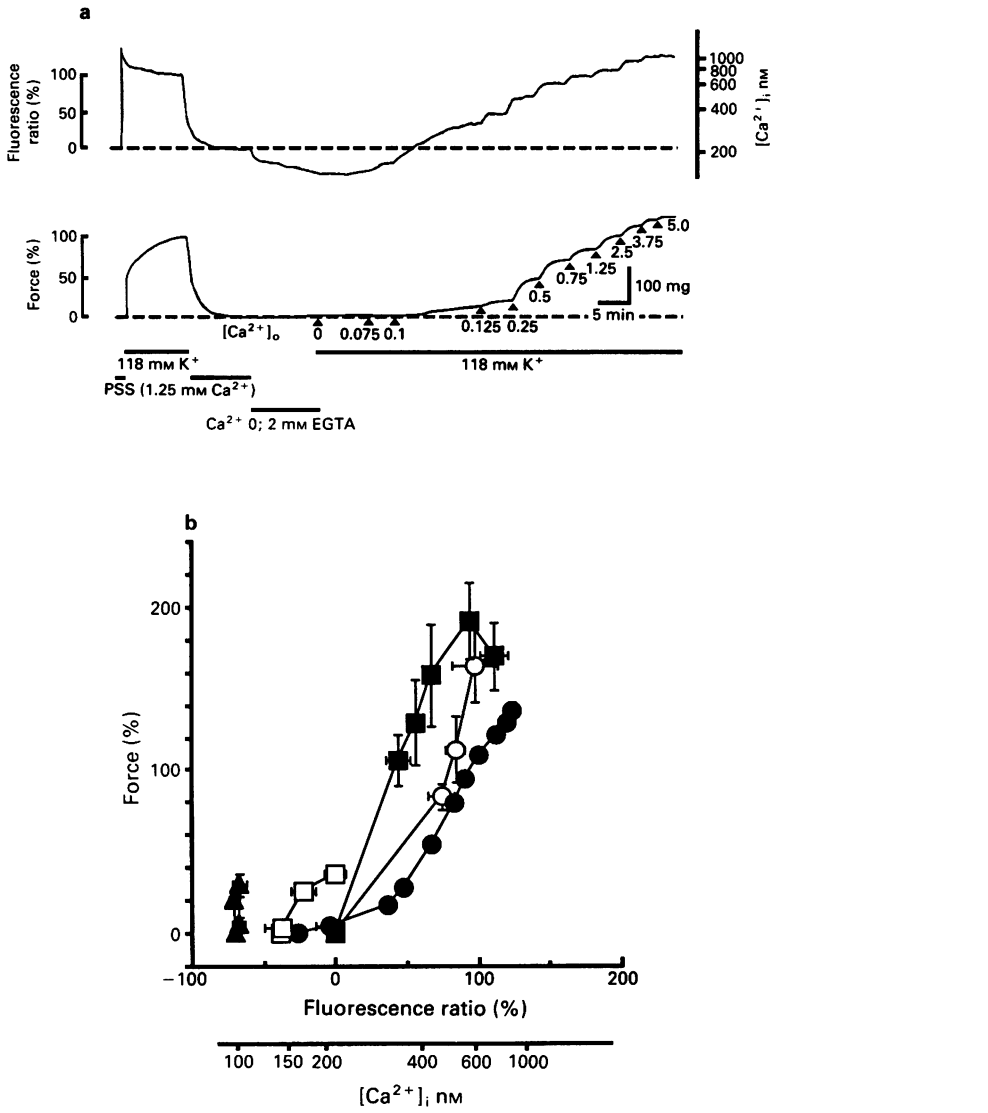


Figure 7 Effects of sarafotoxin on the $[Ca^{2+}]_i$-force relationship. (a) Representative recording of the changes in fluorescence ratio (upper panel) and force (lower panel) induced by cumulative applications of extracellular Ca^{2+} (0–5 mM) during exposure to 118 mM K^+-containing depolarizing solution. Numbers at each triangle (▲) indicate concentrations (in mM) of extracellular Ca^{2+}. (b) The $[Ca^{2+}]_i$-force relationships obtained from responses obtained in experiments as depicted in (a) (●), and from responses elicited by various concentrations of sarafotoxin in 1.25 mM Ca^{2+}-containing solution (○; from Figure 2). Responses to 0.1 μM sarafotoxin in strips treated with various concentrations of diltiazem in 1.25 mM Ca^{2+}-containing solution (■; from Figure 3), and responses to 0.1 μM sarafotoxin after exposure to Ca^{2+}-free solution for 15 (□) or 60 (▲) min (from Figure 2). Vertical bars represent s.e.mean.

Thus the caffeine-sensitive Ca^{2+} store partially overlaps with the sarafotoxin-sensitive store. The endothelin-1-sensitive intracellular Ca^{2+} store also overlaps with that sensitive to caffeine in rat aortic smooth muscle cells in primary culture (Kai *et al.*, 1989). The vasoconstrictors, sarafotoxin and endothelin-1, probably share similar mechanisms.

The EC_{50} for sarafotoxin-induced force development was about 20 fold higher after treatment of tissues for 15 min in Ca^{2+}-free solution than when measured in the presence of Ca^{2+}. This finding leads to the notion that sarafotoxin evokes force mainly in a manner which depends on extracellular Ca^{2+}. In addition to the importance for force development of extracellular and intracellularly stored Ca^{2+}, sarafotoxin evokes a sustained force in rat aortic strips without elevating $[Ca^{2+}]_i$. The lack of an increase in fluorescence ratio by sarafotoxin (Figures 2a, 4b and 5a) after a 60 min exposure to Ca^{2+}-free physiological salt solution is not due to secretion or to sequestration of fura-2 (Di Virgilio *et al.*, 1988), since a subsequent exposure to 118 mM K^+-containing solution induced increases in the fluorescence ratio similar to those observed before exposure to Ca^{2+}-free solution. This sustained force development was inhibited by H-7, a compound that inhibits protein kinase C *in vitro* with a K_i of 6 μM and myosin light chain kinase with a K_i of 97 μM (Hidaka *et al.*, 1984). Thus our finding that 10 μM H-7 inhibited the above sustained force development by 83.6% suggests that this component of contractility might be linked to the activation of protein kinase C and subsequent protein phosphorylation (Nishizuka, 1984) in vascular smooth muscle cells (Rasmussen *et al.*, 1987). It has been reported that endothelin also evokes a Ca^{2+}-independent, transient contraction in porcine coronary arterial strips (Kodama *et al.*, 1989). The finding that sarafotoxin induced the same force development either after repetitive application of caffeine in the absence of extracellular Ca^{2+} or without caffeine treatment under the same conditions suggests that the amount of Ca^{2+} released from the caffeine-sensitive store does not affect the extent of contraction. The underlying mechanisms which explain these phenomena remain to be elucidated.

Higher concentrations of sarafotoxin caused a proportion-

ally greater force development for a given change in $[Ca^{2+}]_i$ than did incubation in K^+-containing solution and hence the $[Ca^{2+}]_i$-force relationship for sarafotoxin was to the left of that for K^+. In addition, the sarafotoxin-induced $[Ca^{2+}]_i$-force relationship was shifted to the left by diltiazem. Similarly, we have reported that histamine, a receptor stimulating agonist, causes a greater contraction for a given change in $[Ca^{2+}]_i$ than K^+-depolarization, and also that diltiazem induces a leftward shift of $[Ca^{2+}]_i$-force relationship for histamine (Hirano *et al.*, 1990). It was suggested (Schächtele *et al.*, 1989) that diltiazem might have no direct effect on the Ca^{2+}-sensitivity of contractile elements. However, in addition to an increase in Ca^{2+} sensitivity by sarafotoxin, the possibility that diltiazem may increase Ca^{2+} sensitivity of the contractile apparatus would need to be excluded, as this may lead to a leftward shift of the Ca^{2+}-force relationship. By use of *Staphylococcus aureus* α-toxin permeabilized vascular smooth muscle preparations, it has been found that some agonists enhance the sensitivity of contractile elements to $[Ca^{2+}]_i$ through a G-protein-mediated pathway other than via inositol 1,4,5-trisphosphate production (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1989). There is evidence suggesting that the activation of protein kinase C may increase the force developed for a given increase in $[Ca^{2+}]_i$ (Itoh *et al.*, 1988; Nishimura & van Breemen, 1989). In our studies, sarafotoxin induced Ca^{2+}-independent force development after a 60 min incubation of tissues in Ca^{2+}-free solution and H-7 abolished this response. It is therefore concluded that Ca^{2+}-independent components of contractions, which are possibly mediated by protein kinase C activation, may play a role in producing a greater contraction for a given $[Ca^{2+}]_i$.

We postulate that in rat aorta, sarafotoxin induces contractions in a manner which is mainly dependent on extracellular Ca^{2+}. Also, in addition to those components of contraction dependent on extracellular Ca^{2+} and intracellularly stored Ca^{2+}, sarafotoxin can induce contractions without changing $[Ca^{2+}]_i$. This component of contractility is probably linked to activation of protein kinase C and may partly contribute to the leftward shift of the sarafotoxin-induced $[Ca^{2+}]_i$-force relationship relative to that obtained in K^+-containing solution.

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Dependence of endotoxin-induced vascular hyporeactivity on extracellular L-arginine

[1]Cathy A. Schott, [2]Gillian A. Gray & Jean-Claude Stoclet

Université Louis Pasteur de Strasbourg, Laboratoire de Pharmacologie Cellulaire et Moléculaire-CNRS URA600, B.P. 24, 67401 Illkirch, France

1 The dependence on extracellular L-arginine of vascular hyporeactivity induced by bacterial lipopolysaccharide (LPS) was studied *in vivo* in rats infused with LPS and *in vitro* in endothelium-denuded rat thoracic aortic rings exposed to LPS.

2 Infusion of LPS during 50 min at a dose of 10 mg kg^{-1} h^{-1} produced a significant impairment of the pressor effect of noradrenaline, while in tissues collected 60 min after the start of LPS infusion, no significant alteration in either plasma arginine concentration or aortic arginine content was found compared to saline-infused controls (where plasma arginine was 78.5 ± 7 μM and aortic arginine 394 ± 124 nmol g^{-1} tissue).

3 Incubation of isolated, endothelium-denuded aortic rings with LPS (10 μg ml^{-1}) in the absence of L-arginine for 4 h at 37°C produced a 6 fold ($P<0.01$) rightward shift in the noradrenaline concentration-effect curve compared to polymyxin B (1 μg ml^{-1}, a LPS neutralizing agent) and reduced by 15% the maximum observed tension.

4 The presence of L-arginine (100 μM) during the incubation with LPS and throughout the following contraction experiments caused a 15 fold ($P<0.01$) increase in the EC_{50} of noradrenaline and greater depression (45%) of the maximum observed tension compared to polymyxin B-treated controls. Responses in control, non LPS-treated rings were unaffected by the presence of L-arginine.

5 The addition of L-arginine to rings incubated with LPS in the absence of L-arginine and maximally precontracted with noradrenaline (10 μM) induced a dose-dependent relaxation. The EC_{50} of L-arginine was 8.0 ± 0.3 μM.

6 The reactivity of LPS-treated rings to noradrenaline both in the absence and presence of L-arginine was restored to control levels by N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM), an inhibitor of NO production and by methylene blue (3 μM), an inhibitor of guanylate cyclase.

7 Incubation of isolated aortae in the absence of L-arginine did not significantly decrease the tissue arginine content, whether LPS (10 μg ml^{-1}) was present or not. Similarly, the presence of L-arginine (100 μM) in the incubation medium did not modify the tissue arginine content.

8 These results show that the LPS-induced impairment of vasoconstriction elicited by noradrenaline is dependent on extracellular L-arginine, although the tissue arginine content is not depleted after LPS pretreatment, and that circulating L-arginine is sufficient to activate maximally the vascular L-arginine/NO pathway in endotoxaemic rats.

Keywords: Extracellular L-arginine; nitric oxide; NO synthase; lipopolysaccharide; septic shock

Introduction

Loss of vascular responsiveness to several vasoconstrictor agents is associated with high mortality in septic shock (Groenenveld & Thijs, 1986). Administration of *Echerichia coli* lipopolysaccharide (LPS) to rats *in vivo* (Wakabayashi *et al.*, 1987) produces hyporesponsiveness to the pressor effects of catecholamines and other agonists (Schaller *et al.*, 1985) and hyporeactivity in arteries studied *ex vivo* (Julou-Schaeffer *et al.*, 1990; Schneider *et al.*, 1992). Hyporeactivity to catecholamines can also be induced *in vitro* in vessels incubated with LPS (McKenna, 1988; Fleming *et al.*, 1990). Vascular hyporeactivity produced by LPS in these different conditions is reversed by inhibitors of nitric oxide (NO) production from L-arginine (Julou-Schaeffer *et al.*, 1990; Fleming *et al.*, 1990; Gray *et al.*, 1991), suggesting that NO, which stimulates cytosolic guanylate cyclase, is a major mediator of the hyporeactivity. This hypothesis has been further supported by the finding that LPS induces an NO synthase activity in the rat medial- adventitial layer (Knowles *et al.*, 1990; Rees *et al.*, 1990) and subsequent guanosine 3′:5′cyclic-monophosphate (cyclic GMP) accumulation in vascular smooth muscle cells (Fleming *et al.*, 1991a). Unlike the constitutive endothelial NO synthase, the induced NO synthase activity was calcium/calmodulin independent in smooth muscle cells.

L-Arginine, the substrate of NO synthase, is present in endothelial cells, where NO production does not require the addition of exogenous L-arginine. In addition to a system capable of transporting L-arginine from the extracellular space (Mann *et al.*, 1989; 1990), endothelial cells have the ability to synthesize L-arginine (Hecker *et al.*, 1990). In these cells, NO production by the constitutive NO synthase is therefore not limited by the supply of extracellular L-arginine, except after prolonged incubation without L-arginine (Gold *et al.*, 1989). In contrast, relatively little is known about regulation of L-arginine supply in smooth muscle cells. It has been reported that prolonged incubation with L-arginine decreases contraction elicited by phenylephrine in rat aortic rings (Schini & Vanhoutte, 1991). In addition, *ex vivo* and *in vitro* aortic hyporeactivity induced by LPS is enhanced by the addition of L-arginine to endothelium-denuded aortae in the organ bath (Julou-Schaeffer *et al.*, 1990; 1991). Both studies suggest that after NO synthase induction, smooth muscle

[1] Author for correspondence.
[2] Present address: Pharma Division; Preclinical Research, Hoffman-La Roche, Basel 4002, Switzerland.

cells may have a higher dependence on extracellular L-arginine for NO production than endothelial cells containing the constitutive enzyme. However, infusion of L-arginine in LPS-treated rats did not enhance *in vivo* hyporeactivity to intravenously injected noradrenaline (Gray *et al.*, 1991).

The aim of the present work was to investigate the effect of LPS on circulating and tissue levels of L-arginine and the relationship between extracellular L-arginine supply and hyporesponsiveness to noradrenaline (NA) in LPS-exposed aortic rings. Since small amounts of LPS contained in physiological solutions may be sufficient to induce NO synthase in the rat aorta (Rees *et al.*, 1990), some control experiments were run in the presence of polymyxin B in order to neutralize any small contaminant concentrations of LPS (Flynn *et al.*, 1987; Stokes *et al.*, 1989). Activation of the L-arginine/NO pathway and guanylate cyclase was assessed with the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) and the guanylate cyclase inhibitor, methylene blue.

Methods

In vivo *experiments*

Male Wistar rats (12 weeks, 250–300 g) were anaesthetized by intraperitoneal injection of sodium pentobarbitone (60 mg kg^{-1}, Sanofi). Arterial blood pressure was monitored with a pressure transducer (Gould Statham P23 ID) connected via a cannula containing heparinized saline (150 iu ml^{-1}, NaCl 0.9%) to the right carotid artery. The blood pressure was recorded with a pen recorder (Beckmann R511A). The right femoral vein and the right jugular vein were cannulated for NA administration and for LPS infusion (10 mg kg^{-1} h^{-1}) respectively. The body temperature was maintained at 37 ± 0.5°C.

In order to assess the effect of LPS on NA-induced increases of arterial blood pressure, NA (100, 300 and 1000 ng kg^{-1}) pressor responses were measured after a 30 min stabilization period and after 50 min of infusion of LPS (10 mg kg^{-1} h^{-1}) or saline (NaCl 0.9%). In preliminary experiments it was seen that neither blood pressure nor LPS-induced hyporeactivity to NA changed after 50 min infusion. The blood pressure was allowed to return to the basal (pre-NA) value between each dose of NA and before collection of blood samples (2 ml). The return to basal blood pressure took approximately 2 to 3 min and the assessment of reactivity to the three doses of NA was achieved within 10 min.

Blood samples were collected into heparinized tubes 60 min after the start of LPS infusion and immediately centrifuged. At the same time, the animals were exsanguinated and aortae were removed. Trichloroacetic acid (TCA) was added to the plasma and to the aortae at a final concentration of 6% and the samples were frozen until determination of arginine levels.

In vitro *contraction studies*

Male Wistar rats (12 weeks, 250–300 g) were killed by cervical dislocation. Thoracic aortae were removed, cleared of adherent tissue and the endothelium was removed by gently rubbing with blunt forceps. They were used for measurements of arginine content or cut into rings 2 mm in length for contraction studies. Both aortae and rings were incubated for 4 h at 37°C in an incubator gassed with 5% CO_2/95% air in L-arginine-free Eagle's minimal essential medium (MEM) with LPS (10 μg ml^{-1}) or polymyxin B (1 μg ml^{-1}), with or without L-arginine (100 μM). After incubation with LPS or polymyxin B, rings were mounted under 2 g tension in organ baths containing physiological salt solution (PSS) of the following composition (in mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.7 at 37°C bubbled with 95% O_2/5% CO_2 (pH 7.4). L-Arginine (100 μM) was added to the PSS of some rings as indicated. All experiments were carried out in the presence of indomethacin (10 μM), cocaine (30 μM) and corticosterone (40 μM). Indomethacin, cocaine and corticosterone were present to prevent the interference of cyclo-oxygenase products, neuronal and non neuronal NA uptake, respectively. Tension was measured with an isometric force transducer.

After an equilibration period of 60 min, during which time the PSS was changed each 15 min, the basal tension was re-adjusted to 2 g. The absence of functional endothelium was verified by the lack of effect of acetylcholine (1 μM) on rings precontracted with NA (1 μM). After a second washing period of 60 min, during which tension returned to baseline levels, a cumulative dose-response curve to NA (10^{-9}–10^{-5} M) was obtained in some experiments. In other experiments, the rings were maximally contracted with a single dose of NA (10 μM) and activation of the L-arginine/NO pathway was assessed with L-NAME and methylene blue, or a cumulative dose-response curve to L-arginine (1–300 μM) was obtained. Note that all contraction studies were made 6 h after initial contact with LPS, and that tissue arginine content in isolated control and LPS-treated aortae were determined similarly after 6 h (see below).

Determination of arginine levels

Aortae taken from rats infused intravenously with LPS (10 mg kg^{-1} h^{-1}) or saline were homogenized in TCA (6%) with a Potter glass/glass homogenizer, sonicated (Ultrason-Annemasse, Type 75 TS France) and centrifuged at 10,000 *g* for 15 min. Blood samples were also centrifuged at 10,000 *g* for 15 min. The supernatants were extracted with water-saturated diethyl ether, the aqueous layer was lyophilized and reconstituted in 4 ml of 0.1 M citrate buffer, pH 5.3. The samples were then chromatographed on 0.7 × 0.6 cm Dowex-Na^+ columns (Dowex 50 W, 200–400 × 8, H^+). Each column was washed with 20 ml of citrate buffer in order to remove citrulline and ornithine, and arginine was eluted with 4 ml of 0.2 N NaOH (Gold *et al.*, 1989). In controls, >95% of known arginine concentrations were recovered by this method. The arginine containing solutions were lyophilized and reconstituted in 1 or 2 ml distilled H_2O for aortic tissue and plasma, respectively. Arginine was assayed by the Sakaguchi reaction (formation of a coloured complex with α-naphthol) modified by the addition of thymine to stabilize the coloured complex, according to Van Pilsum *et al.* (1956). A 0.5 ml aliquot of a 1:1 (vol/vol) solution of thymine (20 mg ml^{-1} in 10% NaOH) and α-naphthol (0.04% in ethanol) was added to the sample, followed by the addition of 0.2 ml NaOCl (1% solution) with immediate mixing. Exactly 1 min later, 0.2 ml of a $Na_2S_2O_3$ solution (0.03 g ml^{-1} in H_2O) was added. The reaction was monitored at 515 nm (Beckman model 25 Spectrophotometer). Standard titration curves for L-arginine were linear from 0 to 100 μM, with a calculated (Miller & Miller, 1988) threshold level of detection of 4 μM and a sensitivity of 3 μM. Both the weight of individual aortic samples (approximately 90 mg) and appropriate dilutions of plasma samples were chosen to allow determinations of absolute arginine levels to be made in a range close to the midpoint of the control titration curve.

Arginine content of isolated aortae was similarly determined. Aortae were either used immediately after dissection or treated as described for contraction studies, i.e. arginine content was determined after 6 h incubation (see above).

Materials

Noradrenaline bitartrate (Sigma Chemical Co.) was stored as a 10 mM stock solution in buffer containing Na_2SO_3 (7.9 mM) and HCl (34 mM), and diluted as required. Acetylcholine chloride (ACh, 10 mM, Sigma Chemical Co.) and cocaine (30 mM) were stored frozen. Methylene blue (Sigma Chemical Co.) was made up as a 10 mM stock solution, and diluted as required. All other substances were freshly prepared before

each experiment. Polymyxin B sulphate (Sigma Chemical Co.), L-arginine hydrochloride (Calbiochem), and N^G-nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Co.) were dissolved in PSS or in Eagle's minimum essential medium (MEM, Eurobio). Indomethacin (Sigma Chemical Co.) was dissolved in 0.5% Na_2CO_3 solution. Lipopolysaccharide (LPS, *E. coli* 055: B5, Difco) was dissolved in MEM for *in vitro* studies and in saline (NaCl 0.9%) for *in vivo* infusion. Corticosterone (Sigma Chemical Co.) was dissolved in 70% ethanol.

Statistical analysis

NA pressor responses *in vivo* were compared by two-way analysis of variance (ANOVA). Where ANOVA showed a significant difference, the results were further analysed with an *a posteriori* Newmann-Keuls test. Aortic and plasma contents of L-arginine *in vivo* were compared by Student's *t* test. For *in vitro* experiments, EC_{50} (concentration which gives 50% of the maximal response) values were calculated by logit-log regression. EC_{50} and maximal contractile responses were compared by Student's *t* tests for unpaired data. The change in tension induced by L-arginine in NA-contracted aortic rings and the arginine aortic content were analyzed with a one-way ANOVA.

Results

In vivo *experiments*

The mean arterial blood pressure in anaesthetized rats was not significantly changed after a 50 min infusion of LPS (10 mg kg^{-1} h^{-1}) compared to saline-infused controls (Table 1). Infusion of LPS caused a significant decrease in NA pressor responses at all three NA doses used compared to control rats infused with saline (Table 1). Neither the plasma nor the aortic arginine content were altered in a significant way by infusion of LPS (Table 1).

Contraction studies in vitro

Effect of the presence of L-arginine throughout the experiment Concentration-response curves to NA are illustrated in Figure 1 and the corresponding EC_{50} and maximal contraction values are given in Table 2. Controls were run in the presence and in the absence of polymyxin B (1 μg ml^{-1}). Polymyxin B was used to neutralize any contaminant LPS and did not modify significantly the concentration-response curves to NA (Table 2). The presence of L-arginine (100 μM) throughout the experiment, i.e. during the initial 4 h incubation period and the following contraction experiment, did not produce any significant alteration in concentration-response curves to NA obtained in control rings (both with or without polymyxin B).

In the absence of L-arginine, treatment with LPS induced a significant ($P<0.01$) rightward shift of the dose-response curve to NA (EC_{50} was increased 6 fold, compared to polymyxin B, $P<0.01$). However, in this case, the slight decrease in maximal contractile response seen after LPS treatment was not significant (Table 2). When L-arginine was present throughout the experiment, LPS induced a greater rightward shift of the concentration-response curve to NA: EC_{50} was increased 15 fold, $P<0.01$, $n=5$ compared with control rings with or without polymyxin B and 2.5 fold ($P<0.01$) compared with LPS-treated rings without arginine. LPS also induced a significant ($P<0.01$) decrease in the maximal contractile response when L-arginine was present throughout (Figure 1).

Effect of the addition of L-arginine, L-NAME and methylene blue to NA-contracted aortae After completion of NA concentration-effect curves, L-arginine (1 mM) was added to maximally contracted rings (Figure 2). Addition of L-arginine to control rings (incubated with or without polymyxin B) maximally contracted with NA (10 μM) had no effect on

Table 1 Effects of lipopolysaccharide (LPS) *in vivo* on blood pressure, responsiveness to noradrenaline (NA) and arginine content

		Control	*LPS*	
BP (mmHg)	Before infusion	115 ± 8	109 ± 4	
	After infusion	112 ± 3	102 ± 3	
Increase in BP (mmHg)	NA 100 ng kg^{-1}	17 ± 2	8 ± 1	*
	NA 300 ng kg^{-1}	35 ± 4	15 ± 2	**
	NA 1000 ng kg	58 ± 5	33 ± 4	**
Arginine content	Plasma (μM)	78.5 ± 7	81.9 ± 6	NS
	Aortic (μmol kg^{-1})	394 ± 124	279 ± 98	NS

Mean arterial blood pressure (BP, mmHg), responsiveness to NA (increase in BP, mmHg) and circulating and aortic arginine contents in control and LPS-treated rats. Values are the mean ± s.e.mean of 5 experiments.
*$P<0.05$, **$P<0.001$, NS: not significant $P>0.05$ compared to respective controls.

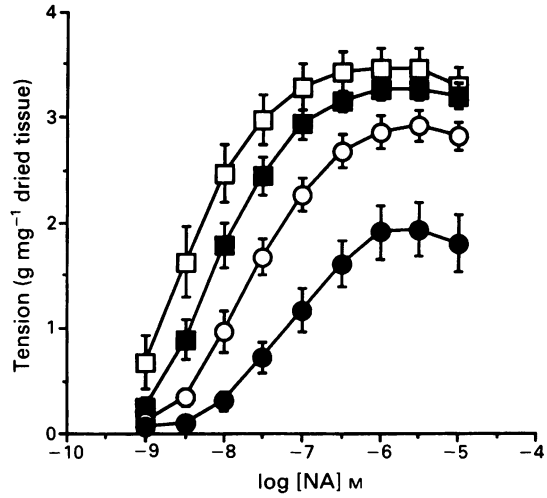


Figure 1 Influence of L-arginine on vascular hyporeactivity to noradrenaline (NA) induced by lipopolysaccharide (LPS). Concentration-response curves elicited by NA in endothelium-denuded rat aortic rings incubated for 6 h without (open symbols) or with (closed symbols) L-arginine (100 μM) and exposed to either polymyxin B (1 μg ml^{-1}, □ ■) or LPS (10 μg ml^{-1}, ○ ●).

Table 2 Effect of L-arginine on aortic contractile responsiveness to noradrenaline

Treatment	*EC_{50}* (μM)		*Maximal contraction* (g mg^{-1} dried tissue)	
None	5.3 ± 1.2		3.0 ± 0.1	
None + L-arginine	12.5 ± 2.3	NS	3.1 ± 0.4	NS
Polymyxin	4.3 ± 1.3		3.3 ± 0.2	
Polymyxin + L-arginine	9.9 ± 2.3	NS	3.2 ± 0.1	NS
LPS	25.0 ± 6.2		2.8 ± 0.1	
LPS + L-arginine	65.9 ± 10.8	**	1.8 ± 0.3	**

EC_{50} values and the maximal tension were determined in aortic rings without endothelium treated with or without polymyxin B or LPS, in the absence or presence of L-arginine. Values are the mean ± s.e.mean of 5 experiments.
**$P<0.01$; NS $P>0.05$ compared to respective values in the absence of L-arginine.

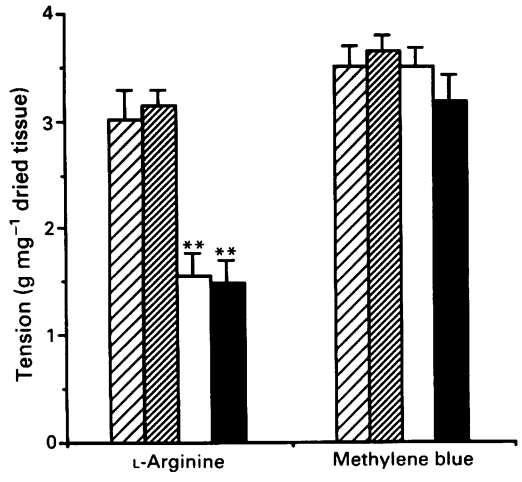


Figure 2 Effect of the consecutive addition of L-arginine (1 mM) and methylene blue (3 μM) to aortic rings exposed to a maximally active noradrenaline (NA) concentration (10 μM), after completion of the concentration-response curves for rings incubated with polymyxin B (1 μg ml^{-1}, ▨ ▨) or with LPS (10 μg ml^{-1}, □ ■), in the absence (▨ □) or in the presence (▨ ■) of L-arginine (100 μM). The effect of L-arginine was measured 5 min after its addition but 30 min after the addition of methylene blue. Values are the mean of 5–7 experiments; vertical lines show s.e.mean. $^{**}P<0.001$ compared to polymyxin B.

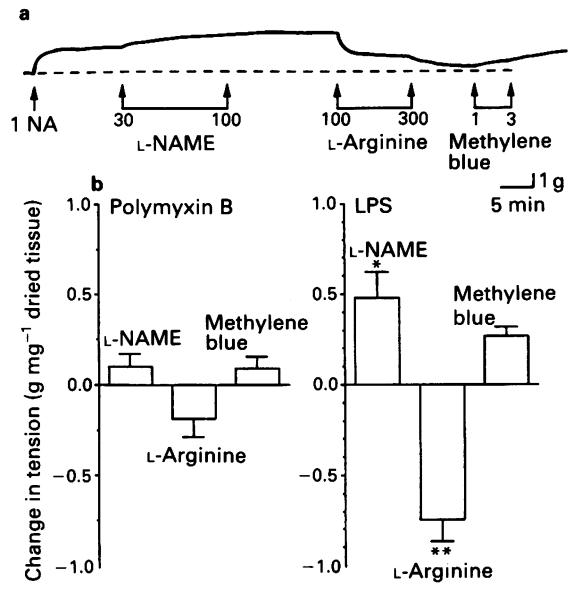


Figure 3 Effects of addition of N^G-nitro-L-arginine methyl ester (L-NAME), L-arginine and methylene blue to noradrenaline (NA)-precontracted rings incubated without L-arginine. (a) Representative tension trace showing the effect of L-NAME (100 μM) and subsequent additions of L-arginine and methylene blue to NA contracted, LPS-treated aortic ring. The numbers indicate the drug concentrations in μM. (b) Mean changes in tension elicited by L-NAME (100 μM) L-arginine (300 μM) and methylene blue (3 μM) in NA-precontracted (1 μM), polymyxin B- (left panel) or LPS-treated (right panel) endothelium denuded aortic rings using the protocol illustrated in (a). The tensions elicited by NA (zero value) were 2.3 ± 0.4 and 2.4 ± 0.2 g mg^{-1} dried tissue in polymyxin B and LPS-exposed rings, respectively. The effects of L-NAME and methylene blue were obtained 30 min after the addition of the drug and 5 min after the addition of L-arginine. Values are the mean of 5–6 experiments; vertical lines show s.e.mean. $^{*}P<0.05$; $^{**}P<0.01$; compared to NA contraction.

tension, as was the case for subsequent addition of L-NAME (1 mM). This was true whether the control rings were incubated with or without L-arginine throughout the experiment. In contrast, the addition of L-arginine (1 mM) to LPS-treated rings incubated without L-arginine reduced tension (by 33%, $P<0.001$) to a stable level equivalent to that obtained in LPS-treated rings incubated in the continuous presence of L-arginine.

Further addition of L-arginine (1 mM) to LPS-treated aortae incubated with 100 μM L-arginine failed to induce any relaxation. The relaxation obtained with L-arginine, present during the incubation or added to contracted aortae, was abolished by addition of L-NAME (1 mM) or methylene blue (1 μM) (Figure 2) in LPS-treated rings. After the addition of either L-NAME or methylene blue, tension was not different in the four groups.

Figure 3 illustrates the effects of L-NAME and further consecutive additions of L-arginine and methylene blue in NA-precontracted aortic rings which were incubated without L-arginine. The recorded tension of maximally NA-contracted aortae was not significantly different between LPS-treated and control rings. In control rings incubated with polymyxin B, L-NAME produced only a slight but non significant increase in tension (Figure 3b). However, it significantly ($P<0.05$) enhanced tension in LPS-treated rings (Figure 3a,b). This effect of L-NAME was reversed by subsequent addition of L-arginine (300 μM), which again was without effect in control rings. Methylene blue reversed the L-arginine induced relaxation in LPS-treated rings, but was without effect in control rings.

Concentration dependence of the effect of L-arginine for tissue incubated without L-arginine Figure 4 illustrates dose-dependent effects of L-arginine in NA-precontracted aortic rings incubated without L-arginine. For LPS-treated rings, increasing concentrations of L-arginine added cumulatively caused relaxation. This relaxation was reversed by subsequent addition of 3 μM methylene blue (tension reached 2.4 g mg^{-1} dried tissue; not shown). In LPS-treated rings, L-arginine-induced relaxation was significant at a concentration as low as 1 μM and reached a maximum at concentrations higher than 30 μM. Half maximal relaxation was obtained at 8.0 ± 3.0 μM (Figure 4).

In control aortae, L-arginine had slight effects, relaxation being significant only in the presence of the highest L-arginine concentrations (> 100 μM) in rings incubated without polymyxin B ($P<0.002$), but not in the presence of polymyxin B. At low concentrations (1–3 μM), L-arginine also produced a small, non significant increase in tension in control rings with or without polymyxin B.

Tissue arginine levels during in vitro *experiments*

Incubation of endothelium-denuded aortae *in vitro* without L-arginine for 6 h induced no significant change in arginine content, whether LPS was present in the medium (for 4 h) or absent (neutralized by polymyxin B). The actual values were 435 ± 32 nmol g^{-1} tissue before incubation, 415 ± 45 and 358 ± 47 nmol g^{-1} tissue, for polymyxin B-treated and LPS-treated aortae ($n = 5$), respectively. In addition, the presence of L-arginine (100 μM) in the incubation medium did not significantly modify the arginine content of control, polymyxin B-treated aortae (498 ± 65 nmol g^{-1} tissue, $n = 5$) compared to LPS-treated aortae (460 ± 38 nmol g^{-1} tissue, $n = 5$).

Discussion

The present work documents a key role for extracellular L-arginine in regulating the extent of vascular hyporeactivity produced by LPS. In endotoxaemic rats, the circulating level

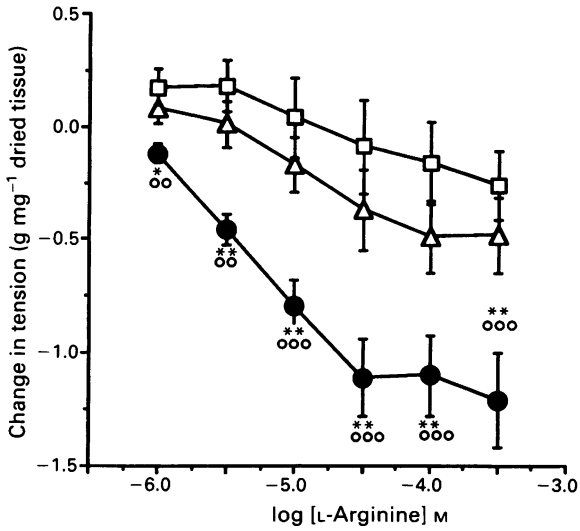


Figure 4 L-Arginine concentration-response curves in maximally noradrenaline (NA)-contracted (10 μM) aortic rings incubated without L-arginine. Control (△); LPS-treated (10 μg ml^{-1}, ●); polymyxin B-treated (1 μg ml^{-1}, □). Cumulative doses of L-arginine were added every 5 min and the maximal effect of each concentration measured 5 min after addition of each dose. Values are the mean of 5–6 experiments; vertical lines show s.e.mean. $^{*}P<0.05$; $^{**}P<0.01$; with respect to control; $^{\circ}P<0.05$; $^{\circ\circ}P<0.01$; $^{\circ\circ\circ}P<0.001$; with respect to polymyxin B.

of arginine (80 μM) was not decreased compared to control animals, and was close to concentrations producing maximal relaxation in LPS-exposed vessels such as the aorta (this study) or a small resistance mesenteric artery (Schneider *et al.*, 1992). The finding that injection of L-arginine failed to decrease blood pressure and to intensify hyporeactivity to NA in LPS-treated rats *in vivo* (Gray *et al.*, 1991) might seem unexpected in view of the relaxant effect of L-arginine in LPS-exposed vessels. This difference between results obtained *in vivo* and *in vitro* is not due to the absence of endothelium in aortic rings used in the present study, as the presence or the absence of endothelium in LPS-treated vessels was previously reported not to change the effect of L-arginine on both cyclic GMP levels and contractile responses to NA (Fleming *et al.*, 1991b). However, this apparent discrepancy may be explained by the present observation that the circulating arginine concentration was unaffected by LPS and sufficient to induce maximal vascular hyporeactivity in LPS-treated rats.

The findings that neither circulating nor tissue arginine contents varied after LPS infusion is not due to a lack of sensitivity of the assay used, as the calculated threshold level of detection and sensitivity of the assay were 4 and 3 μM L-arginine, respectively (see Methods). Furthermore, other amino acids (including ornithine and glutamine) and more than 100 other naturally occurring biological compounds do not interfere with the assay, which is based on detection of guanidine residues (Van Pilsum *et al.*, 1956). While other guanidine-containing compounds might contribute to the determined arginine content, if present and not removed by the chromatographic procedure (see Methods), comparable arginine tissue levels (400–800 μM depending on the tissue) have been reported with this or another technique (Gold *et al.*, 1989; Baydoun *et al.*, 1990).

The continuous presence of L-arginine (100 μM) in the isolated organ bath throughout the experiment or its addition during the contraction experiments, after 6 h in its absence, resulted in the same level of contraction in LPS-treated aortic rings. This relaxation or inhibition of contraction elicited by L-arginine was due to production of NO or a NO-like factor and subsequent cyclic GMP synthesis, since it was inhibited by both the NO synthase inhibitor L-NAME and the guanylate cyclase inhibitor methylene blue, indicating that NO synthase was induced during the 4 h incubation with LPS (note that the contraction studies were made 2 h afterwards, or for control rings, after 6 h). It has also been reported that LPS induces NO synthase activity in the rat aortic medial-adventitial layer (Rees *et al.*, 1990) and in freshly dissociated aortic smooth muscle cells (Fleming *et al.*, 1991a). Consistent with previous data obtained in the aorta (Julou-Schaeffer *et al.*, 1990; Fleming *et al.*, 1991a) and in small resistance arteries (Schneider *et al.*, 1992) from endotoxaemic rats, the present results suggest that extracellular L-arginine concentration is a rate limiting factor for NO synthase activity in vessels exposed to LPS *in vitro*. It was previously reported that these effects of L-arginine on cyclic GMP content and reactivity of arteries are stereospecific since they were not produced by D-arginine (Fleming *et al.*, 1991b).

The presence of low concentrations of LPS in saline solutions may be sufficient to induce the L-arginine/NO pathway (Rees *et al.*, 1990). Controls were therefore run in parallel with and without polymyxin B, which neutralizes LPS but may also produce other effects. No statistically significant difference was found between these two control groups, although a small but significant relaxation was induced by the highest L-arginine concentration in rings incubated without polymyxin B. Thus in the incubation conditions used here, production of an L-arginine derived relaxing factor in control aortic rings was very slight, if at all, consistent with the previously reported lack of constitutive NO synthase activity in aortic smooth muscle cells (Fleming *et al.*, 1991a).

The present results also show that the dependence of smooth muscle relaxation on extracellular L-arginine was not due to exhaustion of tissue arginine content consecutive to NO production from the amino acid or to its release into the extracellular fluid. The tissue arginine content was not significantly altered after 6 h incubation in PSS without L-arginine, whether LPS was present in the bath or not. Furthermore, consistent with previous determinations in the same tissue (Gold *et al.*, 1989), aortic arginine content (300–400 nmol g^{-1} tissue) was high compared to the concentration (30–100 μM) of L-arginine inducing maximal relaxation. This large tissue arginine store was apparently only partly available as substrate for the LPS-induced NO synthase, as shown by the relatively small LPS-induced reduction of the response to NA in rings incubated with exogenous L-arginine (Figure 1). Most of the substrate for maximal activity of the induced NO synthase was of extracellular origin, as shown by the large relaxation induced by the addition of L-arginine in LPS-treated rings incubated without L-arginine (Figure 2). These data support the view that extracellular L-arginine concentration is a rate limiting factor for NO synthase activity in vessels exposed to LPS *in vitro*.

The extracellular L-arginine dependence of NO production observed in endothelium-denuded aortic rings exposed to LPS seems different from what happens in vascular endothelial cells. In these cells, agonists are able to activate NO production by the constitutive NO synthase in the absence of extracellular L-arginine, perhaps because endothelial cells are able to recycle L-citrulline to L-arginine (Hecker *et al.*, 1990). However, it was recently reported that after incubation in absence of L-arginine, agonists that increase NO release also stimulate L-arginine uptake in endothelial cells, even though their intracellular arginine content seems to be high enough to permit maximal NO production by the enzyme (Bogle *et al.*, 1991). Thus, the source of L-arginine used by NO synthase in different cells requires further investigaton.

The major endogenous source of circulating L-arginine *in vivo* is renal synthesis from L-citrulline (Featherston *et al.*, 1973). Upon infusion of citrulline, both renal citrulline uptake and renal production of L-arginine increase (Dhanakoti *et al.*, 1990). During LPS stimulation, induced tissue NO synthase activity converts L-arginine to NO and to L-citrulline. Any L-citrulline released into the circulation might

therefore be used in renal synthesis of L-arginine or locally converted to L-arginine through the pathway described in endothelial cells (Hecker *et al.*, 1990), thus contributing to maintain the arginine content at a constant level.

In summary, the results of this investigation indicate that the vascular NO production induced by LPS is dependent to a large extent on extracellular L-arginine and that the circulating arginine concentration is high enough to induce a maximal L-arginine relaxation in endotoxaemic rats.

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Effects of cholinoceptor and 5-hydroxytryptamine$_3$ receptor antagonism on erythromycin-induced canine intestinal motility disruption and emesis

[1]Xin Yu Qin, [2]Marie-Anne Pilot, [3]Hilary Thompson & Mark Scott

GI Science and Surgical Units, The London Hospital Medical College, Turner Street, London E1 2AD

1 Erythromycin administration is associated with gastrointestinal problems, disturbed gastrointestinal motility and emesis. This study in the dog investigates the underlying mechanisms.

2 Intestinal myoelectrical activity and the occurrence and latency of emesis were recorded in eight conscious dogs. All drugs were administered intravenously.

3 Erythromycin (7 mg kg^{-1}) increased contractions of the proximal small intestine, and caused emesis in all fasted dogs and in 5 dogs after food. Atropine (50 mg kg^{-1} min^{-1}) and hexamethonium (10 mg kg^{-1} h^{-1}) partially inhibited the GI motility effects but did not significantly reduce emesis.

4 Metoclopramide at a high dose (2 mg kg^{-1} h^{-1}) reduced the incidence of emesis in the presence of increased intestinal motility, but a low dose (150 μg kg^{-1} h^{-1}) was ineffective.

5 A 5-hydroxytryptamine$_3$ (5-HT$_3$) receptor antagonist, MDL 72222 (1 mg kg^{-1}), reduced emesis when given alone and combined with metoclopramide (low dose). The 5-HT$_4$ receptor agonist BRL24924 (Renzapride, 1 mg kg^{-1}) had no effect on emesis either alone in combination with metoclopramide.

6 In conclusion, erythromycin-induced GI motility disturbances and emesis are not causally related. Whereas the increase in intestinal smooth muscle activity is possibly cholinergically mediated, emesis occurs at least in part via a 5-hydroxytryptaminergic mechanism, but does not involve the dopamine system.

Keywords: Erythromycin; gastrointestinal motility; 5-hydroxytryptamine; Renzapride; MDL72222; cholinergic; atropine; hexamethonium; emesis; metoclopramide

Introduction

Investigation of the effects of erythromycin on the gastrointestinal (GI) tract began less than a decade ago. These studies were prompted by numerous reports associating cramping, nausea, vomiting and diarrhoea with erythromycin treatment, which in some cases were severe enough to discontinue treatment (Shoemaker & Yow, 1954; Mather *et al.*, 1981; Downey & Chaput de Saintonge, 1986; Hovi *et al.*, 1987; Shanks *et al.*, 1989). Erythromycin was found to have a disruptive effect on gastrointestinal motor patterns (Pilot *et al.*, 1984; Itoh *et al.*, 1984), even when administered at very low, subtherapeutic doses (Tomomasa *et al.*, 1986; Otterson & Sarna, 1990). At therapeutic doses erythromycin interrupts and disrupts the migrating motor complex (MMC) pattern which occurs naturally during fasting, induces increased gastric and upper intestinal motor activity and causes emesis after both oral and intravenous administration (Qin *et al.*, 1985; 1986; Zara *et al.*, 1991). In the past two years, use has been made of these motor GI effects by giving erythromycin to patients with gastroparesis in order accelerate gastric emptying (Janssens *et al.*, 1990).

In spite of these beneficial effects, patients on erythromycin still report adverse GI effects. The aims of the present study in the dog were to investigate the possible pathways by which these alterations in gastrointestinal function occur, and whether there was a causal relationship between GI motility disturbances and emesis.

Methods

Eight dogs of either sex were used in the experiments. Under halothane/nitrous oxide anaesthesia, twelve silver electrodes were implanted on the serosal surface of the small intestine, four on the duodenum and eight on the jejunum and ileum. The electrode leads were exteriorized laterally (Qin, 1989). After operation, two weeks were allowed for recovery before beginning experiments.

Before each recording the dogs were fasted for 18 h. The experiments were performed in either the pre or post prandial state when the dog was fed a tin of 390 g 'PAL' dog's meat (Pedigree Petfoods Ltd) 10 min before the recording began. During the recording the dog stood on a table, supported by a canvas sling. Intestinal myoelectrical signals were recorded on two cassette tapes by use of two four-channel Medilog MR-10 recorders (Oxford Medical Systems), and replayed on a 4-channel PB-2 Replay Unit (Oxford Medical Systems). During replay the data were sent to an Apple II microcomputer via an interface, and saved as a sequential text file for further analysis (Barnett *et al.*, 1989).

Experimental protocol

Erythromycin After 3 hours of basal recording erythromycin lactobionate (Erythrocin, Abbott Laboratories Ltd) at the dose of 7 mg kg^{-1} was infused intravenously for 30 min. The recording was continued for a further 3 h after the end of the infusion.

Atropine, hexamethonium and metoclopramide The following protocol was used to study the effects of atropine, hexamethonium and metoclopramide on GI motility, and to investigate whether these compounds have an effect on the erythromycin-induced motility and emesis. After a basal period of 3 h, one of these three drugs was infused intravenously for one hour, following which there was a recovery period of 3 h. Erythromycin was infused (7 mg kg^{-1}) during the last 30 min of each drug infusion.

[1] Present address: Department of Surgery, Zhong Shan Hospital, 136 Yi Xue Yuan Road, Shanghai, 100032, China.
[2] Author for correspondence.
[3] Present address: Lister Hospital, Coveys Mill Lane, Stevenage, Herts SG1 4AB.

MDL 72222 and BRL 24924 These experiments were conducted primarily to investigate the effect of the two compounds on erythromycin-induced emesis. Because the emetic response was more pronounced when the animals were fasted, this series of experiments was performed on fasted dogs only. Half of the dose (5 ml) of the compound under study was injected intravenously after a 3 h basal period. Thirty minutes later an infusion of erythromycin (7 mg kg^{-1}) was started and at the same time the remaining 5 ml of the compound under investigation was injected. The infusion of erythromycin lasted for 30 min. In order to investigate the possibility of potentiation these experiments were repeated in the presence of metoclopramide at a low dose of 150 $\mu g\ kg^{-1}\ h^{-1}$ infused for 1 h starting at the time of the first injection of MDL2222 or BRL24924.

Drugs and dosages

All drugs were given by the i.v. route. Erythromycin lactobionate (7 mg kg^{-1} 30 min^{-1}; Abbott Laboratories), was dissolved in 10 ml water, and the volume of infusion was increased to 50 ml with 0.9% saline. Atropine sulphate (50 mg $kg^{-1}\ min^{-1}$) was obtained in ampoule form from Antigen Ltd., and 0.9% saline was added to obtain the infusion volume. Hexamethonium (10 mg $kg^{-1}\ h^{-1}$, Sigma Chemical Co., Poole, Dorset) was dissolved in 0.9% saline. Metoclopramide (low dose: 150 $\mu g\ kg^{-1}\ h^{-1}$, high dose: 2 mg $kh^{-1}\ h^{-1}$) was obtained in ampoule form from Smith-Kline Beecham, and the infusion volume was obtained by adding 0.9% saline. MDL 72222 (1 mg kg^{-1}), a gift from Merrel Dow, and BRL 24924 (1 mg kg^{-1}), a gift from Smith-Kline Beecham, were dissolved into 10 ml of 0.9% saline.

Data analysis

Electromyographic signals from the intestine were filtered so that only the fast potentials (spikes) were retained. The spikes were changed into TTL compatible pulses which were counted by an Apple microcomputer (Barnett *et al.*, 1989). Because of the cyclical nature of the preprandial motor patterns, comparison of spike numbers before and after treatment were done only in postprandial studies when spike activity is more uniform. In postprandial experiments, therefore, three periods were chosen for comparison. The first period (control) consisted of the 30 min before any drug was given, and the other two (Periods 2 and 3) were the two thirty minute periods of the hour of drug infusion. Erythromycin was always given during the second of these two periods (Period 3). The number of spikes in each period was summed and the values obtained in Periods 2 and 3 were compared to the control values of Period 1, by use of Wilcoxon's signed rank test for statistical analysis. Data are shown as median and interquartile values.

Results

Motility

Erythromycin Erythromycin lactobionate given intravenously interrupted the basal pre- and post-prandial motility patterns which were replaced by increased irregular spike activity in the duodenum and jejunum (Figure 1). The effects of erythromycin were more pronounced and prolonged in the duodenum than in the jejunum. When the infusion of erythromycin was stopped the irregular spike activity gradually decreased but continued to the end of the experiment with no evidence of a return to a basal pattern in either proximal or distal regions of the small intestine. In fed dogs, compared with the control period, the number of spikes during the infusion of erythromycin was significantly increased at the duodenum and jejunum, but not in the ileum (Table 1). Ileal activity appeared to be profoundly inhibited during the recovery period in both fed and fasted states.

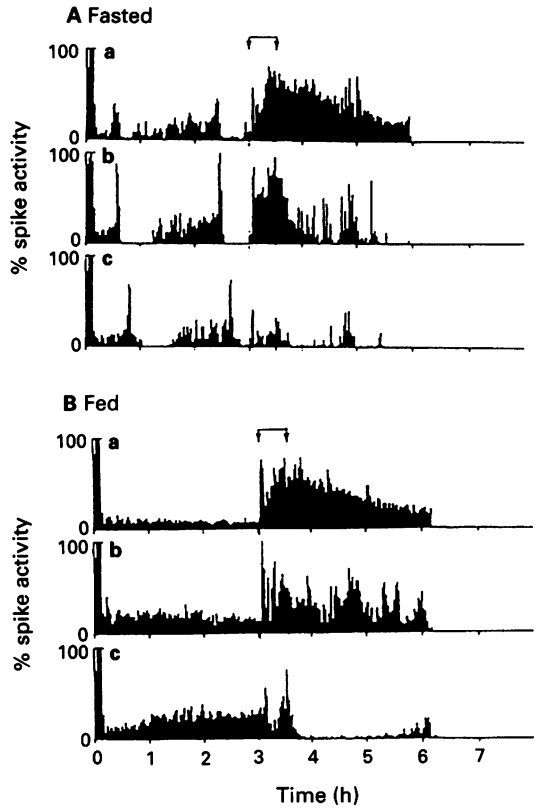


Figure 1 Effects of erythromycin (7 mg kg^{-1} 30 min^{-1}) on pre (A) and post (B) prandial intestinal electrical activity recorded by electrodes implanted at 3 different sites ((a) duodenum, (b) jejunum and (c) ileum) of the canine small bowel. The number of spikes occurring in each minute of recording has been normalised, with the first 10 min showing calibration of 120 pulses min^{-1}. The arrows indicate the period of infusion. Note the increase in spikes in the proximal small intestine during and after the infusion of erythromycin in both the fasted and fed states.

Atropine Although atropine inhibited both the basal pre- and post-prandial motility pattern when given on its own during the first 30 min of infusion (Period 2, Table 1), its effect on erythromycin-induced spike activity depended on whether the animals were fed or fasted (Figure 2). Postprandially, atropine did not abolish the increase in spike activity caused by erythromycin. Thus, in the presence of atropine the number of spikes during the infusion of erythromycin was significantly increased in the duodenum when compared with the control period (Table 1, Figure 2). In eight fasted dogs the only effect seen with erythromycin in the presence of atropine was an increase in spike activity in the duodenum during the 30 min of infusion (Period 3). In the jejunum and ileum during the erythromycin infusion, and at the 3 electrode sites during the recovery period erythromycin was unable to overcome the smooth muscle inhibition consistent with the presence of atropine (Figure 2).

Hexamethonium Unlike atropine, hexamethonium decreased the effects of erythromycin on the proximal intestine in both the fasted and fed states (Figure 3). Postprandially, during the first 30 min of hexamethonium infusion (Period 2, Table 1) spike activity was significantly depressed. The number of spikes increased during the administration of erythromycin (Period 3, Table 1), although they were not significantly different from control values.

Table 1 Postprandial spike activity before and after erythromycin-alone and in combination with drugs in the small intestine of the fed dog

Drugs	n	*Electrode site*	*Median spike number (interquartile)* *Period 1 (control)*	*Period 2*	*Period 3*
Erythromycin 7 mg kg^{-1}	8	Duodenum	565 (416–763)	–	2022 (1275–3213)**
		Jejunum	241 (204–663)	–	763 (288–1670)*
		Ileum	628 (357–1027)	–	739 (544–1146)
Atropine 50 mg kg^{-1} min^{-1}	8	Duodenum	216 (105–272)	64 (54–81)*	642 (411–1078)*
		Jejunum	162 (46–373)	120 (30–280)	195 (68–320)
		Ileum	266 (164–409)	156 (52–189)**	146 (62–275)
Hexamethonium 10 mg kg^{-1} 1h^{-1}	8	Duodenum	223 (100–377)	111 (24–213)**	217 (150–565)
		Jejunum	514 (287–653)	202 (140–295)**	103 (45–222)**
		Ileum	265 (184–369)	97 (66–264)**	144 (64–377)
Metoclopramide 150 μg kg^{-1}	8	Duodenum	390 (271–432)	306 (197–498)	1527 (870–1858)**
		Jejunum	264 (218–543)	233 (183–698)	683 (350–1083)*
		Ileum	478 (268–607)	565 (355–705)	481 (246–613)
Metoclopramide 2 mg kg^{-1}	8	Duodenum	408 (309–492)	467 (333–561)	1216 (716–1755)*
		Jejunum	490 (226–541)	410 (307–680)	654 (295–828)
		Ileum	439 (261–562)	622 (187–795)	532 (280–859)

The 3 periods of 30 min each are consecutive. Period 1: immediately before administration of any drug. Period 2: atropine, hexamethonium and metoclopramide were administered alone. Period 3: the above drugs were given in conjunction with erythromycin. The number of spikes in Periods 2 and 3 were compared to the number in the control period. Values are given as median and interquartile range from *n* studies. *$P<0.05$, **$P<0.01$, Wilcoxon matched pairs test.

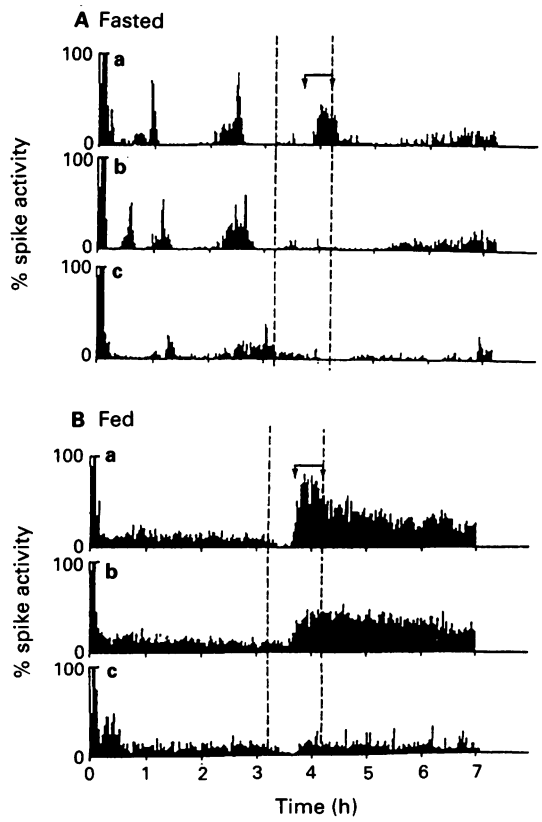


Figure 2 Effect of atropine (50 mg kg^{-1} min^{-1}) on erythromycin (7 mg kg^{-1} 30 min^{-1})-induced spike activity in the duodenum (a), jejunum (b) and ileum (c) in the pre (A) and post (B) prandial states. The dashed lines indicate the atropine infusion and the arrows the erythromycin infusion. The number of spikes occurring in each minute of recording has been normalised, with the first 10 min showing calibration of 120 pulses min^{-1}. For statistical analysis, in each 'fed' trace, the number of spikes was counted during 3 consecutive 30 min periods, before and including the dashed lines.

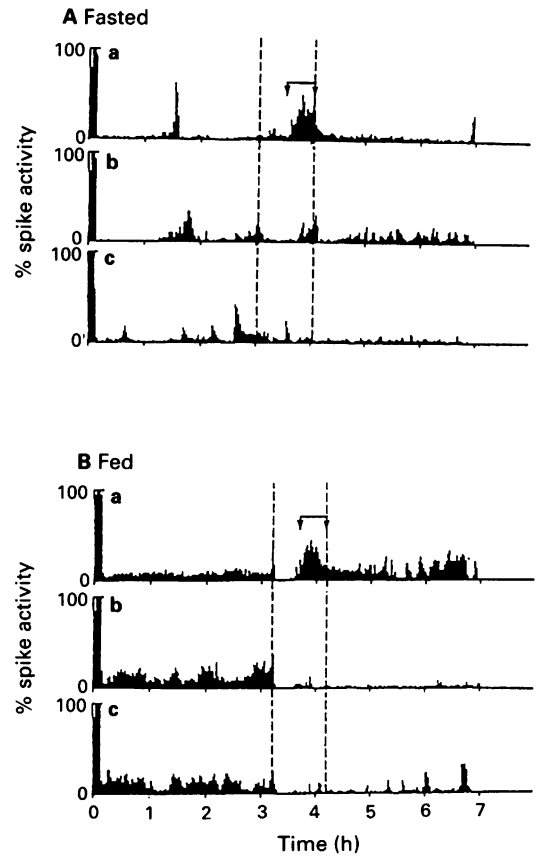


Figure 3 Effect of hexamethonium (10 mg kg^{-1} h^{-1}) on erythromycin (7 mg kg^{-1} 30 min^{-1})-induced spike activity in the duodenum (a), jejunum (b) and ileum (c) in the pre (A) and post (B) prandial states. The dashed lines indicate the period of hexamethonium and the arrows the erythromycin infusion. The number of spikes occurring in each minute of recording has been normalised, with the first 10 min showing calibration of 120 pulses min^{-1}. For statistical analysis, in each 'fed' trace, the number of spikes was counted during 3 consecutive 30 min periods, before and including the dashed lines.

Metoclopramide Neither the low (150 $\mu g\,kg^{-1}\,h^{-1}$) nor the high dose (2 $mg\,kg^{-1}\,h^{-1}$) of metoclopramide had an effect on motility during the first 30 min of infusion (Period 2). The control MMC pattern of the fasted state was not disturbed (Figure 4), and the number of postprandial spikes was the same as control (Table 1). When erythromycin was infused (Period 3), the spike number in the proximal intestine was significantly increased (Figure 4, Table 1), although the higher dose of metoclopramide appeared to reduce the erythromycin-induced effect on spike activity.

Emesis

Erythromycin After the infusion of erythromycin, all 8 dogs vomited when fasted, and five out of eight when fed (Table 2). One fed dog which did not vomit showed signs of nausea and retching, and this animal was categorised as having no emetic episodes. Table 2 illustrates the effects of the five drugs given in conjunction with erythromycin to investigate the emetic mechanism, showing the number of subjects with a positive emetic response as well as the latency of onset of the response. Atropine and hexamethonium tended to reduce the number of animals with emesis, although this was not statistically significant ($P > 0.05$). Metoclopramide at a low dose and BRL24924 (either alone or in combination with low dose metoclopramide) were even less effective than the cholinoceptor drugs in decreasing the incidence of emesis. Only metoclopramide at a high dose and the 5-hydroxytryptamine$_3$ (5-HT$_3$) receptor antagonist MDL72222 significantly reduced the emesis caused by erythromycin. The combination of MDL72222 and a low dose of metoclopramide did not further decrease the erythromycin-induced emesis.

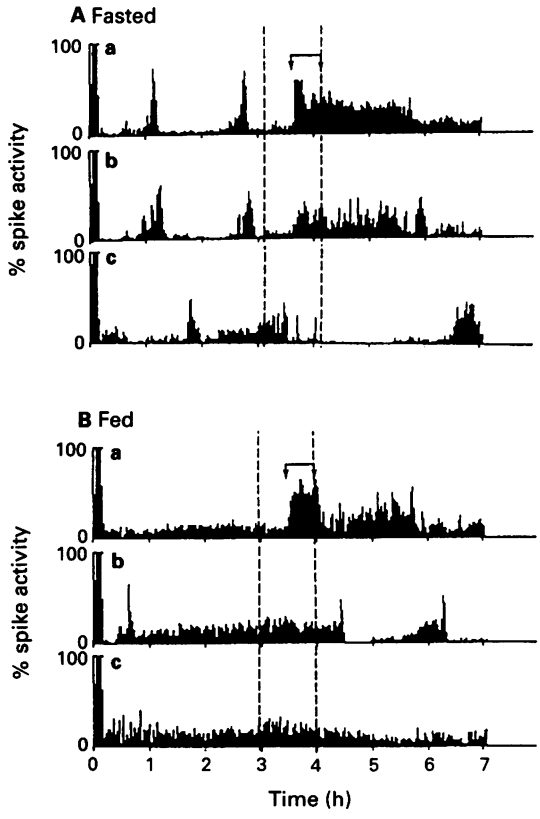


Figure 4 Effect of high dose metoclopramide (2 $mg\,kg^{-1}$) on erythromycin (7 $mg\,kg^{-1}$ 30 min^{-1})-induced spike activity in the duodenum (a), jejunum (b) and ileum (c) in the pre (A) and post (B) prandial states. The dashed lines indicate the metoclopramide infusion and the arrows the erythromycin infusion. The number of spikes occurring in each minute of recording has been normalised, with the first 10 min showing calibration of 120 pulses min^{-1}. For statistical analysis, in each 'fed' trace, the number of spikes was counted during 3 consecutive 30 min periods, before and including the dashed lines.

Discussion

The present study shows that erythromycin interrupted pre and postprandial motility patterns, increased intestinal spike activity and caused emesis in the canine small intestine. The dose of erythromycin used in the present study lies within the therapeutic range used clinically in intravenous therapy.

The involvement of a cholinergic mechanism at both the pre- and postganglionic levels in the erythromycin-induced increase in motility is suggested by the partial inhibition of this activity by atropine and hexamethonium. The higher density of cholinergic nerves in the proximal small intestine (Schulze-Derieu, 1981) could also explain why the motor effects of erythromycin were more pronounced on the proximal small intestine than the distal. However, the differential effect may implicate another receptor system in the light of recent findings that erythromycin competes with the intestinal peptide motilin in binding duodenal receptors (Kondo *et al.*, 1988; Peeters *et al.*, 1989) and that the number and sensivity of these receptor sites are higher in the proximal small bowel (Matsumoto *et al.*, 1986; Bormans *et al.*, 1988; Peeters *et al.*, 1988).

The possibility that the large increase in contractile activity seen in the presence of erythromycin was responsible for the high incidence of emesis was investigated with the use of motor-inhibiting cholinoceptor antagonists and of known anti-emetics. Could the two effects of erythromycin be dissociated? Although the increase in intestinal motility was greatly decreased by atropine and hexamethonium, the incidence of induced emesis was only slightly reduced by the two cholinoceptor agents. Metoclopramide, on the other hand, when given at a high dose, significantly decreased the incidence of emesis elicited by erythromycin but did not prevent the significant increase in intestinal motility. This dissociation of effects suggests that the disruption of GI motility and the emesis observed with erythromycin are not causally related.

All animals had at least one emetic episode when erythromycin was administered pre-prandially, but if erythromycin was infused when the post-prandial pattern was well established in the intestine, fewer animals had emetic episodes, and food delayed the onset of emesis. It is possible that the digestive hormones released by the meal act on receptors located either in the area postrema or the vomiting centre to reduce the emetic effect, or that the segmental peristaltic movements which propel the meal down the bowel counteract a possible reverse peristalsis.

Only metoclopramide at a high dose and MDL72222 significantly reduced erythromycin-induced emesis. Studies with patients receiving cytotoxic drugs have shown that the antiemetic effects of metoclopramide are dose-dependent. Only at a high dose (2 $mg\,kg^{-1}$ every 2 h to 10 $mg\,kg^{-1}$ over 24 h i.v.) does metoclopramide reduce emesis caused by cisplatin (Gralla *et al.*, 1981). A lower dose of metoclopramide (10 to 30 mg i.v.), which antagonizes emesis induced by apomorphine, and is therefore thought to occur through dopamine antagonism, is ineffective against cisplatin-induced emesis. The efficacy of metoclopramide at high doses was confirmed by the present study in the dog while a low dose was without effect, thus suggesting a mechanism other than on dopamine receptors.

It has recently been suggested that the anti-emetic effect of a high dose of metoclopramide is related to its action on 5-hydroxytryptamine (5-HT) receptors rather than to the dopamine antagonism obtained with lower doses (Miner & Sanger, 1986). Subsequently specific 5-HT$_3$ receptor antagonists were shown to be potent anti-emetics both experimentally and clinically (Fozard, 1987; Sanger, 1990). In the present study we used MDL 72222, which reduces emesis caused by cisplatin in ferrets (Miner & Sanger, 1986). Similar

Table 2 Emetic response to erythromycin and to drugs modulating the response in the fasted and fed dog

Drug		n (total)	n (emesis)	n Onset latency (min) [0–10	11–20	>20]
Erythromycin alone	Fasted	8	8	5	3	–
	Fed	8	5	1	2	2
Atropine	Fasted	8	5	3	1	1
	Fed	8	5	–	4	1
Hexamethonium	Fasted	8	5	3	2	–
	Fed	8	3	–	2	1
Metoclopramide (Low dose)	Fasted	8	7	5	2	–
	Fed	8	5	–	5	–
Metoclopramide (High dose)	Fasted	8	3*	1	2	–
	Fed	8	3	2	1	–
MDL72222	Fasted	8	4*	3	–	1
MDL + low dose metoclopramide	Fasted	8	4*	2	2	–
BRL24924	Fasted	8	7	6	1	–
BRL + low dose metoclopramide	Fasted	8	6	3	2	1

Erythromycin (7 mg kg^{-1}) was given either alone or in combination with all the drugs listed in the first column. For each treatment the number (n) of animals with emesis was compared with the number when erythromycin was given alone where $*P<0.05$, Fisher's exact test, and onset of latency is also shown.

results on emesis were obtained with another purported 5-HT_3 receptor antagonist, BRL 24924 (Renzapride), also in ferrets (Andrews *et al.*, 1986). The anti-emetic effect of these compounds was confirmed in the dog (Fitzpatrick *et al.*, 1990; Gullickson *et al.*, 1991) and man (Cunningham *et al.*, 1987).

In the present study the actions of MDL 72222 and BRL 24924 on erythromycin-induced emesis were studied because of their reported anti-emetic potential. Because emesis occurred more readily in the fasted than in the fed state, the two compounds were only studied in fasted dogs. They were used either on their own to test the 5-hydroxytryptaminergic component of any anti-emetic action, or in combination with a low dose of metoclopramide to investigate whether the emetic mechanism involved at least in part a dopamine pathway. MDL 72222 and other 5-HT_3 receptor antagonists have previously been shown to have no significant effect on pre and post prandial intestinal motility patterns (Davidson *et al.*, 1990). In the present study, only MDL 72222 was effective against the emesis caused by erythromycin, both alone and in combination with low-dose metoclopramide, confirming the anti-emetic efficacy of 5-HT_3 receptor antagonists and indicating that a 5-hydroxytryptaminergic mechanism is involved in the emesis provoked by erythromycin. BRL 24924 did not reduce emesis triggered by erythromycin even when combined with a low dose of metoclopramide.

Following the definition of a new class of 5-HT receptors (Dumuis *et al.* 1988), it is now suggested that substituted benzamides like BRL24924 act on 5-HT_4 receptors, rather than on 5-HT_3 receptors as was first reported. The main action of these drugs is said to be 'prokinetic', they accelerate gastric emptying, increase antro-duondenal coordination, and are mild anti-emetics. It is thus possible that the anti-emetic effect of 5-HT_4 receptor agonists may be secondary to enhanced peristaltic coordination due to the activation of the 5-HT_4 peripheral receptor on the gastrointestinal tract (Craig & Clarke, 1989; 1991). It is not surprising that BRL24924 was not effective in preventing or decreasing emesis caused by erythromycin in the present study. Erythromycin has recently been shown to enhance gastric emptying by increasing antro-duodenal coordination (Janssens *et al.*, 1990; Otterson & Sarna, 1990), and any compound that would decrease emesis by accelerating gastric emptying and coordinating peristalsis would be ineffective against erythromycin-induced emesis.

In summary, at clinically therapeutic doses, erythromycin causes large increases in small intestinal motility as well as emesis in the dog, these two effects occurring independently. The increase in contractile activity appears to be partly under cholinergic control, whereas emesis appears to occur via the activation of 5-HT_3 receptors without evidence for a dopamine pathway involvement.

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Na^+/H^+ exchange inhibitors reverse lactate-induced depression in postischaemic ventricular recovery

Morris Karmazyn

Department of Pharmacology and Toxicology, University of Western Ontario, London, Ontario N6A 5C1, Canada

1 By use of pharmacological approaches, the present study examined the hypothesis that the deleterious effect of lactate on postischaemic ventricular recovery may be mediated, at least in part, by enhanced activation of the Na^+/H^+ exchanger at the time of reperfusion.

2 Spontaneously beating isolated hearts of the rat were subjected to 15 min zero-flow global ischaemia followed by 30 min reperfusion. The effects of lactate (10, 20 or 40 mM) were studied by adding it 20 min before ischaemia whereas reperfusion was carried out with lactate-free buffer.

3 Pretreatment with 20 or 40 mM lactate significantly reduced postischaemic recovery of developed force to $17 \pm 3\%$ and $16 \pm 4\%$ of preischaemic values ($P < 0.05$) compared to a $78 \pm 4\%$ recovery in control hearts. Similarly, recovery in ventricular rate was significantly reduced to $34 \pm 7.6\%$ and $38 \pm 12\%$ with 20 and 40 mM lactate, respectively compared to $97.5 \pm 6.4\%$ recovery in control hearts. At a concentration of 10 mM, lactate was without effect on either force or ventricular rate recovery.

4 Coadministration of either of two Na^+/H^+ exchange inhibitors, amiloride (174 μM) or 5-N,N-hexamethylene amiloride (HMA, 1 μM) with lactate and inclusion of the two drugs during the first 5 min of reperfusion resulted in reversal of lactate-induced inhibition of force recovery with observed recoveries of $69 \pm 6.7\%$ and $64 \pm 5\%$ with amiloride and HMA, respectively. Similarly, recovery in ventricular rate was significantly enhanced to $92 \pm 10\%$ and $89 \pm 6\%$ with amiloride and HMA, respectively compared to $38 \pm 12\%$ recovery in control hearts.

5 In electrically paced hearts (5 Hz) subjected to 15 min ischaemia and 30 min reperfusion, 40 mM lactate significantly reduced force recovery to $36 \pm 5.5\%$ compared to $57 \pm 6\%$ in control hearts. In the presence of amiloride or HMA, force recovery in lactate-treated hearts was significantly increased to $68 \pm 16\%$ and $72 \pm 4.7\%$ of preischaemic values, respectively.

6 In spontaneously beating hearts, resting tension changes during both ischaemia and reperfusion were not statistically different between treatment groups. However, in paced hearts pretreated with 40 mM lactate the elevation in resting tension during the first 5 min of reperfusion, was significantly reduced by both amiloride and HMA.

7 Changes in functional recoveries produced by either lactate or Na^+/H^+ exchange inhibitors were unrelated to alterations in high energy phosphate depletion during ischaemia or to repletion of these compounds after 30 min reperfusion either in spontaneously beating or electrically paced hearts.

8 The results suggest that stimulated Na^+/H^+ exchange activation at reflow contributes, at least partially, to lactate-induced depression of postischaemic recovery.

Keywords: Rat heart; ischaemia; reperfusion; lactate; Na^+/H^+ exchange; contractile recovery; amiloride

Introduction

Many factors are thought to regulate functional recovery of the reperfused myocardium following prolonged ischaemia including blood borne constituents such as neutrophils or endogenous myocardial principles such as defective energy metabolism or ionic cellular homeostatis. An interesting hypothesis proposed a number of years ago (Neely & Grotyohann, 1984) suggested that enhanced accumulation of glycolytic products, particularly lactate, at the end of the ischaemic period can contribute significantly to diminished recovery of ventricular function after reperfusion. The evidence for lactate involvement originated primarily from two observations; (1) prevention of lactate accumulation during ischaemia by glycogen depletion resulted in enhanced postischaemic recovery whereas, (2) exogenous lactate administration to isolated hearts significantly attenuated recovery (Neely & Grotyohann, 1984).

The mechanisms underlying lactate-induced depression in postischaemic recovery are unknown, nonetheless its inclusion in ischaemiamimetic solutions is required for the production of manifestations of tissue dysfunction in various experimental models (Ferrier *et al.*, 1985; Northover, 1987; 1989). One possibility is that lactate directly depresses contractility and thus recovery following reperfusion or inhibits energy substrate utilization (Tennant, 1935; Armiger *et al.*, 1974; Neely & Grotyohann, 1984). Alternatively, enhanced intracellular lactate accumulation and subsequent cellular acidification may contribute to diminished recovery. In this regard, it has been suggested that Na^+/H^+ exchange activation at the time of reflow would result in enhanced tissue injury as stimulation of this exchanger would elevate intracellular Na^+ concentrations and, subsequently, cellular Ca^{2+} content via the Na^+/Ca^{2+} exchanger (Lazdunski *et al.*, 1985). The possibility that Na^+/H^+ exchange inhibition provides a salutary influence on the reperfused ischaemic heart was supported by an initial report from the author's laboratory (Karmazyn, 1988), and that of others (Tani & Neely, 1989) that inhibitors of this exchanger significantly reduced reperfusion injury and enhanced functional recovery following ischaemia. Recently, we have reported that this protection is associated with a marked reduction in arrhythmias, preservation of cellular ultrastructure and reduction in mitochondrial injury (Duan & Karmazyn, 1992). The antiarrhythmic property of amiloride and amiloride analogues in reperfused myocardium has also been demonstrated by Dennis *et al.* (1990) in rat isolated hearts subjected to 15 min low-flow ischaemia followed by 2 min reperfusion. Moreover, the antiarrhythmic property of these drugs was similarly demonstrated in a canine model of coronary artery ligation and reperfusion (Duff *et al.*, 1991). In both of these studies, the antiarrhythmic effects were attributed to inhibition of the

Na^+/H^+ antiport (Dennis *et al.*, 1990; Duff *et al.*, 1991).

Because of the potential importance of the Na^+/H^+ exchanger to recovery following ischaemia, it was hypothesized that inhibition of postischaemic recovery by lactate could be mediated by an initial lactate-induced acid load followed by enhanced Na^+/H^+ exchange activation at the time of reflow. To examine this possibility indirectly, we evaluated the effect of amiloride (Frelin *et al.*, 1984; Simchowitz & Cragoe, 1986), a non specific Na^+/H^+ exchange inhibitor and 5-N,N-hexamethylene amiloride, an inhibitor of the exchanger demonstrating markedly greater specificity and potency than the parent compound (Simchowitz & Cragoe, 1986), on lactate-induced functional and metabolic changes in the ischaemic and reperfused rat heart.

Methods

Male Sprague-Dawley rats weighing between 250 and 300 g were used in these studies. Animals were purchased from Charles River Canada Ltd. (St. Constant, Quebec, Canada). The animals were maintained in the Animal Care Facilities of the Faculty of Medicine, University of Western Ontario with free access to food and water in accordance with guidelines of the Canadian Council on Animal Care (Ottawa, Ontario).

For perfusion, the animals were killed by decapitation and the heart was rapidly excised, placed in a small crucible containing ice-cold Krebs-Henseleit buffer (composition below) and squeezed a few times with fingers to dislodge any clotted blood in the coronary vasculature. The heart was then mounted by the aorta on a stainless steel cannula and arranged for retrograde perfusion via the coronary arteries according to the Langendorff method as described in detail previously (Karmazyn, 1988; Watson & Karmazyn, 1991). The perfusion fluid was Krebs-Henseleit buffer (pH 7.4) of the following composition (in mM): NaCl 120, $NaHCO_3$ 20, KCl 4.63, KH_2PO_4 1.17, $MgCl_2$ 1.2, $CaCl_2$ 1.25 and glucose 8 as substrate. The buffer was continuously gassed with a 95% O_2/5% CO_2 mixture. Flow rate was set at 10 ml min^{-1} and controlled by a Watson-Marlow peristaltic pump. Contractile force (apicobasal displacement) was obtained by connecting a force displacement transducer (Grass FT.03) to the apex of the heart at an initial preload stretch of 2 g. For most experiments, hearts were allowed to contract spontaneously. In order to remove the contribution of rate to postischaemic recovery, some experiments were also done in which hearts were electrically stimulated at 5 Hz with a Grass S44 stimulator via platinum electrodes inserted into the ventricular epicardium. The entire system was regulated at 37°C. All recordings were obtained with a Grass polygraph.

The hearts were initially equilibrated for a 30 min perfusion period before induction of ischaemia. Where the effects of lactate were examined, sodium lactate (Sigma Chemical Co., St. Louis, MO, U.S.A.) was administered 20 min before ischaemia at a final concentration of either 10, 20 or 40 mM; for these experiments NaCl was reduced by equimolar concentrations, as appropriate. Only 40 mM lactate was examined in paced hearts. To test the effects of Na^+/H^+ exchange inhibitors, either amiloride (Sigma) dissolved in water after gentle warming (174 μM final concentration) or 5-N,N,-hexamethylene amiloride (HMA, 1 μM, Research Biochemicals Inc., Natick, MA, U.S.A.), dissolved in methanol (0.004% final buffer concentration) was added concomitantly with lactate. Initial experiments showed that neither of the two Na^+/H^+ exchange inhibitors or the HMA vehicle had a direct effect on cardiac function when added on their own.

Following the equilibration period, hearts were subjected to zero-flow ischaemia with or without a further 30 min reperfusion period. Zero-flow global ischaemia was initiated by turning off the perfusion pump for 15 min, care being taken to ensure temperature was maintained at 37°C during the ischaemic period. It should be emphasized that hearts initially treated with lactate were reperfused without lactate present in the buffer. However, when either amiloride or HMA was studied, these drugs were also present in the buffer for the first 5 min of reperfusion after which reperfusion was continued with drug-free buffer.

To obtain a full profile of energy metabolite content, hearts were clamped between tongs precooled in liquid nitrogen while on the perfusion cannula either immediately before the start of ischaemia, at the end of the 15 min ischaemic period or after 5 or 30 min of reperfusion. The frozen tissue was pulverized in liquid nitrogen and extracted with 6% perchloric acid for determination of high energy phosphate, adenine nucleotide or lactate content by use of enzymatic techniques as described previously (Bergmeyer, 1963; Watson & Karmazyn, 1991).

Statistical analyses were carried out by use of Analysis of Variance followed by Student-Newman-Keuls test to locate significant differences between treatment groups. Treatments were considered significant when $P<0.05$.

Results

Basal function

Table 1 demonstrates functional parameters of rat isolated hearts prior to initiation of ischaemia. No direct effect of 10 or 20 mM lactate on developed force or ventricular rate was observed whereas at 40 mM, lactate significantly depressed contractile force in the presence or absence of either amiloride or HMA. This cardiodepressant effect of 40 mM lactate was observed in both spontaneously beating or paced hearts, although with respect to the latter, a significant effect was seen only in the presence of HMA. Although the highest concentration of lactate exerted no effect on spontaneous ventricular rate, a significant negative chronotropic effect was observed in the presence of either amiloride or HMA (Table 1). No direct effect of lactate on resting tension was ever observed (not shown).

Tissue lactate contents

Profiles of lactate content are shown in Table 2. Lactate values increased markedly following 15 min ischaemia and, expectedly, these values were further increased by lactate treatment in a concentration-dependent fashion. Thus, in hearts pretreated with 40 mM lactate, tissue lactate at the end of ischaemia was approximately 80% higher than seen in the absence of lactate. Elevations in lactate content were unaffected by either amiloride or HMA treatment. After reperfusion, lactate values rapidly (within 5 min) returned to values which were essentially similar to those seen in non-ischaemic hearts. This recovery was unaffected by either lactate pretreatment or by amiloride or HMA.

Effects of lactate on the ischaemic and reperfused heart

Figure 1 shows the recovery of reperfused spontaneously contracting isolated hearts of the rat following a 15 min zero-flow ischaemic period, under control conditions or when pretreated with either 10, 20 or 40 mM lactate and reperfused with lactate-free buffer. In control hearts, force recovered to 78 ± 4% of preischaemic values after 30 min reperfusion which was not significantly affected by 10 mM lactate. However, 20 and 40 mM lactate significantly ($P<0.05$) attenuated force recovery to 17 ± 3% and 16 ± 4% of preischaemic values, respectively.

Recovery in ventricular rate was similar for control hearts and hearts treated with 10 mM lactate (96.5 ± 6.4% and 86 ± 7.6%, respectively, Figure 1). However, treatment with both 20 and 40 mM lactate significantly ($P<0.05$) depressed recovery of ventricular rate to 34 ± 7.6% and 38 ± 12% of preischaemic values, respectively (Figure 1). It should be noted that in hearts treated with lactate ($n = 15$), 4 (26.6%)

Table 1 Function data before the initiation of ischaemia in spontaneously contracting and paced hearts

Treatment	*Force (g)*	*Ventricular rate* (beats min^{-1})
	Spontaneously contracting	
Control	7.5 ± 0.4	329 ± 16
Lactate 10 mM	7.2 ± 0.6	309 ± 12
Lactate 20 mM	5.7 ± 1.0	311 ± 26
Lactate 40 mM	3.6 ± 0.5*	321 ± 18
Lactate 40 mM + amiloride	3.5 ± 0.8*	269 ± 23*
Lactate 40 mM + HMA	4.0 ± 0.5*	284 ± 13*
	Paced	
Control	5.8 ± 0.5	–
Lactate 40 mM	3.9 ± 0.4	–
Lactate 40 mM + amiloride	3.7 ± 0.5	–
Lactate 40 mM + HMA	3.5 ± 0.3*	–

Values indicate means ± s.e.mean of $n = 15$ except data for 10 mM lactate and paced hearts where $n = 5$. *$P < 0.05$ from control. HMA = hexamethylene amiloride. Data were obtained immediately before the initiation of ischaemia.

Table 2 Lactate content in control, ischaemic and reperfused hearts

Treatment	*Buffer lactate concentration* (mM) 0	10	20	40	40 + Amiloride	40 + HMA
	Tissue lactate content (μmol g^{-1} dry wt.)					
30 min normal perfusion	8.3 ± 1.6	5.9 ± 2.5	19.8 ± 7.3	47.8 ± 16.5*	–	–
15 min ischaemia	97.8 ± 11.6	111.6 ± 23.6	136.9 ± 17.5	173.8 ± 21.5*	197.6 ± 17.7*	183.0 ± 27.4*
5 min reperfusion	5.9 ± 2.4	4.7 ± 0.9	4.9 ± 2.7	6.1 ± 1.1	4.61 ± 1.8	5.8 ± 1.7
30 min reperfusion	7.8 ± 3.6	5.2 ± 0.7	6.3 ± 2.9	3.8 ± 1.2	5.9 ± 1.6	3.4 ± 2.1

Values indicate means ± s.e.mean of $n = 15$, except 5 min reperfusion and 10 mM lactate data where $n = 5$. *$P < 0.05$ from respective values obtained in the absence of exogenous lactate. HMA = hexamethylene amiloride. –; not done.

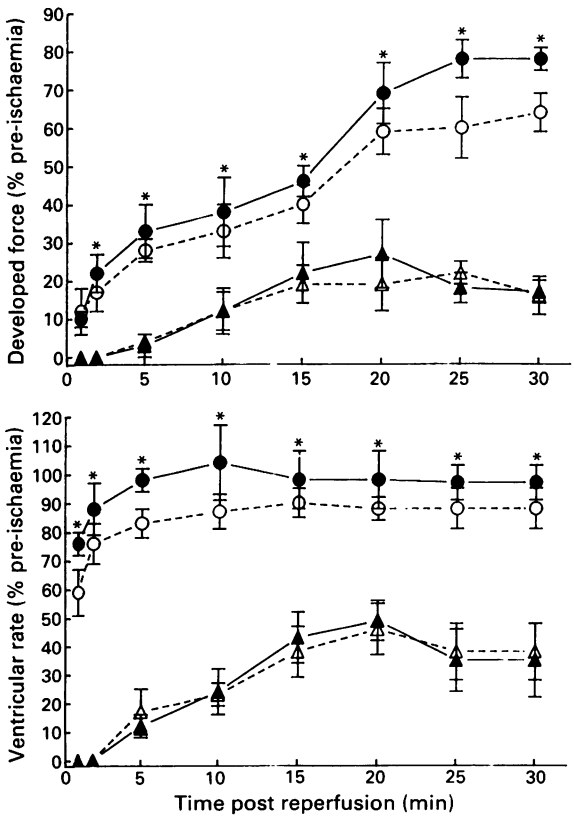


Figure 1 Effect of pretreatment with 10 (○), 20 (▲) or 40 (△) mM lactate on developed force and ventricular rate recovery in reperfused isolated spontaneously beating rat hearts following 15 min zero-flow ischaemia. See Methods for description of drug addition protocols. Values indicate means of $n = 15$ except for 10 mM lactate data where $n = 5$; s.e.mean shown by vertical bars. *$P < 0.05$ from values obtained with 20 or 40 mM lactate. (●) Control data.

and 6 (40%) totally failed to recover when treated with 20 or 40 mM lactate, respectively, whereas all control hearts or hearts treated with 10 mM lactate demonstrated functional recovery.

The effects of amiloride and HMA on diminished post-ischaemic recovery induced by either 20 or 40 mM lactate were examined. The results obtained with either inhibitor were similar irrespective of lactate concentration; thus, for conciseness results are presented only for those studies in which 40 mM lactate was used (Figure 2). As shown in Figure 2, both amiloride and HMA significantly improved recovery of force to 69 ± 6.7% and 64 ± 5% of preischaemic values, respectively, compared to 17 ± 2% seen with lactate alone. In addition, the 38 ± 12% recovery in ventricular rate after 30 min reperfusion was significantly increased to 92 ± 10% and 89 ± 6% in hearts treated with amiloride or HMA, respectively (Figure 2).

Because of the substantial effects of various treatments on ventricular rate, experiments were also done to assess the influence of 40 mM lactate and Na^+/H^+ exchange inhibitors in hearts in which ventricular rate was maintained by electrical pacing. As shown in Figure 3, control hearts subjected to this protocol recovered 57 ± 5.5% contractility, markedly lower than described above for spontaneously beating hearts. Nonetheless, lactate significantly ($P < 0.05$) depressed recovery to 36 ± 6% after 30 min reperfusion. Both amiloride and HMA improved recovery to 68 ± 16% and 72 ± 5%, respectively, values which were not different from control but significantly higher than those seen with 40 mM lactate alone (Figure 3).

Table 3 shows a summary of resting tension data in ischaemic and reperfused hearts and the influence of lactate. Under control conditions, resting tension was unaffected by ischaemia but was rapidly increased by reperfusion with a progressive return to close to preischaemic values thereafter. At all concentrations studied, lactate failed to alter significantly this response in hearts which were allowed to contract spontaneously. However, in paced hearts the eleva-

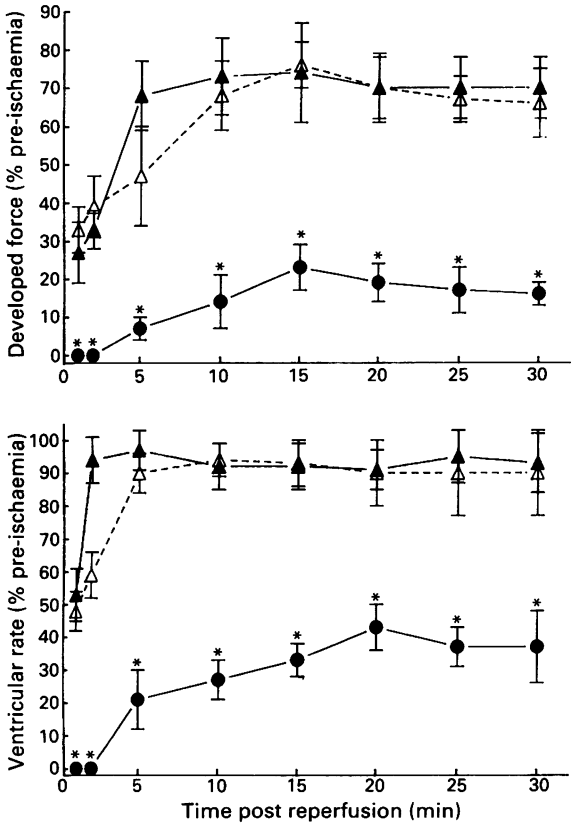


Figure 2 Effect of amiloride (▲) or 5-N,N-hexamethylene amiloride (△) on depression in postischaemic developed force and ventricular rate recovery produced by 40 mM lactate in reperfused isolated spontaneously beating rat hearts following 15 min zero-flow ischaemia. See Methods for description of drug addition protocols. Values indicate means of $n = 15$; s.e. mean shown by vertical bars. $*P < 0.05$ from values obtained with lactate alone. (●) Effect of lactate alone.

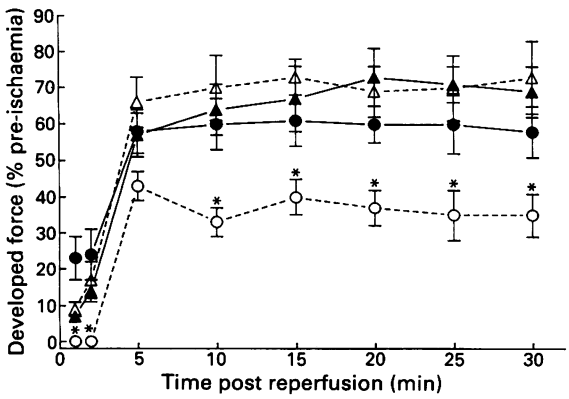


Figure 3 Effect of pretreatment with 40 mM lactate in the presence or absence of amiloride or 5-N,N-hexamethylene amiloride (HMA) on developed force recovery in reperfused isolated paced rat hearts following 15 min zero-flow ischaemia. See Methods for description of drug addition protocols. Values indicate means of $n = 5$; s.e. mean shown by vertical bars. $*P < 0.05$ from other treatment groups except the first two points which indicate a significant difference from control group only. (●) Control; effect of lactate alone (○) and in the presence of amiloride (▲) or HMA (△).

tion in resting tension in the presence of 40 mM lactate was significantly attenuated by both amiloride and HMA (Table 3).

Profile of myocardial energy metabolism

Table 4 shows a summary of changes in energy products in terms of high energy phosphates and adenine nucleotide contents under the various experimental conditions. Fifteen minutes of zero flow ischaemia resulted in a large and significant reduction in tissue high energy phosphates, particularly creatine phosphate (CrP). These changes were accompanied by increases in adenosine 5'-diphosphate (ADP) levels. The magnitude of these changes was unaffected by any treatment. Reperfusion for 30 min resulted in an incomplete recovery in high energy phosphate and adenine nucleotide contents. Interestingly, there were no significant differences in these values between any of the treatment groups studied, despite the substantial effects on contractile recovery produced by lactate pretreatment and Na^+/H^+ exchange inhibitors.

Discussion

Myocardial ischaemia results in numerous biochemical aberations among which are the accumulation of various products derived from anaerobic glycolytic metabolism (Neely & Feuvray, 1981). It has been suggested that accumulation of these products during ischaemia may contribute to diminished functional recovery upon reperfusion (Neely & Grotyohann, 1984). Of these, lactate has been suggested to represent a particularly important factor in limiting recovery. This contention is supported by the finding that prevention of lactate accumulation through glycogen depletion results in improved recovery of isolated hearts following reperfusion whereas addition of exogenous lactate results in markedly diminished recovery (Neely & Grotyohann, 1984). Nevertheless, the involvement of lactate and approaches aimed at preventing its accumulation still remain controversial. For instance, with respect to lactate accumulation, it has been shown that preischaemic hypoxia and subsequent glycogen depletion in rabbit hearts does not prevent lactate accumulation during ischaemia and indeed actually results in diminished recovery after reperfusion (Lagerstrom *et al.*, 1988). Furthermore, it has recently been demonstrated that exogenous lactate (10 or 50 mM) failed to increase damage in rat cardiac myocytes subjected to anoxia and reoxygenation (Geisbuhler & Rovetto, 1990). The reasons for such apparent discrepancies still remain to be elucidated although they may be related to such factors as species or experimental model.

The present results are supportive of previous studies demonstrating deleterious effects of lactate in various models of myocardial ischaemia (Neely & Grotyohann, 1984; Ferrier *et al.*, 1985; Northover, 1987; 1989) which demonstrate that exogenous lactate, at concentrations similar to those used in the present study, can inhibit postischaemic recovery. It is important to emphasize that although lactate is a cardiodepressant on its own, the ability of lactate to depress recovery is most likely due to cellular mechanisms initiated by ischaemia and reperfusion since reflow was carried out in the absence of lactate in the perfusion medium. Indeed, as early as 5 min after reperfusion, lactate contents were similar in all treatment groups despite markedly different degrees of functional recovery. In addition, it should be emphasized that 20 mM lactate was equally effective in reducing postischaemic recovery in the absence of a significant direct negative inotropic influence. The inability of 10 mM lactate to depress recovery was surprising, although it may suggest that critically high tissue lactate levels must be achieved in order for the deleterious effect of lactate to be manifested.

Differences in depletion in high energy phosphate stores as a consequence of ischaemia or the degree of repletion of these compounds after reperfusion were also unlikely to represent contributing factors, since values did not differ between treatment groups under any of the conditions studied. This finding is in agreement with various other studies showing dissociation between depletion-repletion of high energy phosphates and postischaemic ventricular recovery under various experimental conditions (Higgins & Blackburn, 1984; Neely & Grotyohann, 1984; Karmazyn & Neely, 1989; Currie & Karmazyn, 1990; Murphy *et al.*, 1991). Not

Table 3 Effects of treatments on resting tension in ischaemic and reperfused hearts

Time	Control	Lac 40 mM	*Treatment* Lac 40 mM + Amil	Lac 40 mM + HMA
			Resting tension (g)	
			Spontaneously beating hearts	
0 min	2.0 ± 0	2.6 ± 0.4	2.0 ± 0.3	2.0 ± 0.1
15 min isch	2.0 ± 0	2.3 ± 0.5	2.0 ± 0	2.0 ± 0
Reperfusion				
5 min	4.0 ± 0.7	4.4 ± 1.1	2.6 ± 0.5	3.3 ± 0.5
10 min	2.7 ± 0.4	3.7 ± 0.8	2.5 ± 0.5	3.2 ± 0.5
15 min	2.4 ± 0.4	2.7 ± 0.6	2.2 ± 0.2	2.7 ± 0.3
20 min	2.3 ± 0.3	2.5 ± 0.4	2.3 ± 0.1	2.2 ± 0.2
30 min	2.4 ± 0.5	2.7 ± 0.8	2.0 ± 0	2.2 ± 0.2
			Paced hearts	
0 min	2.1 ± 0.2	2.0 ± 0.3	2.0 ± 0.1	2.0 ± 0.2
15 min isch	3.1 ± 0.4	4.3 ± 0.4	2.7 ± 0.4	2.1 ± 0.5
Reperfusion				
5 min	5.5 ± 0.9	7.1 ± 1.2	4.1 ± 0.3*	3.6 ± 0.5*
10 min	3.9 ± 1.3	4.7 ± 0.8	2.9 ± 0.5	3.0 ± 0.4
15 min	2.7 ± 0.5	3.6 ± 0.6	2.5 ± 0.6	2.5 ± 0.4
20 min	2.5 ± 0.4	3.0 ± 0.3	2.7 ± 0.2	2.3 ± 0.4
30 min	2.4 ± 0.4	3.0 ± 0.3	2.6 ± 0.3	2.3 ± 0.4

Values indicate means ± s.e.mean, $n = 15$ for spontaneously beating hearts and 5 for paced hearts. *$P < 0.05$ from values obtained with 40 mM lactate alone. Amil = amiloride, HMA = hexamethylene amiloride, Lac = lactate, isch = ischaemia. Zero min data refer to values immediately before ischaemia.

Table 4 High energy phosphate and adenine nucleotide contents in control, ischaemic and reperfused hearts

Treatment	ATP	*Metabolite* (μmol g^{-1} dry wt.) CrP	ADP	AMP
30 min normal perfusion				
Control	22.4 ± 2.7	28.3 ± 3.9	7.3 ± 1.0	3.4 ± 0.5
Lactate 10 mM	24.6 ± 4.3	32.7 ± 4.0	6.8 ± 0.7	3.1 ± 0.6
Lactate 20 mM	23.7 ± 2.9	31.6 ± 1.0	7.0 ± 0.6	2.7 ± 0.6
Lactate 40 mM	23.3 ± 1.3	30.8 ± 5.2	6.6 ± 1.0	3.0 ± 0.7
15 min ischaemia				
Control	7.1 ± 1.6	3.1 ± 0.7	11.9 ± 2.0	1.6 ± 0.5
Lactate 10 mM	7.1 ± 0.9	2.7 ± 1.3	12.4 ± 3.3	2.4 ± 0.6
Lactate 20 mM	9.8 ± 2.1	4.0 ± 0.7	13.3 ± 0.8	1.9 ± 0.8
Lactate 40 mM	10.8 ± 3.8	4.2 ± 0.4	8.8 ± 1.0	5.3 ± 0.7
Lactate 40 mM + amiloride	11.9 ± 1.3	6.9 ± 1.8	10.3 ± 2.8	4.7 ± 0.8
Lactate 40 mM + HMA	12.1 ± 3.4	5.8 ± 1.5	9.9 ± 1.3	5.2 ± 0.9
5 min reperfusion				
Control	11.6 ± 1.5	9.8 ± 1.2	5.1 ± 0.6	2.5 ± 0.8
Lactate 10 mM	9.7 ± 1.3	8.7 ± 0.8	4.9 ± 1.1	3.0 ± 0.7
Lactate 20 mM	12.3 ± 1.8	13.6 ± 2.8	5.8 ± 1.2	3.1 ± 0.9
Lactate 40 mM	10.7 ± 2.3	11.8 ± 1.7	4.9 ± 0.8	2.7 ± 0.6
Lactate 40 mM + amiloride	13.5 ± 1.6	12.9 ± 1.3	5.7 ± 0.7	2.5 ± 0.3
Lactate 40 mM + HMA	12.2 ± 3.0	14.6 ± 1.8	5.8 ± 1.0	2.9 ± 0.7
30 min reperfusion				
Control	14.7 ± 3.1	19.8 ± 4.0	4.9 ± 0.8	2.8 ± 0.7
Lactate 10 mM	17.3 ± 2.9	19.0 ± 4.8	5.3 ± 1.0	3.1 ± 0.5
Lactate 20 mM	11.9 ± 2.7	15.3 ± 3.2	5.0 ± 0.9	2.6 ± 0.7
Lactate 40 mM	12.5 ± 3.1	14.7 ± 3.6	4.9 ± 1.0	2.5 ± 0.8
Lactate 40 mM + amiloride	13.4 ± 4.1	15.7 ± 3.0	4.5 ± 0.9	2.3 ± 1.0
Lactate 40 mM + HMA	12.9 ± 3.4	16.8 ± 5.7	3.9 ± 0.6	2.0 ± 0.6

Values indicate means ± s.e.mean of $n = 15$ except 10 mM lactate data where $n = 5$. HMA = hexamethylene amiloride.

unexpectedly therefore, the only significant metabolic consequence of lactate pretreatment that was measured in this study was the significantly elevated lactate content at the end of ischaemia in the absence or presence of Na^+/H^+ exchange inhibitors.

Previous studies from our laboratory have demonstrated a protective effect of amiloride against reperfusion injury (Karmazyn, 1988; Duan & Karmazyn, 1992). It has been shown that this protective effect of amiloride was associated with an inhibition of the early (2 min after reperfusion) rise in tissue Na^+ concentrations and the subsequent attenuation of Ca^{2+} accumulation (Tani & Neely, 1989). In addition, these authors have shown that the protective effect of amiloride can be mimicked by attenuation of lactate accumulation at the end of ischaemia (Tani & Neely 1989). Thus, the importance of Na^+/H^+ to reperfusion injury appears to be adequately documented.

The present study was done to examine pharmacologically if Na^+/H^+ exchange contributes to lactate-induced depression in postischaemic recovery. Amiloride exerts numerous inhibitory effects on other cellular processes including the Na^+/K^+ ATPase and the Na^+/Ca^{2+} exchange at concentrations substantially higher than those required to inhibit Na^+/H^+ exchange or that were used in the present study (Floreani *et*

al., 1987). To strengthen the concept of Na^+/H^+ exchange involvement, we also tested the effects of HMA, a markedly more specific and potent inhibitor of Na^+/H^+ exchange (Simchowitz & Cragoe, 1986). The ability of both agents to potently reverse lactate-induced depression of ventricular recovery suggests that a potential mechanism for the latter may involve stimulation of Na^+/H^+ exchanger. For instance, cellular lactate uptake (Dennis *et al.*, 1984; Mann *et al.*, 1985) could result in increased intracellular acidification during ischaemia and, since the pH gradient represents a primary determinant of Na^+/H^+ exchange activation (Lazdunski *et al.*, 1985; Piwnica-Worms *et al.*, 1986), increased Na^+/H^+ exchange activation upon reperfusion. It is also interesting to note that the beneficial effects of both amiloride as well as HMA were observed even though the drug treatment was restricted only to the initial 5 min of reperfusion, suggesting that early events during reflow modulate recovery. Nonetheless, it should be noted that pH_i was not measured in the present study and hence the degree of intracellular acidification following lactate preperfusion cannot be established. In addition, although tissue lactate content was measured, this represents total content found both intracellularly as well as interstitial space. Thus, the precise degree of lactate uptake into myocytes cannot be established with certainty. Taken together, an alternative explanation for the protective effects of amiloride or HMA may exist: for example that both drugs prevented ischaemia-induced changes in cellular homeostasis. For instance, it has been shown that amiloride can block the elevations in both $[Na^+]_i$ and $[Ca^{2+}]_i$ that occur during ischaemia in the absence of adenosine 5'-triphosphate (ATP) preservation resulting in improved postischaemic recovery (Murphy *et al.*, 1991). In the present study, neither amiloride nor HMA exerted significant beneficial effects when administered at the time of reflow (data now shown), a finding in agreement with a previous study by the author (Karmazyn, 1988) at least with respect to amiloride. Thus, the possibility exists that modulation of cellular events occurring during ischaemia represents an important constituent for the salutary effects of Na^+/H^+ exchange inhibitors. It is also noteworthy that the deleterious effects of exogenous lactate and the protective effects of amiloride or HMA were not restricted to developed force since lactate also produced diminished recovery in rate which was reversed by the Na^+/H^+ exchange inhibitors. The mechanism for the rate effects is uncertain particularly in an isolated heart preparation which precludes detailed investigations into cellular mechanisms, especially in terms of electrophysiological changes. It is interesting that intracellular acidification inhibits the calcium channel (Kaibara & Kameyama, 1988) and it could therefore be speculated that combined ischaemia plus exogenous lactate results in prolonged inhibition of pacemaker activity upon reflow. However, this possibility cannot explain the ability of either amiloride or HMA to reverse the effects of lactate on rate in the reperfused myocardium. Moreover, as only ventricular rate was measured, it cannot be stated with certainty whether reduced rate recovery represented effects on ventricular or atrial conduction. Mechanistic-based studies are currently being planned using myocyte preparations, where intracellular events such as ionic and electrophysiological changes could be precisely monitored.

Irrespective of precise mechanisms involved, the results of the present study add to the emerging body of evidence that Na^+/H^+ exchange inhibitors may represent an important approach towards the salvage of the ischaemic myocardium following reperfusion procedures.

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Comparison of the effects of selective inhibitors of phosphodiesterase types III and IV in airway smooth muscle with differing β-adrenoceptor subtypes

Adrian Tomkinson, Jan-Anders Karlsson & [1]David Raeburn

Rhône-Poulenc Rorer Ltd., Dagenham Research Centre, Rainham Road South, Dagenham, Essex RM10 7XS

1 The relaxant properties of the type IV adenosine 3',5'-cyclic monophosphate phosphodiesterase (cyclic AMP PDE) inhibitor, rolipram and the β_2-selective and non-selective β-adrenoceptor agonists salbutamol and isoprenaline, were compared on the guinea-pig, bovine, and mouse trachea and porcine bronchus all precontracted with methacholine (EC_{30}).

2 Rolipram and both β-agonists produced concentration-dependent reversal of the methacholine-induced tone in the four airway preparations.

3 Isoprenaline and salbutamol were similar in potency on the guinea-pig ($-\log_{10}IC_{50}$:8.43, 8.06) and bovine ($-\log_{10}$ IC_{50}:8.52, 8.40) airways. In contrast, salbutamol was much less potent than isoprenaline on the mouse trachea (>1000 fold) and the porcine bronchus (>100 000 fold).

4 The potency of rolipram approached that of isoprenaline on the guinea-pig and bovine trachea (β_2-adrenoceptors predominate). However, rolipram was significantly less active than isoprenaline on the porcine bronchus (1000 fold) and mouse trachea (>2000 fold) where β_2-adrenoceptors predominate.

5 Siguazodan, the type III cyclic AMP PDE inhibitor, produced concentration-dependent relaxations of the porcine bronchus and guinea-pig trachea contracted with methacholine. Siquazodan was 100 fold more active than rolipram in pig tissues indicating the type III isoenzyme may be of greater functional significance in this tissue. In contrast, siguazodan was 15 times less potent that rolipram in guinea-pig airways suggesting a greater role for the type IV PDE.

6 These findings may reflect a possible relationship between the β_2-adrenoceptor subtype and the functional importance of the type IV PDE isoenzyme. A similar relationship may exist between β_1-adrenoceptors and the PDE type III isoenzyme.

Keywords: Airway smooth muscle; phosphodiesterase inhibitors; β-adrenoceptors; rolipram; siguazodan

Introduction

β-adrenoceptor agonists are potent relaxants of smooth muscle, both *in vitro* and *in vivo*. The alteration in contractility may be mediated by selective stimulation of the β_1 or the β_2 adrenoceptor subtypes or by a non-selective stimulation of both depending on the receptor subtype(s) present (see review by Bülbring & Tomita, 1987).

In airway smooth muscle from several experimental animal species there are differences in the density and the relative proportions of the β-adrenoceptor subtypes present. The predominant subtype present in guinea-pig (O'Donnell, 1972; Zaagsma *et al.*, 1983) and bovine (Lemoine *et al.*, 1989) tissues is the β_2 whereas in the pig (Goldie *et al.*, 1983) and mouse (Henry & Goldie, 1990) the β_1 subtype predominate. In human airway smooth muscle only β_2-adrenoceptors have been demonstrated (Richardson & Ferguson, 1979; Carstairs *et al.*, 1985).

It has been shown that both the β_1 and β_2-adrenoceptors may coexist in cardiac muscle, although functionally the β_1-adrenoceptor subtype predominates (Heitz *et al.*, 1983; Molenaar & Summers, 1987). Thus, by the use of selective β_2-agonists it is possible to achieve a significant bronchodilatation without concomitant cardiovascular effects (Tattersfield & Britton, 1988).

It is widely accepted that β-adrenoceptor agonists produce their effects on airway and other smooth muscle by the elevation of tissue adenosine 3',5'-cyclic monophosphate (cyclic AMP) (see review by Giembycz & Raeburn 1991 and references therein), the level of which is regulated by the family of isoenzymes known as the phosphodiesterases (PDEs, Beavo & Reifsnyder, 1990; Torphy & Undem, 1991; Giembycz & Dent, 1992). Biochemical assay of the PDE isoenzyme profile has been performed in airway smooth muscle from a number of different species. In human airway smooth muscle PDE types I, II, III, IV and V have been resolved (Cieslinski *et al.*, 1988; Giembycz *et al.*, 1992; Shahid *et al.*, 1992). Types I, II, IV and V have been demonstrated in bovine trachealis (Giembycz & Barnes 1991; Shahid *et al.*, 1991) whereas only the type III and IV have been recognized in guinea-pig trachealis, although two additional fractions of PDE were not characterized (Silver *et al.*, 1988). All of these studies suggest that the type IV PDE may be the major isoenzyme present. In contrast to airways, in atrial and ventricular muscle and vascular smooth muscle the PDE type III isoenzyme appears to predominate (Silver *et al.*, 1988; Muller *et al.*, 1990 a, b).

In a preliminary study *in vitro* (Tomkinson *et al.*, 1992) we found that in the guinea-pig airways (predominantly β_2) the selective PDE type IV inhibitor rolipram approached the potency of salbutamol and isoprenaline (a selective and non-selective β-agonist respectively). On the other hand in porcine airways (predominantly β_1) isoprenaline was markedly more potent than either the selective β_2-adrenoceptor agonist or the type IV PDE inhibitor. Based on these findings we have hypothesized that there may be a relationship between β-adrenoceptor subtype and PDE isoenzyme in reducing airway smooth muscle contractility. In the present study, we have attempted to test this hypothesis by comparing the smooth muscle relaxing properties *in vitro* of salbutamol, isoprenaline, rolipram and siguazodan (SKF 94836, a PDE type III inhibitor) in airway preparations which have been shown to contain different β-adrenoceptor populations.

[1] Author for correspondence.

Methods

Tissue preparation

Guinea-pigs (male, Dunkin-Hartley, 400–500 g. Interfauna U.K.) were killed by cervical dislocation followed by exsanguination and mice (male, 20–25 g, BALBc, Charles river, U.K.) were killed by exposure to a rising concentration of carbon dioxide. Tracheae were removed and placed in Krebs-Ringer Bicarbonate (KRB) solution and fat and connective tissue dissected away. Bovine and porcine tissues obtained from the local abattoir were placed immediately in KRB for transport. Bovine tracheae and porcine bronchi were cleaned as for the other tissues. All tissues were denuded, by mechanical abrasion, of their epithelium to remove the influence of epithelial-derived factors (Raeburn, 1990) and because of the high density of β-adrenoceptors present (Goldie et al., 1986a; Henry et al., 1990). Tracheal strips (guinea-pig, cow), tracheal rings (mouse) and bronchial strips (pig primary bronchus) were suspended under an applied load such that they were at their respective optimal length (Lo), derived from preliminary experiments, and equilibrated for 90 min washing at 15 min intervals. The KRB was maintained at 37°C and gassed with 5% CO_2 plus 95% O_2, pH was 7.4. The composition of the KRB (mM) was as follows: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, dextrose 10.1.

Contractility studies

Cumulative concentration-response curves were constructed to methacholine (10 nM–100 μM) in each tissue and the concentration producing 30% of maximum contraction (EC_{30}) was determined by computerized linear regression analysis. This concentration of spasmogen was chosen to reduce complications of functional antagonism seen at higher concentrations of the agonist. For investigation of their relaxant properties, tissues were contracted with methacholine (EC_{30}) and when the response had plateaued isoprenaline, salbutamol, rolipram, siguazodan (all from 10 nM to 100 μM) or vehicle control (saline, isoprenaline and salbutamol; DMSO, rolipram and siguazodan) were each added cumulatively. Ascorbic acid (0.57 mM) was added to the isoprenaline solution to prevent oxidative degradation. The concentration of relaxant producing 50% inhibition (IC_{50}) of the methacholine response was calculated in each tissue by linear regression. Results are expressed as the negative log of the IC_{50} ($-\log_{10} IC_{50}$).

Drugs and solutions

The following substances were used: (−)-salbutamol hemisulphate, (−)-isoprenaline (+)-bitartrate, histamine diphosphate, methacholine chloride (all Sigma); rolipram and siguazodan were synthesized by Rhône-Poulenc Rorer, Dagenham.

Statistical evaluation

Results are expressed as mean ± s.e.mean from n animals. EC_{30} values for methacholine and $-\log_{10} IC_{50}$ values for the relaxants were examined by one-way analysis of variance (ANOVA). Where a difference was found across the groups, Student's t test (unpaired, two-tailed) was performed to assess significance of difference. $P<0.05$ was accepted as significant. Least squares linear regression analysis was performed for comparison of tissue sensitivity (EC_{30}, IC_{50}) with contractility.

Results

Methacholine contractions

Cumulative concentration-response curves to methacholine in airway smooth muscle preparations from the various species are depicted in Figure 1. EC_{30} values and maximum force of contraction are listed in Table 1. The rank order of tissue sensitivity to methacholine was bovine trachealis = guinea-pig trachealis > mouse trachealis > pig bronchus. As expected, the maximum force of contraction was greater in the tissues with more muscle mass. There was, however, no correlation between maximum contractile force and tissue sensitivity to methacholine ($r = -0.29$).

Isoprenaline-induced relaxations

Isoprenaline produced concentration-dependent relaxations of the methacholine (EC_{30})-contracted airway smooth muscle

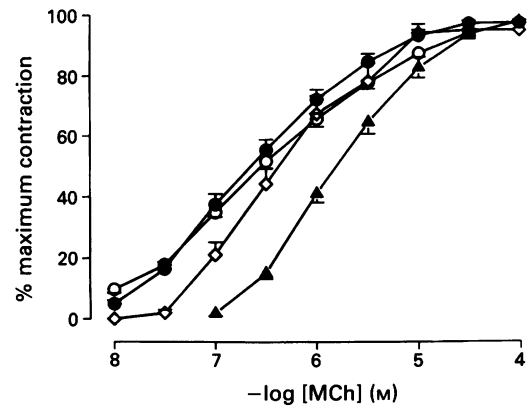


Figure 1 Comparison of the methacholine (MCh) cumulative concentration-response curves in airway smooth muscle preparations from four different species. (◇, $n = 6$) Mouse trachea, (●, $n = 10$) guinea-pig trachea, (○, $n = 5$) bovine trachea, (▲, $n = 6$) porcine bronchus. Abscissa scale: −log molar MCh concentration, ordinate scale: % maximum contraction. Values are the mean and vertical lines show s.e.mean.

Table 1 EC_{30} values and maximum force of contraction for methacholine in different airway smooth muscle preparations

Tissue	n	EC_{30} (nM)				Max. contraction (g)
Bovine trachea	5	81 ± 6	N.S.			10.74 ± 1.14
Guinea-pig trachea	10	93 ± 16		*		2.73 ± 0.30
Mouse trachea	6	210 ± 60			**	0.26 ± 0.03
Porcine bronchus	6	710 ± 60		*		3.04 ± 0.50

Data shown are means ± s.e.mean
N.S. $P>0.05$; *$P<0.05$; **$P<0.01$.

preparations from all four species (Figures 2–5). Relaxation of the methacholine-induced response was complete (>100%) in all tissues except the mouse trachea where maximum relaxation achieved was 86 ± 6% ($n = 3$). The potency of isoprenaline was similar in all tissues except the porcine bronchus (18 fold less potent) as indicated by the $-\log_{10} IC_{50}$ values quoted (Table 2).

Salbutamol-induced relaxations

Salbutamol caused concentration-dependent relaxations of the methacholine-contracted airway preparations (Figures 2–5). As with isoprenaline, relaxations to salbutamol were complete in all tissues except the mouse trachea where the maximum relaxation observed was 32 ± 14% ($n = 3$). The potency of salbutamol on the different airway preparations is indicated by the $-\log_{10} IC_{50}$ values shown in Table 2. Salbutamol was similar in potency to isoprenaline on bovine and guinea-pig tracheal preparations in which β_2-adrenoceptors predominate, the $-\log_{10} IC_{50}$ values for salbutamol being 8.40 ± 0.10 ($n = 5$) and 8.06 ± 0.10 ($n = 5$) respectively. In contrast, on mouse trachea and porcine bronchus, which have been shown to contain a predominance of β_1-receptors, salbutamol was much less potent (>1000 fold, >100,000 fold respectively) than isoprenaline.

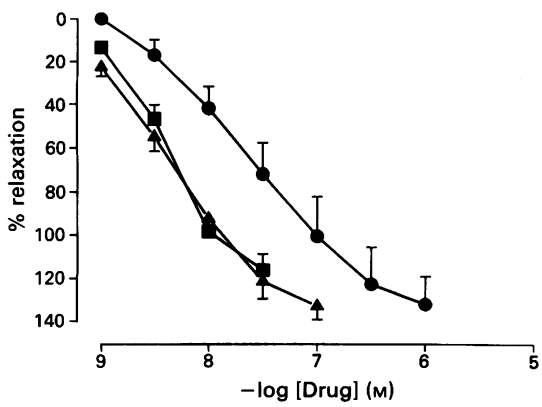


Figure 2 Relaxation of the methacholine (MCh, EC_{30})-contracted bovine tracheal smooth muscle preparation by isoprenaline (▲, $n = 5$), salbutamol (■, $n = 5$), and rolipram (●, $n = 5$). Abscissa scale: −log molar drug concentration, ordinate scale: % relaxation MCh contraction. Values are the mean and vertical lines show s.e.mean.

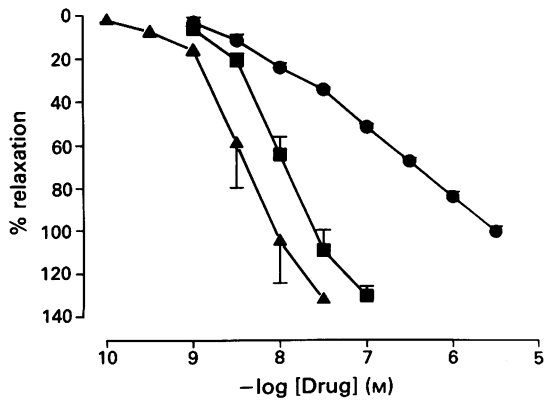


Figure 3 Relaxation of the methacholine (MCh, EC_{30})-contracted guinea-pig tracheal smooth muscle preparation by isoprenaline (▲, $n = 5$), salbutamol (■, $n = 5$), and rolipram (●, $n = 5$). Abscissa scale: −log molar drug concentration, ordinate scale: % relaxation MCh contraction. Values are the mean and vertical lines show s.e.mean.

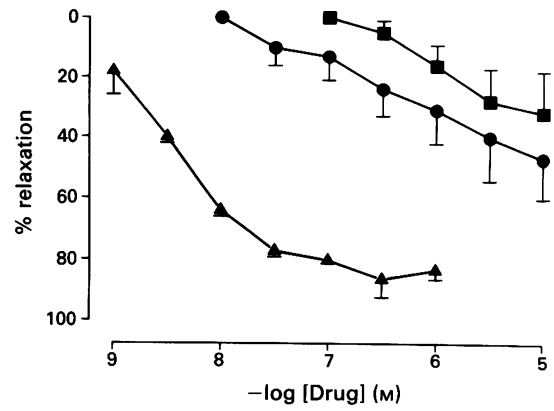


Figure 4 Relaxation of the methacholine (MCh, EC_{30})-contracted mouse tracheal smooth muscle preparation by isoprenaline (▲, $n = 3$), salbutamol (■, $n = 3$), and rolipram (●, $n = 5$). Abscissa scale: −log molar drug concentration, ordinate scale: % relaxation MCh contraction. Values are the mean and vertical lines show s.e.mean.

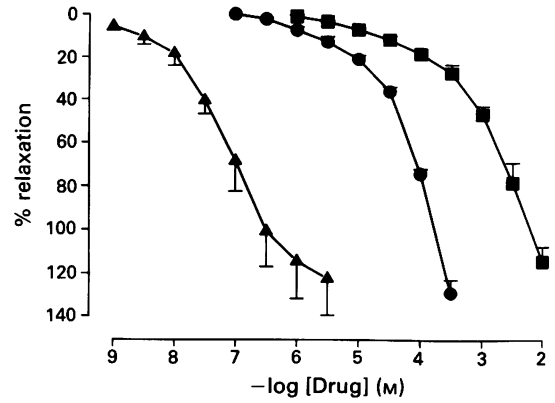


Figure 5 Relaxation of the methacholine (MCh, EC_{30})-contracted porcine bronchus smooth muscle preparation by isoprenaline (▲, $n = 5$), salbutamol (■, $n = 5$), and rolipram (●, $n = 5$). Abscissa scale: −log molar drug concentration, ordinate scale: % relaxation MCh contraction. Values are the mean and vertical lines show s.e.mean.

Rolipram-induced relaxations

Rolipram caused concentration-dependent relaxation of the methacholine-contracted airway preparations (Figures 2–5). Rolipram produced complete reversal of the methacholine-induced response in all tissues except the mouse trachea (47 ± 13%, $n = 5$). The $-\log_{10} IC_{50}$ values shown in Table 2 indicate the relative potency of rolipram on the preparations used. In bovine and guinea-pig tracheal preparations the potency of rolipram was similar and approached that of salbutamol and isoprenaline. In contrast, in porcine bronchus and mouse trachea rolipram was significantly less active than isoprenaline (1000 fold, >2000 fold respectively).

Siguazodan-induced relaxations

Siguazodan produced concentration-dependent relaxation of methacholine-contracted airway smooth muscle from pig bronchus and guinea-pig trachea. In each case reversal was complete (Figure 6). The $-\log_{10} IC_{50}$ for siguazodan in pig bronchus was 6.28 ± 0.13 ($n = 7$) indicating the type III PDE inhibitor to be 100 fold more potent than rolipram on this tissue (Figure 6a). Siguazodan-induced reversal of the methacholine-induced contraction of the guinea-pig trachea (Figure 6b) was considerably less potent (approximately 15 times) than that seen with rolipram ($-\log_{10} IC_{50}$: 5.87 ± 0.07, $n = 5$).

Table 2 $-\log_{10} IC_{50}$ values for isoprenaline, salbutamol and rolipram in methacholine (EC_{30})-contracted airway smooth muscle preparations

Tissue	n	Predominant β-receptor subtype	$-logo_{10} IC_{50}$ Isoprenaline	Salbutamol	Rolipram
Bovine trachea	5	β_2	8.52 ± 0.08	8.40 ± 0.10	7.71 ± 0.23
Guinea-pig trachea	5	β_2	8.43 ± 0.15	8.06 ± 0.10	7.01 ± 0.04
Mouse trachea	3-5	β_1	8.30 ± 0.03	< 5.00	< 5.00
Porcine bronchus	5	β_1	7.22 ± 0.20	2.94 ± 0.08	4.25 ± 0.02

Data shown are mean ± s.e.mean

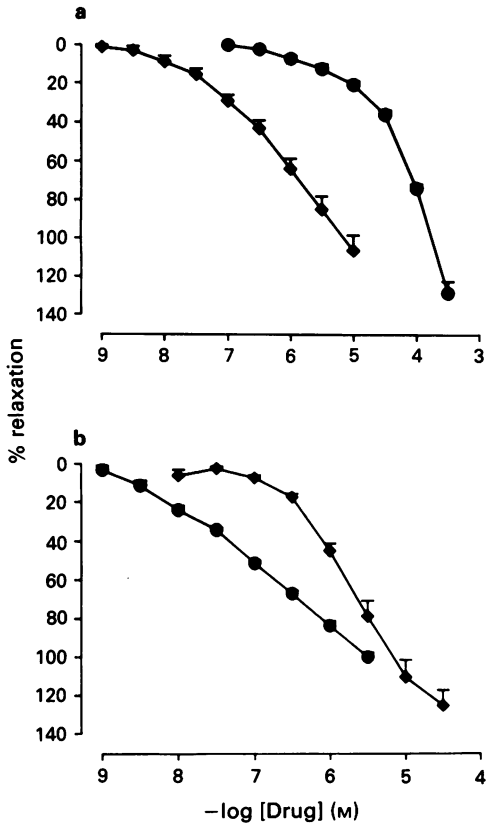


Figure 6 Comparison of the relaxant properties of rolipram (●) and siguazodan (◆) on the porcine bronchus (a) and guinea-pig trachea (b) contracted with methacholine (MCh, EC_{30}). Abscissa scale: −log molar drug concentration, ordinate scale: % relaxation MCh contraction. Values are the mean and vertical lines show s.e.mean ($n > 5$).

Discussion

In guinea-pig and bovine tracheal preparations the potency of salbutamol was similar to that of isoprenaline, confirming the predominance of the β_2-adrenoceptor subtype previously demonstrated functionally and by radioligand binding studies (Carswell & Nahorski 1983). In contrast to the guinea-pig and bovine trachea, salbutamol was significantly less potent than isoprenaline on the mouse trachea. Functional and autoradiography studies have recently demonstrated, quite comprehensively, the β_1-adrenoceptor subtype as being the predominant functional adrenoceptor in this tissue, supporting our observations (Henry & Goldie 1990; Henry *et al.*, 1990).

As with the mouse trachea, salbutamol was significantly less potent than isoprenaline on the porcine bronchus. The difference in potency of the β-agonists was particularly evident in this preparation where the potency of salbutamol was approximately 100 fold less than that seen on the mouse trachea. Surprisingly, given its low potency, salbutamol eventually produced complete reversal of the methacholine response. This probably reflects non-selectivity of salbutamol at these very high concentrations. Our findings support those of Goldie *et al.* (1983) who demonstrated an homogeneous population of β_1-adrenoceptors on the porcine bronchus.

The varied effects of the type IV PDE inhibitor, rolipram, revealed a marked difference between guinea-pig and bovine tracheal preparations on the one hand and the mouse tracheal and porcine bronchial preparations on the other, as observed for the selective β_2-adrenoceptor agonist salbutamol. Rolipram produced complete reversal of the methacholine-induced response on both the guinea-pig and bovine preparations. The potency approached that of isoprenaline and salbutamol indicating the type IV PDE isoenzyme to be of significant functional importance. These observations are consistent with previous profile and functional studies (Silver *et al.*, 1988; Harris *et al.*, 1989; Osborn *et al.*, 1990; Shahid *et al.*, 1991). In contrast, on the mouse trachea and porcine bronchus rolipram was significantly less potent than isoprenaline suggesting the type IV PDE to be of less functional significance in these tissues.

Although the PDE isoenzyme profile of the porcine bronchus and mouse trachea has yet to be described, the very low potency of rolipram on these tissues would suggest that a different PDE isoenzyme to the type IV isoenzyme may be of greater functional significance. In the present study the relaxant effect of siguazodan, a type III cyclic AMP PDE inhibitor, was determined on the porcine bronchus where it was approximately 100 fold more active than rolipram. In contrast, siguazodan was approximately 15 times less potent than rolipram on the guinea-pig trachea. These findings suggests that the type III isoenzyme may be the major functional PDE isoenzyme in pig airways whilst confirming the functional significance of the type IV isoenzyme in the guinea-pig airways. Based on these observations, we feel that as was the case with pig airways the type III isoenzyme may be the major functional isoenzyme in the mouse trachea but this has yet to be confirmed.

Collectively, these results may be interpreted as suggesting a possible functional relationship between the β_2-adrenoceptor subtype and the type IV cyclic AMP PDE isoenzyme, as proposed in our hypothesis. Furthermore, a similar relationship between β_1-adrenoceptors and the type III PDE may also occur. The existence of such a relationship would predict a low potency for rolipram on the mouse and pig tissues previously shown to contain a predominance of β_1-adrenoceptors.

In conclusion, the relatively high potency of rolipram in the guinea-pig and bovine trachea, in which β_2-adrenoceptors predominate over β_1-adrenoceptors, and the relatively low potency in the mouse trachea and porcine bronchus, in which β_1-adrenoceptors predominate over β_2-adrenoceptors, suggests a possible relationship between the β_2-adrenoceptor subtype and the functional importance of the type IV cyclic AMP PDE isoenzyme. A Similar relationship may exist between the β_1-adrenoceptor and the type III cyclic AMP PDE isoenzyme as suggested by the potency of siguazodan, relative to rolipram, on the guinea-pig and porcine airway preparations. However, since in every preparation examined rolipram and

siguazodan relaxed the tissues, irrespective of the predominant β-adrenoceptor subtype present, this suggests that both the type III and type IV PDE isoenzymes are involved in regulating airway smooth muscle contractility. It is possible that rolipram and siguazodan could be acting additionally at the type III and type IV isoenzymes respectively, that is to say that at the higher concentrations used we may be seeing some non-selective effects. However, given their respective IC_{50} values for inhibiting PDE type III and IV (Torphy & Undem, 1991; Giembycz & Dent, 1992) this seems less likely. Theophylline, a non-selective PDE inhibitor, is similar in potency on the porcine bronchus (Goldie *et al.*, 1986a), bovine trachea (Lohmann *et al.*, 1977; Grandordy *et al.*, 1987) and guinea-pig trachea (Goldie *et al.*, 1986b) suggesting that it is unlikely that the differences in potency of rolipram in these tissues represents a difference in phosphodiesterase activity *per se*.

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The local anti-inflammatory action of dexamethasone in the rat carrageenin oedema model is reversed by an antiserum to lipocortin 1

[1]G.S. Duncan, S.H. Peers, *F. Carey, *R. Forder & R.J. Flower

Department of Biochemical Pharmacology, The William Harvey Research Institute, The Medical College of St. Bartholomew's Hospital, Charterhouse Square, London, EC1M 6BQ and *Bioscience 2, ICI Pharmaceuticals, Macclesfield, Cheshire, SK10 4TG

1 A local pre-injection of 1 μg dexamethasone sodium phosphate strongly inhibited (>60% inhibition at 3 h; $P<0.001$ at all time points) the development of carrageenin-induced paw oedema in the rat induced by a subplantar injection of 0.1 ml, 2% carrageenin.

2 Coinjection of a polyclonal rabbit antiserum raised against human 1-188 recombinant lipocortin 1, which also recognised the rat protein, reversed the inhibitory action of dexamethasone ($P<0.05$ at 4 h and 5 h). At the highest volume used (40 μl) control antisera were without any effect.

3 These data further support the concept that lipocortin 1 is involved in the anti-inflammatory mechanism of action of the glucocorticoids.

Keywords: Lipocortin 1; dexamethasone; carrageenin oedema

Introduction

Lipocortin 1 (also called annexin 1) is a member of a super family of proteins (Pepinsky *et al.*, 1988) characterized by the presence of multiple copies of a 17 amino acid motif thought to be responsible for the ability of these proteins to bind negatively charged phospholipids and calcium (Saris *et al.*, 1986).

Lipocortin 1 is induced by glucocorticoids in several systems both *in vivo* (Fuller & Verity, 1989; Ambrose & Hunninghake, 1990; Goulding *et al.*, 1990a) and *in vitro* (Errasfa *et al.*, 1985; Piltch *et al.*, 1989; Browning *et al.*, 1990; Solito *et al.*, 1991). Administration of purified human recombinant lipocortin 1 produces anti-inflammatory and anti-pyretic effects in rats and rabbits (Cirino *et al.*, 1989; Carey *et al.*, 1990; Davidson *et al.*, 1991), eicosanoid suppressive actions in cells *in vitro* (Cirino *et al.*, 1987; Cirino & Flower, 1987) and ameliorates ischaemic damage (infarct size and cerebral oedema) produced in rat brain by occlusion of the middle cerebral artery (Relton *et al.*, 1991).

In some systems neutralising antibodies to lipocortin 1 have been shown to reverse the actions of glucocorticoids. For example, Violette *et al.* (1990) were able to abrogate the effect of dexamethasone on squamous carcinoma cell differentiation by addition of the neutralising anti-lipocortin 1 monoclonal antibody 1A. Likewise, Carey *et al.* (1990) observed that the inhibitory action of dexamethasone on interleukin 1β-induced thermogenesis in the rat was reversed following an intracerebroventricular injection of a polyclonal rabbit antibody raised against 1-188 lipocortin 1. Relton and her colleagues (1991) also observed that this antibody exacerbated the ischaemic damage to the rat brain, pointing to a protective effect of endogenous lipocortin 1.

The rat hind paw carrageenin oedema model is a popular acute model of inflammation which is very sensitive to the inhibitory action of both local and systemic steroids as well as to lipocortin 1 (Cirino *et al.*, 1989). Here we show that the local inhibitory action of dexamethasone is abrogated by the anti-lipocortin 1 1-188 antiserum but not by sera drawn randomly from a panel of control rabbits. The data support the concept that exogenous steroids exert part of their anti-inflammatory effect through the induction of extracellular lipocortin 1.

Methods

Male Wistar rats (150–200 g) were purchased from Olac (U.K.) and retained in the animal unit for one week before use. They were transferred to the procedure room at least 24 h before the start of the experiment and accustomed to the experimental protocol. Hind paw oedema was induced by the injection of 0.1 ml carregeenin (2% w/v in sterile saline) and the paw volume measured hourly by plethysmometry for 5 h as previously described (Cirino *et al.*, 1989) using a hydroplethysmograph (model 7150, Ugo Basile, Camerio, Italy). Dexamethasone (1 μg: Decadron; MSD, Hoddesdon, UK) and/or antisera was injected locally in a volume of 0.05 ml, 10–15 min before the carrageenin injection. Oedema was expressed as volume (ml) increase above original volume of the paw as measured immediately before the first injection.

The lipocortin antiserum was raised in rabbit to human recombinant lipocortin 1 residues 1-188 which had been expressed in *E. coli* as previously described (Carey *et al.*, 1990).

SDS-PAGE and immunoblotting was performed by conventional techniques and lipocortin 1 was visualised using a highly specific antisera (Ab842) which showed no cross reactivity with other members of the lipocortin family (J. Browning, personal communication). Both full length human recombinant lipocortin 1 and Ab 842 were kindly supplied by Dr J. Browning, Biogen Research Inc., Cambridge, U.S.A.

Statistics

Student's t test (unpaired) was used for comparisons of individual data points when comparing control with dexamethasone-treated rats and when comparing antiserum and treated rats with vehicle-treated controls.

[1] Author for correspondence.

Results

In a series of preliminary experiments we established a dose-response curve to locally injected dexamethasone in the rat paw. Injection of 0.1 μg dexamethasone was without effect whereas injection of 10 μg per paw produced a near maximal (>85%) inhibition. For the purposes of this experiment a maximal effect is not desirable and therefore we reduced the dose to 1 μg per paw which produced significant ($P<0.001$) inhibition at all time points>1 h with an approximate inhibition of 65% (at the time of peak oedema) or 60% (area under the curve).

Figure 1 shows the effect of coinjection of 10, 20 and 40 μl of the rabbit polyclonal antiserum which recognises 1–188 lipocortin 1. The antiserum reverses the effect of the dexamethasone although the effect does not show a graded response to different doses. In practice 40 μl produced the maximum inhibition possible whilst 5 μl (data not shown) was without effect.

To determine whether this was a function of normal rabbit serum we tested a panel of 5 other batches of non-immune rabbit serum drawn at random from our colony. All were without effect at the highest dose used (40 μl). Figure 2 shows a matched experiment in which 40 μl of a control antiserum was contrasted with 40 μl of immune antiserum. Inhibition in the presence of the control antiserum was not significantly different from the dexamethasone alone, whilst 40 μl of immune antiserum almost completely abrogated the action of dexamethasone.

From Figures 1 and 2 it can be seen that both control and anti-lipocortin 1 antiserum appeared to enhance the oedematous response during the first hour. To test whether this was an effect of mast cell degranulation caused by serum alone, we performed an experiment in which rats were treated with 10 μg methysergide simultaneously with 20–40 μl serum injection into the paw. The treatment reduced substantially the oedema produced by serum injection at 30 min from 0.44 ± 0.03 ml ($n=5$) to 0.14 ± 0.03 ml ($n=5$), when 40 μl of serum was injected and from 0.30 ± 0.03 ml ($n=5$) to 0.14 ± 0.05 ml ($n=5$) when 20 μl was injected.

To ensure that the rabbit polyclonal anti-human recombinant lipocortin 1 1–188 antiserum recognised rat lipocortin, we performed a simple test in which we compared the ability of this antiserum and another highly specific antiserum (Ab842) to recognise rat lipocortin extracted from lavage macrophages and used Western blotting analysis. Both antibodies recognised an identical pattern of products in the rat macrophage extracts with a major band at 36 kDa (data not shown) corresponding to the native species of lipocortin 1.

The ability of the rabbit polyclonal antiserum to reduce the activity of lipocortin 1 could be caused by proteolytic activity in the serum. To eliminate this possibility we conducted an experiment in which immune and non-immune serum was incubated together with samples of authentic human lipocortin 1 for 1 h at 37°C after which the samples were extracted and subjected to Western blotting analysis. Figure 3 shows the results of this experiment from which it can be seen that a small amount of metabolism occurred in all samples tested, but that there was no difference between the immune and control sera in this respect.

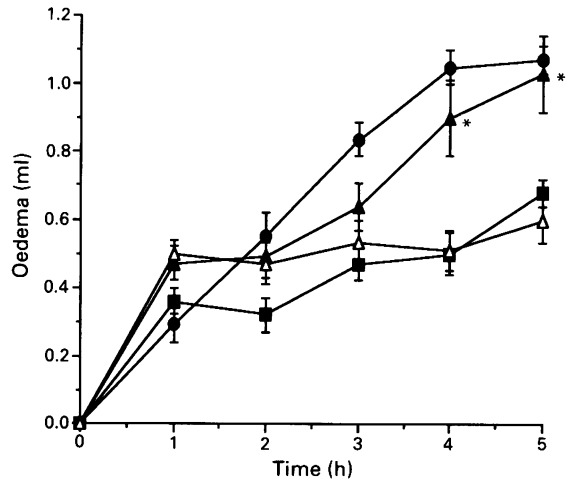


Figure 2 Dexamethasone inhibition of carrageenin-induced rat paw oedema: reversal by anti-lipocortin 1 antibody and lack of effect of control antiserum. Dexamethasone (1 μg) (■) or saline vehicle (●) was pre-injected with 40 μl anti-lipocortin 1 antiserum (▲) or non-immune rabbit serum (△) 15 min before carrageenin, $n=4$. *Significantly different from dexamethasone alone ($P<0.05$).

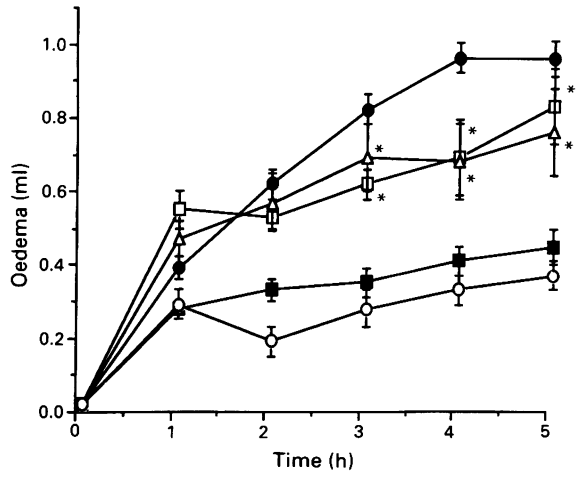


Figure 1 Inhibition of carrageein-induced rat paw oedema by local dexamethasone and reversal by anti-lipocortin 1 antisera. Dexamethasone (1 μg) (■, $n=26$) or saline vehicle (●, $n=27$) was pre-injected with 10 μl (○, $n=9$), 20 μl (△, $n=10$) or 40 μl (□, $n=10$) of antibody or vehicle 15 min before carrageenin, and the paw volume recorded for 3 h. Vertical bars indicate s.e.mean, *significantly different from dexamethasone alone ($P<0.05$).

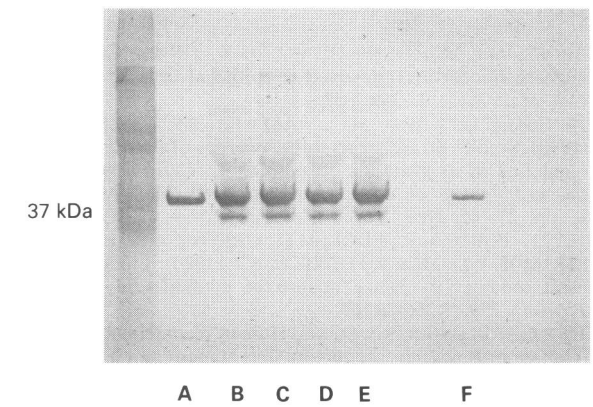


Figure 3 A Western blot shows the degree of proteolytic degradation of lipocortin 1 by the antisera used in this experiment.

Extreme left hand lane molecular weight markers. Lanes (A) = lipocortin 1 + buffer only; (B) = lipocortin 1 + anti lipocortin antiserum (C–E) = non immune control sera. (F) = lipocortin 1 standard, not incubated.

Discussion

The classical approach to the investigation of the function of endogenous compounds is the use of antagonists or antibodies. In the absence of antagonists to the protein lipocortin 1, one is compelled to rely upon experiments with immunological reagents which recognise the purified protein. Data described in this paper using this approach strongly suggest that the acute local anti-inflammatory action of dexamethasone is at least partially dependent upon the generation of lipocortin 1.

Carey *et al.* (1990) were able successfully to demonstrate that the same antiserum as was used in this study, reversed the action of dexamethasone as an anti-pyretic agent in the rat. Lipocortin 1 itself was active as an antipyretic in this model. Lipocortin 1 was also protective in the ischaemic brain damage model of Relton *et al.* (1991) and central administration of the same antiserum exacerbated the damage caused by ischaemia, suggesting a protective role for endogenous lipocortin 1.

Other antisera to lipocortin 1 have also been shown to reduce or block the action of glucocorticoids. Violette *et al.* (1990) demonstrated that hydrocortisone caused the terminal differentiation of a human squamous cell carcinoma line and that this effect was neutralised by the co-presence of a monoclonal anti-lipocortin antibody but not by a non-neutralising anti-lipocortin 1 monoclonal (i.e. an antibody which binds the protein but does not prevent its biological effect). A similar phenomenon was observed by Croxtall & Flower (1992) in which dexamethasone-induced inhibition of A549 cell proliferation was blocked in the presence of the monoclonal anti-lipocortin 1 antibody 1A.

Before the advent of purified recombinant human lipocortin 1 and the appropriate antisera, some highly suggestive experiments which pointed in the same direction were performed with the monoclonal antibody RM23 (Flower *et al.*, 1984) raised against the 'anti-phospholipase' activity generated by steroid treatment of the rat macrophage. This factor was almost certainly a member of the lipocortin family and RM23 inhibited the reduction in macrophage prostaglandin synthesis by hydrocortisone and reversed the local anti-inflammatory effect of dexamethasone in the rat carrageenin-induced pleurisy model.

Although highly suggestive these results could not be taken as definitive since the actual nature of the protein target recognised by the antibody was not completely characterized. We have ensured in these experiments that the rabbit anti-human 1–88 lipocortin 1 antibody does in fact recognise the rat protein. The protein sequence of rat and human lipocortin 1 is almost 90% homologous (Kovacic *et al.*, 1991) so such a cross-reactivity is probably to be anticipated.

The idea that externally applied antisera can reverse the action of hormones such as the glucocorticoids implies strongly that the lipocortin 1 target is extracellular. This indeed is congruent with other evidence. For example the external application of lipocortin to cells or its intravenous administration to animals (Cirino *et al.*, 1989; Carey *et al.*, 1990; Davidson *et al.*, 1991) produces a prompt and dramatic effect on eicosanoid generation, inflammation or fever. Furthermore, the existence of a protein binding site with a high affinity for lipocortin has been detected on the surface of some human cells with the obvious implication that this is a receptor or a target enzyme (Goulding *et al.*, 1990b).

The injection of serum itself, in doses of 20–40 µl, into the carrageenin-inflamed paw caused a non-significant but repoducible increase in the oedema responses at the 1 h time point. This seemed to be caused by mast cell degranulation as a small oedema produced by injection of the serum alone was blocked substantially with methysergide.

As lipocortin 1 produces many effects by acting on the outside of cells it is susceptible to the action of proteases and indeed treatment of cells with proteases has been reported to abrogate the actions of steroids in some systems (Hirata *et al.*, 1980). To exclude the fact that our rabbit serum contained proteolytic activity which might destroy lipocortin, we compared the ability of control and immune serum to degrade authentic human lipocortin 1. There was no apparent difference in the ability of the sera to degrade this protein over a long period of incubation and indeed only a fraction of the protein was degraded at all. This argues against the possibility of non-specific proteolytic mechanisms.

The glucocorticoids can alter the expression of almost 1% of the mammalian genome and it is therefore only to be expected that they have pleiotropic actions on the inflammatory response. Nevertheless it should be possible to identify key effectors which mediate particular aspects of their action. We believe lipocortin 1 to be one of these effector proteins and consider that the evidence presented in this paper further strengthens this concept by demonstrating the specific reversing activity of the antisera against local administered dexamethasone.

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Glucocorticoid – and non-glucocorticoid induction of lipocortins (annexins) 1 and 2 in rat peritoneal leucocytes *in vivo*

[1]Susan H. Peers, [2]Frazer Smillie, Amber J. Elderfield & [1,3]Roderick J. Flower

Pharmacology Group, School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, Avon BA2 7AY

1 We have studied the occurrence, distribution and disposition of lipocortins (annexins) 1, 2 and 5 in mixed peritoneal leucocytes obtained from rats in which glucocorticoid levels were altered by adrenalectomy, administration of the glucocorticoid antagonist, RU486, or by injection of dexamethasone or hydrocortisone, as well as from rats in which the peritoneal cells were elicited by inflammatory stimuli.

2 In cells obtained from untreated rats with an intact adrenal cortex, lipocortins 1, 2 and 5 were readily detectable: the majority of each of the proteins was apparently located intracellularly with much smaller amounts in the membrane. Lipocortin 1 and to a lesser extent lipocortin 5 were also seen in a Ca^{2+}-dependent association with the external plasma membrane. Following administration of RU486 (2×20 mg kg^{-1}) the amounts of lipocortin 1 and 2 in cells were greatly reduced. Conversely, injection of hydrocortisone (1 mg kg^{-1}) or dexamethasone (0.08 mg kg^{-1}) caused an increase in the amount of lipocortin 1 and 2 in peritoneal cells within 30 min. Lipocortin 5 was unchanged by any manipulation of glucocorticoid levels.

3 Lipocortins 1 and 2 were elevated in both intracellular and membrane-associated fractions of macrophages elicited by intraperitoneal injection in inflammogens. This phenomenon also occurred in adrenalectomized animals.

4 Our data indicate that glucocorticoids control the synthesis of some members of the lipocortin family in rat mixed peritoneal cells but also suggest the existence of a separate system for controlling the generation of this protein. The significance of these observations is considered in relation to the mechanism of glucocorticoid hormone action on eicosanoid production.

Keywords: Lipocortin 1; annexins; glucocorticoids; peritoneal leucocytes; RU486

Introduction

The glucocorticoids can exert a rapid and powerful suppression of eicosanoids and platelet activating factor (PAF) synthesis by cells and in many systems a considerable body of evidence exists to suggest that this inhibitory effect is caused by the glucocorticoid–stimulated synthesis or release of a second messenger protein originally termed 'macrocortin', 'lipomodulin' or 'renocortin' (see Flower, 1988). It was surmised that these proteins were either identical or at least very similar and the name 'lipocortin' was coined to replace the earlier terminology (Di Rosa *et al.*, 1984). Biological evidence supported the concept that these proteins, or highly purified preparations containing them, duplicated certain effects of the glucocorticoids, in particular inhibition of eicosanoid synthesis and (some) anti-inflammatory effects (Blackwell *et al.*, 1982). It was also observed, again by use of biological and enzyme-assay techniques, that the glucocorticoids caused the 'induction' by cells of lipocortin and that this effect could be blocked by inhibitors of protein or RNA synthesis (Blackwell *et al.*, 1982). In rat peritoneal macrophages, however, the situation was complex, with glucocorticoids apparently influencing the 'release' as well as the synthesis of the protein (Carnuccio *et al.*, 1981).

Ion-exchange chromatography revealed that 'lipocortin' obtained from rat peritoneal lavage fluid was actually a mixture of at least four proteins with apparently similar molecular weights and biological activity (Blackwell *et al.*, 1982). There were also reports of larger proteins having similar properties (Coote *et al.*, 1983). Recently several of these have been purified, sequenced and cloned revealing a family of 6 proteins with substantial homologies (Wallner *et al.*, 1986; Pepinsky *et al.*, 1988). A surprising finding was that the calpactins, a family of proteins thought to be involved with the maintenance of the cytoskeleton or with exocytosis, were in some cases identical to the lipocortins, as were members of the 'chromobindin' family. Recognition of this fact has opened many new avenues of thought concerning the role of these proteins in cellular physiology. It has been suggested recently that this family should be renamed 'annexins' (Crumpton & Dedman, 1990).

Lipocortin 1 (annexin 1 or calpactin II) is a 37 kDa member of this family and when prepared in its correctly folded form the recombinant human protein has eicosanoid-inhibitory and anti-inflammatory properties strongly reminiscent of the original biological extracts (Cirino & Flower, 1987a; Cirino *et al.*, 1987; 1989). Although biological evidence suggested that (at least) some members of the lipocortin family were 'induced' or 'released' by glucocorticoids, the question of whether lipocortin 1 is regulated in this way has proved to be more complex than expected. Some workers have observed increases in lipocortin 1 or its mRNA following exposure of cultured cells to steroids (Wallner *et al.*, 1986; Fuller & Verity, 1989; Piltch *et al.*, 1989) whereas other groups have not (Hullin *et al.*, 1989; Bienkowski *et al.*, 1989). It has been suggested that the concurrent presence of growth factors is required for steroid induction (Phillips *et al.*, 1989) or that cells must be in a differentiated state before lipocortin 1 induction can occur (Solito *et al.*, 1991).

[1] Present address: Department Biochemical Pharmacology, William Harvey Research Institute, St Bartholomew's Hospital Medical College, Charterhouse Square, London, EC1M 6BQ. [2]Department of Biochemistry, School of Biological Sciences, University of Bath. [3]Author for correspondence.

The situation *in vivo* or in cells *ex vivo* also seems complex: Northup *et al.*, (1988) were unable to detect an increase in lipocortin 1 protein in mouse macrophages following glucocorticoid treatment but Mitchell *et al.* (1988) detected an increase in lipocortin 1 in human amnion cells following dexamethasone treatment. In man, Goulding *et al.* (1990) reported an increase in monocyte lipocortin 1 following intravenous injection of hydrocortisone and Browning *et al.* (1990) described *de novo* synthesis of lipocortin 1 in primary cultures of peripheral monocytes maintained in the presence of glucocorticoids. Glucocorticoids also increase the lipocortin concentration of bronchoalveolar lavage fluid in man (Ambrose & Hunninghake, 1990). Recently (Vishwanath *et al.*, 1992) adrenalectomy has been shown to reduce the levels of lipocortin 1 mRNA and protein in several tissues in the rat.

Using Western blotting techniques we have now investigated the effects of glucocorticoids upon lipocortin 1 synthesis in rat peritoneal leucocytes, one of the systems in which lipocortin 'induction' was first described. Some of these results have appeared in abstract form (Smillie *et al.*, 1989).

Methods

General note on cell collection

Care was taken in all these experiments to minimize artefacts attributable to the fluctuating endogenous steroid background of the rats. All cell collections were made at the same time of day (early p.m., to avoid the corticosterone surge) from rats which were acclimatized and accustomed to the regular animal house routine for at least 2 weeks. Positive action was taken to minimize disturbances to the colony which might arise while removing rats from stock cages.

Collection of cells

Glucocorticoids or saline were administered subcutaneously in a volume of 0.1 ml; saline or paraffin oil (1 ml) was injected intraperitoneally. RU486, prepared as an aqueous suspension, was administered by mouth (1 ml).

Following appropriate treatment, rats were killed by exposure to CO_2 gas and 15–20 ml of either ice-cold Krebs solution or saline (as indicated) containing heparin (5 u ml^{-1}) was injected intraperitoneally and the cavity and its contents lightly massaged. The cell-rich fluid ($10-15 \times 10^6$cells/rat; >90% macrophages) was removed and immediately placed on ice. The pooled cell number was assessed with a haemocytometer and the numbers adjusted to 50×10^6 cells in each separate sample before collection of the cells by centrifugation (1000 *g*, 15 min, 4°C). The cell-free supernatant was removed (and in some cases retained, see below) and lipocortins were then extracted as described below. If not extracted immediately the pellet was snap-frozen by immersion of the tube in dry ice and stored at −20°C.

Extraction of lipocortin

Two points of importance were established in preliminary experiments. Firstly, lipocortins tend to precipitate out of Ca^{2+}-containing solutions particularly when these are stored for any length of time. To guard against such losses of the protein, EDTA was routinely incorporated with all extraction buffers. Secondly, in the case of lipocortins 1 and 5, a proportion of the total cellular protein can, under some conditions, become externalised onto the external surface of the cell. This can be removed following suspension of the cells in a Ca^{2+}-free solution and centrifugation.

Our initial investigation revealed three distinct 'pools' of lipocortins.

(a) *Cell surface extracellular lipocortins ('E')* which were recovered by washing cells in saline and transferring the cell-free fluid after centrifugation directly into tubes containing a molar excess of EDTA. These samples were vortex-mixed, snap-frozen and used in the analytical procedures without further manipulation.

(b) *Intracellular lipocortins ('P').* Cell pellets were resuspended in ice-cold Tris buffer pH 7.4 (50 mM) containing 10 mM EDTA. The cells were lysed by freeze-thawing, the samples were centrifuged (2 min, 13,000 r.p.m.) and the supernatant decanted and retained for analytical work.

(c) *Membrane-bound lipocortins ('M').* The broken cell debris from the previous step was re-extracted with ice cold detergent-containing buffers (Tris pH 7.4 (50 mM), EDTA (10 mM), 2% NP40). The pellets were resuspended and allowed to stand for 30–60 min at 4°C centrifugation for 5 min at 13,000 r.p.m. The supernatant was retained.

An aim of this study was to compare the amounts of lipocortins in different compartments of the cell and to detect any changes in location that might occur following steroid treatment. For this reason the three different 'fractions' obtained as described above were always diluted to the same final volume (200 µl) so that a direct comparison of relative abundances could be made on Western blots.

Gel electrophoresis and immunoblotting of lipocortin

Samples (representing the extracts of 0.5×10^6 cells) were boiled for 5 min in buffer containing 2% sodium dodecyl sulphate (SDS) and 5% 2-mercaptoethanol and then applied to a 1.5 mm 10% polyacrylamide minigel. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by conventional methods (Laemmli, 1970), the samples were transferred electrophoretically onto nitrocellulose and lipocortins detected by use of antibodies raised in rabbits against human recombinant lipocortin 1 (Ab 842), human native lipocortin 2 (Ab 774) and rat lipocortin 5 (Ab 890) respectively. No cross reactivity between these three species was observed with the particular batches of antisera used in these experiments (personal communication from Dr J. Browning, Biogen Research Inc). Bands were visualized by the deposition of peroxide-oxidized diaminobenzidine following incubation with peroxidase-linked anti-rabbit antibody. Data are shown as Western blots from representative experiments ($n > 3$).

Materials

Dexamethasone sodium phosphate (Oradexon-Organon) was obtained from Organon (Cambridge, UK) and heparin (25,000 u ml^{-1}) from Evans (Greenford, UK). Bovine serum albumin (fatty acid-free), hydrocortisone sodium phosphate, buffer salts and 3, 3 diaminobenzidine tetrahydrochloride dihydrate (DAB) were supplied by Sigma (Poole, UK). Sodium dodecyl sulphate, acrylamide and bis-acrylamide were purchased from BDH (Dagenham, UK) and 2-mercaptoethanol from Fisons (Loughborough, UK).

Surgically adrenalectomized Wistar rats were obtained from Interfauna UK Ltd or Olac and maintained in the animal house for 7–14 days with saline drinking water prior to use. Sham-operated control animals were purchased with each batch for purposes of comparison.

The glucocorticoid antagonist RU486 (mifepristone) was a generous gift of Dr R. Deraedt of Roussel-Uclaf, Romainville, France. Authentic human recombinant lipocortin 1 and all the antisera used here (all of which were highly specific rabbit polyclonal preparations) were the generous gift of Dr J. Browning of Biogen Research Inc (Cambridge MA, U.S.A.).

Results

Recovery of lipocortins from cells

Our first observation demonstrated that the mass of lipocortin 1 recovered from the cells of control rats (i.e. rats receiving no exogenous steroid) was extremely variable between batches of rats and that different cell handling techniques produced different profiles of lipocortin and its major metabolites. The variations observed between batches of rats could, at least in part, be attributed to different hormonal backgrounds, although it is clear that there are other factors which are also important.

Figure 1 shows immuno blots of the EDTA-extractable (see below) lipocortin 1 obtained from an identical number of cells from different batches of rats picked to illustrate this point. When extracted and processed under identical conditions the amount of lipocortin 1 and the range of species present in the samples ranges from 'undetectable' (lane A, B) to 'easily detectable' (lanes, C,D,E,). Lanes F and G are from cells taken from a matched 'sham-operated' group (lane F) and an adrenalectomized (lane G) group. It is clear that endogenous corticosteroids profoundly alter the amount found in resting cells in the peritoneal cavity. However the effect of adrenalectomy observed so clearly in Figure 1 was by no means an invariable finding and we encountered instances where the amount present in cells from adrenalectomized rats was high, strongly suggesting that in addition to the steroid background other humoral factors such as acute phase proteins may also be important (see below).

The molecular weight of authentic recombinant human lipocortin 1 by SDS-PAGE is 37–38 kDa, and rat protein has approximately the same mass (Pepinsky *et al.*, 1986). The polyclonal antiserum used here detected intact (37 kDa) lipocortin 1 as well as metabolites of calculated molecular weights of 33.3 and 25 kDa. A high mol. wt. metabolite sometimes seen (e.g. in Figure 3) is probably the 45 kDa asymmetrically-clipped homodimer or adduct referred to by Pepinsky *et al.* (1989). A lipocortin 2 metabolite was also routinely detected. The reasons for variations in the amount of recovered intact lipocortins and metabolites are not clear. Collection of cells or extraction of lipocortin in buffers containing soybean trypsin inhibitor (1 mg ml^{-1}), trasylol (100 u ml^{-1}) or phenylmethylsulphonyl fluoride (PMSF) (200 mM) did not affect the distribution of intact protein and metabolites (data not shown). Other high molecular weight immunoreative species were frequently observed with all of our antisera.

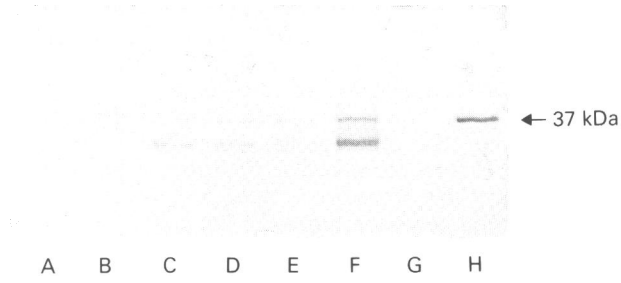


Figure 1 Variable content of lipocortin 1 in rat 'resting' mixed peritoneal leucocytes. The immunoblot shows the range (and molecular weight species) of lipocortin 1 recovered by EDTA extraction in 0.5×10^6 lysed cells from several control (lanes A–E), sham-operated (F) and adrenalectomized (G) rats. Lane H is authentic recombinant human lipocortin.

Location of cellular lipocortins

Table 1 summarises the overall disposition of lipocortins in the mixed peritoneal cells as observed during these experiments. Lipocortin 1 is readily detectable in the intracellular lysate of cells from steroid-treated rats and in some samples from control rats also and may be detected extracellularly attached to the membrane when peritoneal lavage is performed in Ca^{2+}-containing medium. However under routine conditions little was found in the membrane (Figure 2). However, if this latter pool is concentrated 4–5 fold small amounts of the protein may be detected. Lipocortin 2 is less abundant intracellularly, could not be detected extracellularly and was not easily detectable in the membrane under normal conditions. Lipocortin 5 is present predominantly in the cell lysate, but could also be detected in smaller amounts in the cell surface fractions.

Effect of glucocorticoids in vivo

Injection of dexamethasone (0.08 mg kg^{-1}) causes a time-dependent increase in the intracellular content of the lipocortins 1 and 2 (Figure 3) in peritoneal leucocytes. By microdensitometry, the intensities of the lipocortin 1 bands (relative to the control) at 15, 30, 60 and 120 min following dexamethasone injection were 0.48, 3.69, 4.16 and 6.52 respectively. The corresponding figures for the lipocortin 2

Table 1 The disposition of lipocortins 1, 2, and 5 (L1, L2 and L5) in rat mixed peritoneal leucocytes following saline or dexamethasone treatment

	Relative abundances of lipocortins in cells from					
	Saline-treated rats			*Dexamethasone treated rats*		
Fraction	L1	L2	L5	L1	L2	L5
Cell surface (E)	(+)	UD	(+)	+	UD	+
Intracellular (I)	+/++	+	++	+++/++++	+++	++
Membrane bound (M)	(+)	(+)	UD	(+)	(+)	UD

The scores were assessed by visual examination of the Western blots generated during these experiments (data collected from >15 individual experiments). Rats received either saline vehicle or dexamethasone 0.08 mg kg^{-1} and cells were collected, processed and extracted as described in the text.
UD: Protein undetectable area following ×4 concentration.
(+): Protein undetectable in the normal dilution range but just detectable following ×4 concentration.
+ Protein present in small amounts but visually detectable.
++ Protein present in moderate amounts and easily detectable.
+++ Protein present in large (or very large ++++) amounts and strongly visible.

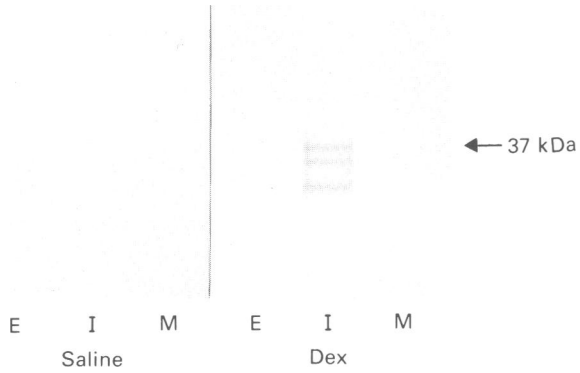


Figure 2 Disposition of lipocortin 1 in rat leucocytes. Recovery of lipocortin 1 from the cell surface (external-'E'), the intracellular (internal-'I') or cell membrane ('M') fractions of rat mixed peritoneal leucocytes obtained in a matched experiment from rats treated with saline or dexamethasone (Dex) for 1 h. Each lane represents the extract obtained from 0.5×10^6 cells.

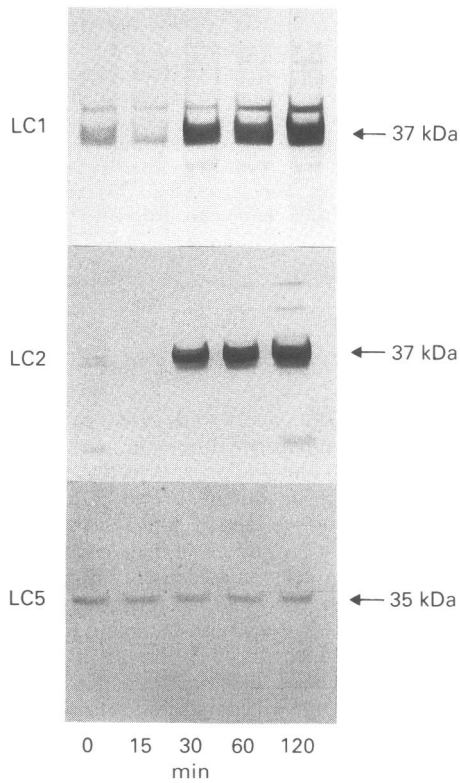


Figure 3 Time course of glucocorticoid induction of lipocortins 1 and 2. Lipocortins 1, 2 and 5 in EDTA extracts of 0.5×10^6 peritoneal mixed cells obtained from adrenalectomized rats receiving dexamethasone (0.8 mg kg^{-1}, s.c.) at different times following steroid treatment. Western blots were prepared in triplicate and probed with antisera to lipocortin 1, 2 and 5. Lipocortin 1 appears as a doublet: the lower band represents the 37 kDa species but the upper band may be an asymmetrically clipped adduct such as that described by Pepinsky *et al.* (1989).

bands were 1.3, 28.9, 39.5 and 43.2. The amount of immunoreactive lipocortin 5 was unchanged at any time.

Early experiments indicated that lipocortin 1 was attached to the exterior of peritoneal cells by a Ca^{2+}-dependent mechanism (Cirino & Flower, 1987b), and indeed lipocortin-like biological activity was discovered in the peritoneal lavage fluid of steroid-treated rats (Blackwell *et al.*, 1982). Figure 4 shows that immunoreactive lipocortin 1 is present in increased amounts in both peritoneal cells (37 kDa band 3.3 relative to control) and in concentrated ($\times$ 10) Ca^{2+}-free lavage fluid (37 kDa band 5.8 relative to control) following dexamethasone injection.

The effect of glucocorticoid receptor antagonists

Pretreatment of rats with the steroid receptor antagonist, RU486, not only prevents dexamethasone-induced increases in peritoneal leucocyte intracellular lipocortins 1 and 2, but also reduces the amount of these proteins in cells from rats which had not received exogenous steroid (Figure 5). Microdensitometry measurements of the bands gave the following relative intensities: lipocortin 1–control (1.0), RU486 (0), dexamethasone (1.67) and hydrocortisone (2.32):lipocortin 2–control (1.0), RU486 (0), dexamethasone (1.98) and hydrocortisone (2.74). Lipocortin 5 was unaffected by RU486. These findings suggest that endogenous steroids are important in regulating the cellular content of lipocortins 1 and 2, as suggested by previous findings of reduced lipocortin activity from adrenalectomized rats (Blackwell *et al.*, 1982). To achieve maximum effect, rats were pretreated with RU486 at 18 h and 2 h before cell harvesting; shorter pretreatment reduced, but did not completely eliminate the effects of exogenous steroids (data not shown).

Hydrocortisone also induces lipocortin 1 and 2 in peritoneal cells, but a higher dose (1 mg kg^{-1}; 1 h pretreatment) was required, consistent with its lower anti-inflammatory potency compared to dexamethasone (Figure 5).

Steroid-independent regulation of rat peritoneal cell lipocortins

Our experiments indicated variability in the basal amounts of lipocortins in cells from control animals despite our attempts to minimize stress to the colony (see Figure 1). In addition, although cells from adrenalectomized rats generally contained less lipocortin 1, some batches of animals acquired very high amounts following surgery, and clearly this could not be caused by endogenous steroids. These observations suggested an additional non-glucocorticoid control mechanism for regulation of lipocortin synthesis.

In a previous paper, Wallner *et al.* (1986) noted that 'elicited' macrophages contained large amounts of lipocortin 1 compared to 'resident' peritoneal cells, suggesting that cell

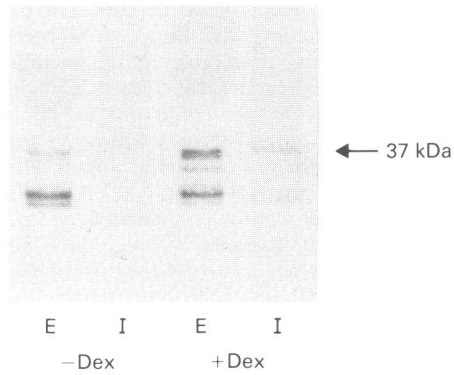


Figure 4 Dexamethasone-induced increase in lipocortin 1 in peritoneal lavage fluid. Mixed peritoneal cells were collected by saline lavage (20 ml) from rats receiving vehicle (− Dex) or dexamethasone 0.8 mg kg^{-1} s.c. 1 h beforehand (+ Dex). The cells were extracted by EDTA-lysis and the saline lavage fluid was concentrated $\times$ 10 prior to Western blotting. 'E' = external (lavage) lipocortin 1; 'I' = internal lipocortin 1. The samples represent the extracts from 0.5×10^6 cells or 1% of the total lavage fluid.

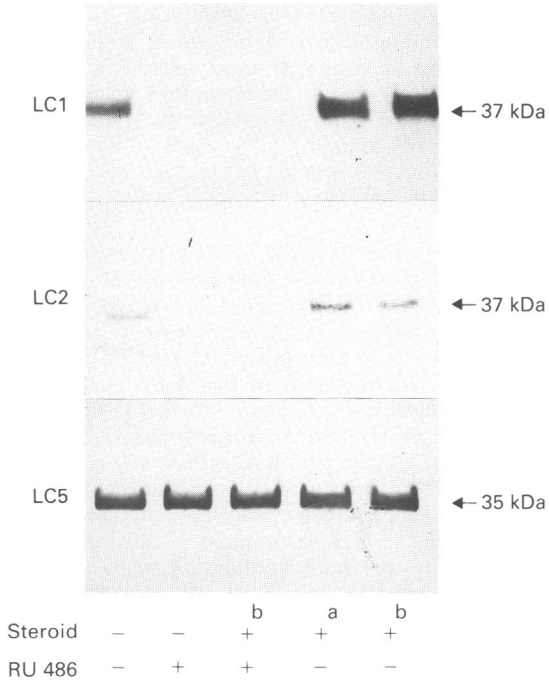


Figure 5 The effect of pretreatment with the glucocorticoid antagonist, RU486, on the content of lipocortins 1, 2 and 5 in rat mixed peritoneal cells. EDTA extracts of cells (0.5×10^6) were obtained from rats receiving saline, RU486 ($20\ mg\ kg^{-1} \times 2$) or steroid (a = hydrocortisone, $1\ mg\ kg^{-1}$; b = dexamethasone, $0.08\ mg\ kg^{-1}$ 1 h before collection) either alone or in combination. Western blots were prepared in triplicate and probed with antisera to lipocortins 1, 2 and 5.

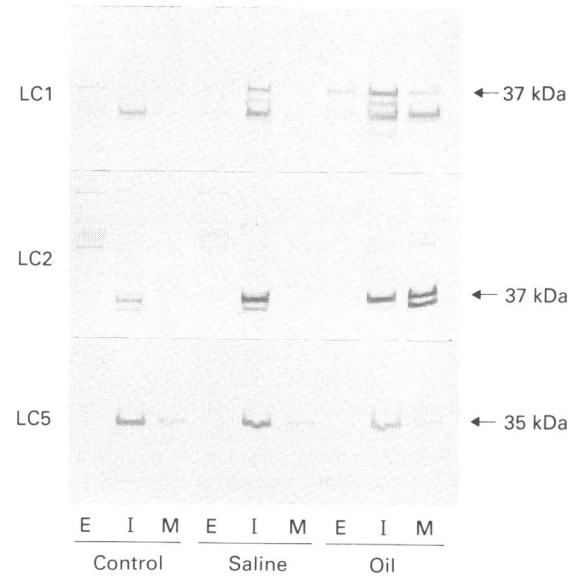


Figure 6 The effects of inflammogens upon lipocortin 1, 2 and 5 in rat leucocytes. The immunoblot shows increased synthesis and differential distribution of lipocortins 1, 2 and 5 caused by the i.p. injection into adrenalectomized rats of 1 ml saline or 1 ml paraffin oil. Control adrenalectomized rats were untreated. Cells were collected from rats 24 h after injection by peritoneal lavage in Ca^{2+}-containing fluid and counted. Equivalent numbers of cells (0.5×10^6) from each group of rats were used to prepare the 'extracellular' (E), 'intracellular' (I) and 'membrane soluble' (M) fractions. Western blots were prepared in triplicate and probed with antisera to lipocortins 1, 2 and 5.

'activation' might increase lipocortin 1 levels in some way; alternatively a second signal resulting from the inflammatory reaction might induce lipocortin synthesis. Intraperitoneal injection of inflammogens, such as zymosan or paraffin oil, results in a sterile inflammatory response featuring infiltration initially of neutrophils and subsequently of monocytic cells. Twenty-four hours following the injection, the elicited cells are predominantly monocyte/macrophages (>90%) although these cells differ phenotypically from resting, resident macrophages.

In cells elicited by injection of 1 ml paraffin oil, or to a lesser extent 1 ml sterile saline, lipocortin 1 and 2 levels are greatly increased compared to resident cells. The distribution of lipocortins was different, with lipocortin 1 being readily, and lipocortin 2 faintly, detectable in the membrane pool from these elicited cells. Lipocortin 5 levels and distribution were unaltered. These changes could not be due to an increase in endogenous corticosteroids following injection of inflammogen, since essentially similar observations were made in adrenalectomised rats (Figure 6). A similar 'induction' of lipocortins was seen in cells elicited by i.p. injection of zymosan (10 mg) or glycogen (10 mg, data not shown) indicating that this was not a feature only of oil-elicited cells, or an artefact of extraction following phagocytosis of oil droplets.

Discussion

The data presented in this paper clearly demonstrate that glucocorticoids can promote an increase in the mass of immunoreactive lipocortins 1 and 2 in extracts from the mixed peritoneal leucocytes of rats. This effect is rapid, is not unique to dexamethasone and is dependent upon glucocorticoid receptor occupation and therefore presumably upon gene modulation.

The doses of the glucocorticoids used in this study are moderate anti-inflammatory doses in the rat and the experiments in which the glucocorticoid antagonist, RU486, was used imply that lipocortin 1 and 2 are sensitive to changes in endogenous levels of glucocorticoids. The fact that cells from adrenalectomized animals generally contained less lipocortin 1 than cells from control animals would also tend to confirm this. No change in the mass of immunoreactive lipocortin 5 was seen with changing endocrine status of the animal or glucocorticoid treatment implying that unlike the closely related species, lipocortins 1 and 2, the lipocortin 5 gene is not glucocorticoid-sensitive.

We have not measured the amount of cellular mRNA for these lipocortins so we cannot pinpoint precisely the mechanism of 'induction': the most obvious possibility is direct enhancement of gene transcription but there could be other explanations. For example, glucocorticoids could promote the translation of a pre-existing pool of lipocortin mRNA or could stimulate movement of the protein from one protein compartment of the cell into the EDTA-extractable phase, perhaps by causing changes in phosphorylation state of the protein, although the reverse redistribution has been proposed (Sheets *et al.*, 1987). We could not detect a substantial pool of any lipocortin elsewhere in the cell using conventional extraction techniques, implying that the most likely explanation for the increase is *de novo* synthesis following gene activation or translation of a pre-existing pool of mRNA. The results obtained here support the biological and enzyme assay data obtained in earlier studies (Blackwell *et al.*, 1982) and are in line with a recent publication demonstrating a reduction in lipocortin mRNA in the rat following adrenalectomy (Vishwanath *et al.*, 1992).

Kovacic *et al.* (1991) have cloned and sequenced the rat

lipocortin 1 gene and compared it with the human gene. A consensus nucleotide sequence for glucocorticoid receptor binding is located in both human and rat genes in intron 1. This finding provides a notional explanation at least for the effect of glucocorticoids on the expression of this protein. Furthermore, the human gene contains a motif characteristic of response elements regulated by substances such as interferons, mitogenic agents and serum factors. Once again, this could explain the ability of cells to respond to substances other than glucocorticoid hormones. A similar observation was made by Browning and his colleagues (1990) who pointed out that the human lipocortin 1 gene also contains an AP1 site consistent with the inducibility of the gene in some cells by phorbol esters (cf. Beinkowski *et al.*, 1989). The lipocortin 1 promoter region also revealed a homology with regulatory elements in the haptoglobin promoter region (Oliviero & Cortese, 1989), a gene which is induced by a combination of IL-6 and steroids, as well as another resembling an acute phase recognition element (APRE-2β), which might prove to be important in gene regulation during chronic inflammatory episodes. Although the synthesis of many acute phase proteins are driven by cytokines and steroids in combination it is also possible to achieve an acute phase response in the absence of glucocorticoids: this could be triggered by surgery (such as adrenalectomy or sham adrenalectomy) or by intercurrent infections.

We will not know precisely what influence other than glucocorticoids are definitely associated with an induction of lipocortin 1 until the molecular biology of the promoter site is more carefully worked out. From the data presented in this paper however it is clear that there is a prominent non-glucocorticoid driven pathway for lipocortin 1 induction in the rate peritoneal macrophage.

Of interest is the discovery that lipocortin 1 is externalized by cells. In solutions containing physiological calcium concentrations this material seems predominantly associated with the cell surface by a Ca^{2+}-dependent mechanism and can be easily detached in low Ca^{2+} solutions. This phenomenon almost certainly explains the observation of Blackwell *et al.* (1982) that the 'polypeptide I' component of 'macrocortin' in crude peritoneal lavage fluid (now known to be lipocortin 1) was elevated by pretreatment of rats by steroids. In these experiments, the cells from treated rats were maintained for a short while in a Ca^{2+}-free environment thus promoting 'release' of lipocortin into the medium. Lipocortin 1 does not possess a signal sequence and the method by which it is externalized is not clear although this phenomenon has been observed by others (Christmas *et al.*, 1991) and lipocortin 5, which also lacks a formal signal sequence, has also been located pericellularly by other groups (Pfaffle *et al.*, 1988). This pool of lipocortin is significant as Carnuccio *et al.* (1981) and Cirino & Flower (1987b) reported that it is the external membrane-associated lipocortin 1 that produces its biological effects. Furthermore, the models in which lipocortin 1 has been shown to have biological activity are those in which the protein has been applied externally (Cirino & Flower, 1987a; Cirino *et al.*, 1987; 1989; Davidson *et al.*, 1991).

The presence of increased intracellular and membrane lipocortins in activated/elicited cells, even from adrenalectomized animals, may explain some of the variability in lipocortin levels seen in control animals, and almost certainly explains its presence in cells from some batches of adrenalectomized and sham-operated animals. A sub-clinical infection following the operation, or even the surgical trauma itself, could lead to cell infiltration, and hence to high background levels in resident cells.

'Membrane-bound' lipocortins have been noted in human placental membranes (Sheets *et al.*, 1987) and in porcine heart (Pula *et al.*, 1990) and A431 cells (Ando *et al.*, 1991). In the first two examples, lipocortin 1, and 5 and 6 respectively could be extracted by non-ionic detergents but not by EGTA, and hence resemble the membrane-bound pools observed in elicited cells. In the case of placental lipocortin 1, this pool may be modified by phosphorylation. The situation in A431 cells appears to be different, in that here, lipocortin 1 forms a covalent polymer following the action of tissue transglutaminase and cannot be extracted from membranes by Triton X-100, although plasmin treatment is effective. The identity of the cellular membrane containing the 'membrane bound' detergent-extractable lipocortin 1 has not been confirmed, although in placenta, the plasma membrane seems the most likely candidate. It may be that the 'membrane-bound' pool in the elicited cells represents 'nascent' lipocortin 1.

Although many workers have used cell lines to investigate lipocortin 1 synthesis we have always worked with primary cells because we believe these to be more relevant to the study of inflammation *in vivo* and indeed, the situation in such cells seems different from many cultured cell lines. The experiments reported in this study indicate that the lipocortin system is very sensitive to glucocorticoid control and responds very quickly to changes in steroid levels. Elsewhere we have reported an elevation of lipocortin 1 in peripheral blood monocytes taken from human volunteers receiving intravenous hydrocortisone (Goulding *et al.*, 1990). In addition, Browning *et al.* (1990) have reported that human peripheral monocytes cultured in [^{35}S]-methionine labelled media synthesized labelled lipocortin 1 in response to dexamethasone. It seems that primary cells respond well to glucocorticoids both *in vivo* and *in vitro* but that this capacity may be lost or in some way impaired following long term culture. The increase in lipocortin 1 and 2 associated with elicited cells implies glucocorticoid-independent mechanism for induction associated with activation or transformation.

Lipocortin 1 has anti-inflammatory properites (Cirino *et al.*, 1989; Errasfa & Russo-Marie, 1989) and the observation that its synthesis is regulated by endogenous and exogenous corticosteroids is therefore of great significance. Since lipocortin 2 is also regulated by steroids we propose that this molecule should also be considered as a potential mediator of steroid hormone action.

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The actions of nitric oxide donors in the prevention or induction of injury to the rat gastric mucosa

J. Lopez-Belmonte, [1]B.J.R. Whittle & S. Moncada

Department of Pharmacology, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 The protective or damaging actions on the gastric mucosa, of locally infused nitrovasodilators that donate nitric oxide (NO), have been investigated in the pentobarbitone-anaesthetized rat.

2 Local intra-arterial infusion of endothelin-1 (ET-1; 5 pmol kg^{-1} min^{-1} for 10 min) induced extensive, macroscopically apparent, haemorrhagic injury to the rat gastric mucosa. This damage was dose-dependently reduced by concurrent local intra-arterial infusion of glyceryl trinitrate (GTN; 10–40 μg kg^{-1} min^{-1}) which liberates NO on metabolic transformation, or the nitrosothiol, S-nitroso-N-acetyl-penicillamine (SNAP, 2.5–10 μg kg^{-1} min^{-1}) which spontaneously liberates NO.

3 Local infusion of higher doses of SNAP (20 and 40 μg kg^{-1} min^{-1}, i.a.) did not, however, significantly protect against mucosal injury induced by ET-1.

4 Furthermore, local infusion alone of these higher doses of SNAP, as well as sodium nitroprusside (10–40 μg kg^{-1} min^{-1}, i.a.) which also spontaneously liberates NO, induced significant mucosal injury, as assessed macroscopically and confirmed by histology.

5 Local infusion of these higher doses of SNAP and nitroprusside reduced systemic arterial blood pressure (BP), but this was not correlated with the extent of mucosal injury.

6 Furthermore, local infusion of GTN (10–40 μg kg^{-1} min^{-1}, i.a.) alone, which also reduced BP, failed to induce gastric mucosal damage.

7 These findings suggest that exogenous NO can protect the rat gastric mucosa from damage induced by the vasoconstrictor peptide ET-1, which may reflect local microcirculatory interactions. However, the unregulated release of high levels of NO within the microvasculature induces mucosal injury.

Keywords: Nitric oxide; NO-donors; nitrovasodilators; nitrosothiols; glyceryl tritnitrate; nitroprusside; S-nitroso-N-acetyl-penicillamine; gastric damage; endothelin-1

Introduction

The regulation of the mucosal microcirculation is intimately involved in the maintenance of gastric integrity and hence the local release of vasoactive mediators from endothelial cells of the microvasculature has a significant modulatory role. Endothelin-1 (ET-1), a 21 residue vasoconstrictor peptide formed by the endothelium (Yanagisawa *et al.*, 1988; Inoue *et al.*, 1989), induces extensive mucosal injury following local intra-arterial infusion to the rat stomach (Whittle & Esplugues, 1988; Whittle & Lopez-Belmonte, 1991) and can augment mucosal damage induced by intragastric ethanol (Wallace *et al.*, 1989). Endothelial cells also release a labile vasodilator substance (Furchgott & Zawadzki, 1980) now characterized as nitric oxide (NO) biosynthesized from L-arginine by a constitutive NO synthase (Palmer *et al.*, 1987; 1988; Ignarro *et al.*, 1987; Khan & Furchgott, 1987). By the use of inhibitors of NO biosynthesis such as N^G-monomethyl-L-arginine and N^G-nitro-L-arginine methyl ester, a role of endogenous NO in the regulation of the rat gastric microcirculation under both resting and stimulated conditions has been established (Pique *et al.*, 1989; 1992; Walder *et al.*, 1990; Tepperman & Whittle, 1992). Furthermore, an interaction of endogenous NO with prostanoids and sensory neuropeptides in the regulation of mucosal integrity has also been demonstrated (Whittle *et al.*, 1990).

The release of NO from nitrovasodilator agents either following metabolic transformation as with glyceryl trinitrate (GTN) and isoamyl nitrite, or spontaneously as with nitroprusside, is responsible for their ability to activate guanylate cyclase and relax vascular smooth muscle (Gruetter *et al.*, 1979; Ignarro *et al.*, 1981; Feelisch & Noack, 1987; Feelisch, 1991). Following intragastric application, these nitrovasodilators can protect against acute haemorrhagic mucosal injury induced by topical irritants (MacNaughton *et al.*, 1989; Kitagawa *et al.*, 1990). The nitrosothiol, S-nitroso-N-acetyl-penicillamine (SNAP), which spontaneously liberates NO (Ignarro *et al.*, 1981) has also been demonstrated to protect against acute microvascular injury in the stomach and small intestine induced by platelet-activating factor (PAF) or endotoxin following intravenous administration (Boughton-Smith *et al.*, 1990; 1992b).

To explore further the interaction between endothelium-derived vasodilator and vasoconstrictor mediators in the mucosal microcirculation, the influence of the local intravascular administration of the NO donors, GTN and SNAP on the gastric damage induced by local intra-arterial administration of ET-1 has been evaluated. In addition, the effects of close-arterial infusion of these nitrovasodilators alone and of nitroprusside, on gastric mucosal integrity have also been investigated.

A preliminary account of this study has been presented to the British Pharmacological Society (Lopez-Belmonte *et al.*, 1992).

Methods

Gastric damage induced by local infusion of endothelin-1

Male Wistar rats (230–260 g body weight) were deprived of food but not water for 18–20 h before the experiments. The animals were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and the stomach exposed by a mid-line incision. The left gastric artery was cannulated with a short 24 gauge teflon cannula (Esplugues & Whittle, 1988). The oesophagus and pylorus were ligated and 2 ml of 0.1 M hy-

[1] Author for correspondence.

drochloric acid (HCl) were instilled into the gastric lumen via the forestomach. ET-1 (2.5–20 pmol kg^{-1} min^{-1}) or its vehicle, 0.1% bovine serum albumin in saline was infused locally for 10 min (13 µl min^{-1}), and the stomachs removed 20 min later.

Effect of nitrovasodilators on endothelin-1 damage

In these studies, the short gastric intra-arterial 24 gauge teflon cannula was attached to a bifurcated catheter which allowed the concurrent administration of two substances under study. Each substance was infused at a rate of 13 µl min^{-1}, reaching the vasculature within 5 s of entering the cannula. The local intra-arterial infusion of the nitrovasodilators, SNAP (2.5–40 µg kg^{-1} min^{-1}) or GTN (5–40 µg kg^{-1} min^{-1}) or isotonic saline, was started 5 min before the infusion of ET-1 and then continued for a further 10 min together with ET-1, being thus administered for a total of 15 min. After a further 20 min following termination of the infusions, the stomachs were removed for assessment of macroscopic damage.

Gastric damage induced by local infusion of nitrovasodilators

In these studies, following instillation of 2 ml 0.1 M HCl into the gastric lumen, GTN (10–40 µg kg^{-1} min^{-1}), nitroprusside (10–40 µg kg^{-1} min^{-1}), SNAP (5–40 µg kg^{-1} min^{-1}) or isotonic saline were infused close-arterially for 15 min (13 µl min^{-1}) and the stomachs removed 20 min later.

Measurement of systemic blood pressure

Mean systemic arterial blood pressure (BP) was measured continuously from a cannula in the right carotid artery, connected to a pressure transducer (Elcomatic) and a chart recorder (Grass model 7D polygraph).

Assessment of mucosal damage

Twenty minutes after terminating local infusion of the drugs, the stomachs were removed from the abdominal cavity, cut open along the greater curvature and gently rinsed with isotonic saline. They were then pinned, mucosal side up, to a wax block and immersed in neutral buffered formalin. Each stomach was photographed on colour transparency film (Kodak EPY 50). Each photograph was then coded to allow for subsequent unbiased assessment. The transparencies were projected and both the overall areas and the damage areas (observed as vasocongestion, haemorrhagic necrosis and epithelial desquamation with mucus precipitation) assessed in a randomized manner by computerised planimetery (Apple IIe) and expressed as % of the total mucosal area.

For histological confirmation of the nature of the mucosal injury, samples of the corpus mucosa were excised from standardized regions of the dorsal and ventral sides of the mucosa. These sections were processed by routine techniques before being embedded in paraffin. Sections (4 µm) were stained with haematoxylin and eosin and examined under a light microscope.

Statistical analysis

All data are expressed as mean ± s.e.mean. Comparisons between groups were made by Student's t test for paired or unpaired data as appropriate, where P values of less than 0.05 were taken as significant.

Materials

Endothelin-1 (human-porcine; Sigma Chemical Co., Poole, Dorset) was dissolved in sterile distilled water and kept frozen (−20°C) in aliquots. Samples were freshly diluted in 0.1% bovine serum albumin in saline.

Glyceryl trinitrate (Du Pont Ltd., U.K., Hertfordshire) was obtained as 0.5 mg ml^{-1} vials, and prepared freshly for infusion by dilution with isotonic saline. Sodium nitroprusside (Sigma Chemical Co., Poole, Dorset) and S-nitroso-N-acetyl-penicillamine (Wellcome Research Laboratories) were dissolved freshly in isotonic saline and kept on ice protected from the light.

Results

Effect of local endothelin-1

In dose-ranging studies, local intra-arterial infusion of ET-1 (2.5–20 pmol kg^{-1} min^{-1} in 0.1% bovine serum albumin) for 10 min induced dose-dependent mucosal damage, assessed macroscopically 20 min later involving 28 ± 4% and 86 ± 3% of the total mucosal area with the lower and high doses respectively (n = 4 for each $P<0.01$), while ET-1 (5 pmol kg^{-1} min^{-1} i.a.) induced a submaximal degree of injury involving 35 ± 5% of the total mucosal area ($n = 7$; $P<0.01$). This damage was characterized macroscopically as regions of vasocongestion, epithelial sloughing, haemorrhage and necrosis, which was confirmed on histological evaluation. At the dose chosen for subsequent studies, local infusion of ET-1 (5 pmol kg^{-1} min^{-1}) had no significant effect on BP (Δ 3 ± 2 mmHg, $n = 4$). Intra-arterial infusion of the vehicle, 0.1% bovine serum albumin in saline, did not significantly damage the mucosa (Figure 2) or affect BP (Δ 2 ± 1 mmHg, $n = 4$).

Effect of glyceryl trinitrate co-administration

Concurrent close-arterial administration of GTN (5–40 µg kg^{-1} min^{-1} for 15 min) caused a significant and dose-dependent reduction in the extent of damage induced by ET-1 (5 pmol kg^{-1} min^{-1}) as shown in Figure 1. Thus, GTN (10, 20 and 40 µg kg^{-1} min^{-1}) inhibited mucosal damage by 53 ± 10% ($n = 5$, $P<0.05$), 69 ± 7% ($n = 7$, $P<0.001$) and 72 ± 6% ($n = 8$, $P<0.001$) respectively.

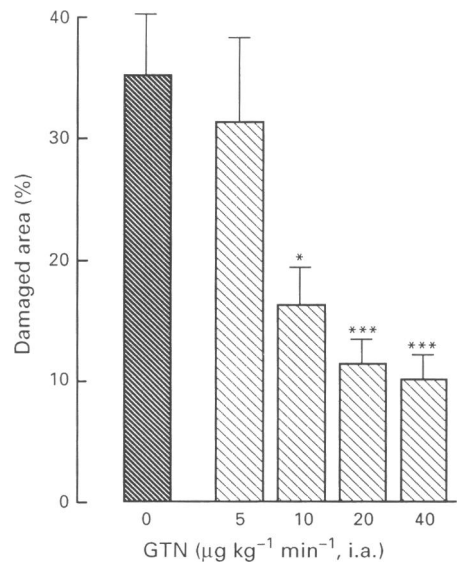


Figure 1 Inhibition by the local intra-arterial infusion (15 min) of glyceryl trinitrate (GTN; 5–40 µg kg^{-1} min^{-1}), of rat gastric mucosal damage induced by concurrent local intra-arterial infusion (10 min) of endothelin-1 (ET-1, 5 pmol kg^{-1} min^{-1}). Results, shown as the area of damage, % of total mucosal area assessed macroscopically 20 min after termination of the ET-1 infusion, are the mean of 5–8 experiments in each group; vertical bars show s.e.mean. Significant difference from the control group (ET-1 alone, first column) is shown as: $*P<0.05$; $***P<0.001$.

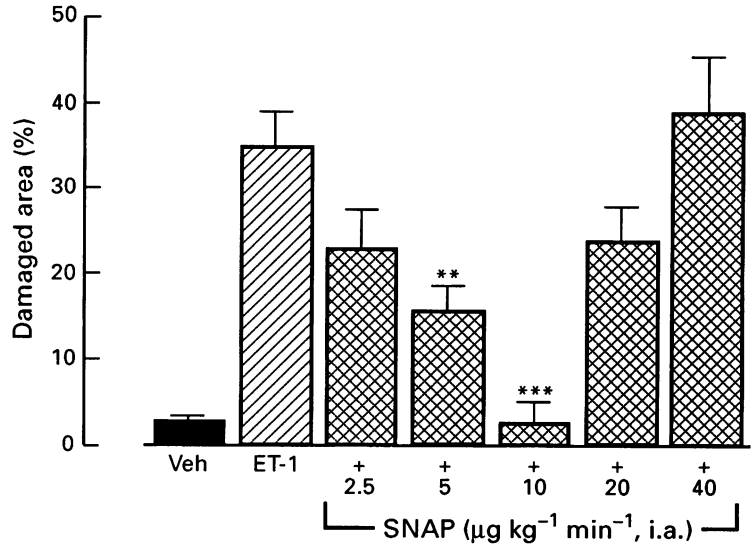


Figure 2 Effects of local intra-arterial infusion (15 min) of S-nitroso-N-acetylpenicillamine (SNAP; 2.5–40 μg kg^{-1} min^{-1}), on rat gastric mucosal damage induced by concurrent local intra-arterial infusion (10 min) of endothelin-1 (ET-1, 5 pmol kg^{-1} min^{-1}). Results, shown as the area of damage, % of total mucosal area assessed macroscopically 20 min after termination of the ET-1 infusion, are the mean of 5–8 experiments in each group; vertical bars show s.e.mean.

Significant difference from the control group (ET-1 alone) is shown as: $^{**}P<0.01$; $^{***}P<0.001$.

Effect of S-nitroso-N-penicillamine co-administration

Co-administration of SNAP (2.5–10 μg kg^{-1} min^{-1}, i.a. for 15 min) caused a significant and dose-dependent inhibition of ET-1-induced damage, reaching a maximum degree of inhibition at a dose of 10 μg kg^{-1} min^{-1} (Figure 2). Thus SNAP (2.5, 5 and 10 μg kg^{-1} min^{-1}) inhibited mucosal damage by 37 ± 10% ($n = 8$), 54 ± 11% ($n = 8$, $P<0.01$) and 79 ± 7% ($n = 8$, $P<0.001$) respectively.

Higher close-arterial does of SNAP (20 and 40 μg kg^{-1} min^{-1}, i.a.), failed to protect significantly against the mucosal injury induced by ET-1 and with the highest dose of SNAP (40 μg kg^{-1} min^{-1}i.a.), the damage was comparable to that induced by ET-1 alone (Figure 2).

Effect of close-arterial infusion of S-nitroso-N-penicillamine, nitroprusside and glyceryl trinitrate alone

Local intra-arterial infusion of SNAP (5–40 μg kg^{-1} min^{-1}) alone for a period of 15 min resulted in a dose-dependent and significant induction of mucosal injury (Figure 3). This damage was macroscopically characterized by extensive areas of vasocongestion and sloughing of epithelium and focal sites of haemorrhage, findings confirmed on histological evaluation (Figure 4). By contrast, local infusion of a solution of SNAP at a dose of 40 μg kg^{-1} min^{-1}, that had been incubated at 37°C in ambient light for 48 h, failed to induce any significant mucosal injury (3 ± 2% of total mucosal area, $n = 7$), being comparable to that induced by intra-arterial saline (3 ± 1% of total mucosal area, $n = 4$).

Close-arterial infusion of nitroprusside (10–40 μg kg^{-1} min^{-1}, 15 min) alone, also induced significant gastric mucosal damage (Figure 3). By contrast, close-arterial infusion of potassium ferricyanide (40 μg kg^{-1} min^{-1} for 15 min) did not induce any significant macroscopic mucosal damage (4 ± 2% of total mucosal area, $n = 5$). Close-arterial infusion of GTN failed to induce any significant gastric mucosal damage at the doses used (10–40 μg kg^{-1} min^{-1} for 15 min) as shown in Figure 3. Administration of higher doses of GTN was hindered by solubility of the commercially available stock solutions.

Effect on mean systemic arterial blood pressure

Local intra-arterial administration of SNAP alone (5–40 μg kg^{-1} min^{-1}) for 15 min induced a dose-dependent significant fall in BP, from the resting BP of 110 ± 5 mmHg ($n = 20$) as shown in Figure 3. By contrast, infusion of a solution of SNAP (at 40 μg kg^{-1} min^{-1}) that had been incubated for 48 h at 37°C in ambient light had no significant effect on BP ($n = 5$).

Local intra-arterial infusion of nitroprusside (10–40 μg kg^{-1} min^{-1}) likewise induced dose-dependent and significant reductions in BP (Figure 3). Furthermore, GTN (20 and

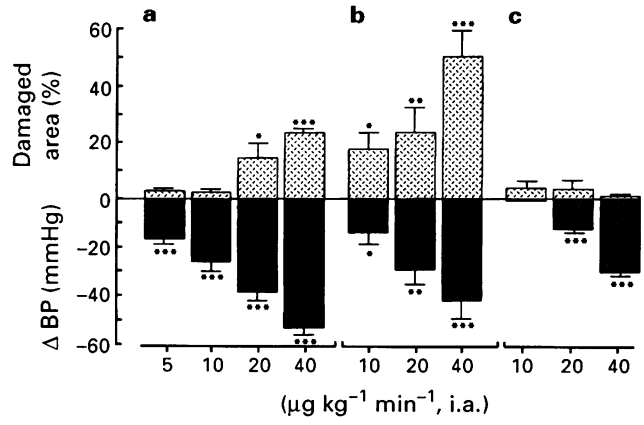


Figure 3 Effect of local intra-arterial infusion (15 min) of (a) S-nitroso-N-acetyl-penicillamine (SNAP; 5–40 μg kg^{-1} min^{-1}) (b) sodium nitroprusside (10–40 μg kg^{-1} min^{-1}) or (c) glyceryl trinitrate (GTN; 10–40 μg kg^{-1} min^{-1}) on rat systemic arterial blood pressure (BP) and the induction of gastric mucosal injury. Macroscopic damage, assessed 20 min after infusion, is shown as % of the total mucosal area, while the change in BP from resting values is given as Δ BP (mmHg). Results are shown as mean of 5–8 experiments; vertical bars represent s.e.mean.

Statistical significance from the change in resting BP (Δ 2 ± 1 mmHg) or from damage area (3 ± 1% total area) in control studies following saline infusion is given as: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

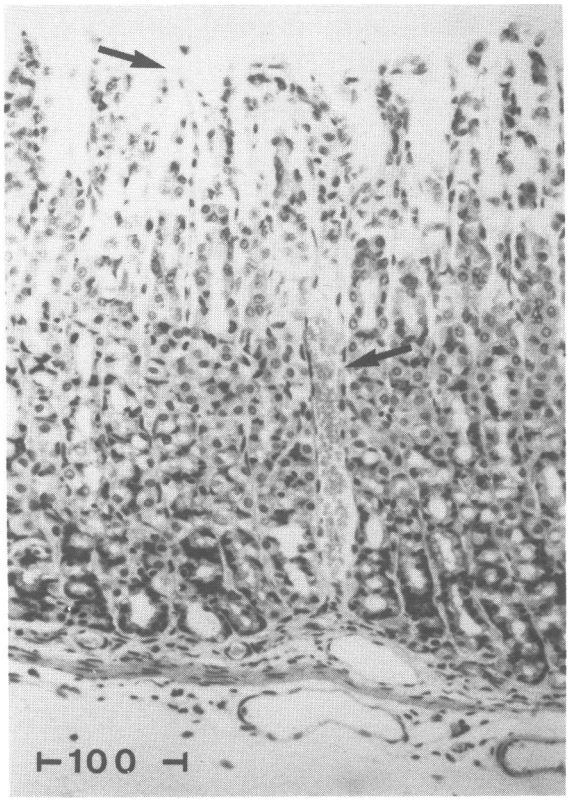


Figure 4 Histological appearance of the rat gastric mucosa, 20 min following local intra-arterial infusion of S-nitroso-N-acetyl-pencillamine (SNAP; 20 $\mu g\ kg^{-1}\ min^{-1}$). Sections (4 μm; wax embedded) were stained with haemotoxylin and eosin; marker bar represents 100 μm. Arrows represent areas of vasocongestion and epithelial disruption.

40 $\mu g\ kg^{-1}\ min^{-1}$, i.a.) also significantly reduced BP (Figure 3). The fall in BP induced by the highest local dose of GTN (40 $\mu g\ kg^{-1}\ min^{-1}$, i.a.) was comparable to that induced by nitroprusside (20 $\mu g\ kg^{-1}\ min^{-1}$, i.a.) and by SNAP (20 $\mu g\ kg^{-1}\ min^{-1}$) being 30 ± 2, 29 ± 5 and 37 ± 3 mmHg respectively ($n = 6-8$), yet as can be seen in Figure 3, GTN unlike SNAP and nitroprusside in these doses, did not induce mucosal damage. Furthermore, the fall in BP induced by the highest dose of nitroprusside (40 $\mu g\ kg^{-1}\ min^{-1}$, i.a.) was less than that induced by SNAP (40 $\mu g\ kg^{-1}\ min^{-1}$, i.a.) being 42 ± 6 mmHg compared with 53 ± 2 mmHg respectively, yet nitroprusside caused two fold greater ($P < 0.05$) damage than SNAP at these doses (Figure 3).

Discussion

In the present study, close-arterial infusion of the NO donor, GTN, dose-dependently prevented the mucosal injury induced by local intra-arterial infusion of ET-1. Likewise at low doses, intra-arterial infusion of the nitrosothiol, SNAP, caused a dose-depenent inhibition of ET-1-induced mucosal damage. These findings thus extend the observations that intragastric instillation of the NO donor, nitroprusside can reduce the haemorrhagic injury induced by a combination of topical ethanol and intravenous infusion of ET-1 (Wallace *et al.*, 1989). The mucosal injury induced by topical ethanol alone has also been shown to be reduced by intragastric GTN or nitroprusside, while that provoked by strong luminal acid was inhibited by intragastric acidified sodium nitrite which liberates NO, and the metabolically activated nitrovasodilator, isoamyl nitrite (MacNaughton *et al.*, 1989; Kitagawa *et al.*, 1990). Intravenous infusion of SNAP likewise protects the stomach from microvascular damage induced by intravascular PAF (Boughton-Smith *et al.*, 1992b).

However, with local intra-arterial doses higher than 10 $\mu g\ kg^{-1}\ min^{-1}$, SNAP did not significantly inhibit the mucosal injury induced by ET-1. This lack of protection appears to reflect the injurious action of SNAP at these higher doses, since its local intra-arterial infusion alone at these doses induced mucosal damage. This damage was macroscopically apparent as sloughing of the epithelium with areas of vasocongestion and focal sites of haemorrhage, findings confirmed on histological evaluation. Such characteristics of mucosal injury have previously been observed with intravascular agents that act by disrupting the microcirculation (Esplugues & Whittle, 1988; 1989), as well as by topical irritants such as ethanol thought to initiate mucosal injury by disruption of endothelial cell continuity (Guth *et al.*, 1984; Szabo *et al.*, 1985). This effect of SNAP appeared to be related to the release of NO and not to the penicillamine moiety of the compound, since incubation of this thermodynamically unstable and photosensitive NO donor for 48 h in ambient light, to deplete its NO content, abolished both its systemic vasoactive effects and its ability to induce mucosal injury.

As found with SNAP, local infusion of nitroprusside which also spontaneously liberates NO, likewise induced extensive mucosal injury following local intra-arterial infusion. The contribution of the concommitant local release of cyanide from nitroprusside to this cytotoxicity cannot be excluded, but it is considered that any such cyanide production *in vivo* will not normally exceed the capacity for its rapid metabolism to the much less toxic thiocyanate (Michenfelder & Tinker, 1977; Kruszyna *et al.*, 1985). Furthermore, close-arterial infusion of potassium ferricynanide, in which the nitroso moiety of nitroprusside is substituted by another cyano group, failed to induce any macroscopically detectable damage, strongly suggesting that NO release accounts for the observed toxicity of nitroprusside.

By contrast to both SNAP and nitroprusside, no mucosal injury was observed following local intra-arterial infusion of GTN. This may reflect the requirement of GTN to be enzymatically activated by a mechanism involving sulphydryls to release NO (Ignarro *et al.*, 1981; Feelisch & Noack, 1987), a process that would be regulated and limited by the metabolic capacity of the microvascular tissue. Gastric intra-arterial infusion of these three NO donors reduced systemic BP, indicating their escape into the systemic circulation following their passage through the mucosal microcirculation. However, such a hypotensive effect on systemic BP, which could possibly reduce microvascular perfusion in the gastric mucosa, does not appear to be the prime mechanism by which tissue injury was initiated. Thus, GTN did not produce mucosal damage in doses producing a comparable fall in systemic BP as injurious doses of nitroprusside, while nitroprusside was substantially more active than SNAP in inducing damage at comparable hypotensive doses.

The mucosal injury associated with the spontaneous release of NO from these agents may indicate cytotoxic actions of high levels of NO on the microvascular endothelium. Indeed, the excessive production of NO by an inducible NO synthase in endothelial cells is considered to underlie the reduction in viability of these cells in culture following exposure over a 48 h period to endotoxin and the cytokine, interferon-γ (Palmer *et al.*, 1992). Furthermore, the microvascular permeability changes, an index of endothelial injury, seen in the rat small and large intestine 4–6 h after endotoxin administration are correlated with the induction of a calcium-independent NO synthase over this period (Boughton-Smith *et al.*, 1992a). The substantial synthesis of NO by the immunologically-induced NO synthase in macrophages also accounts for the cytotoxic effects against tumour cells (Hibbs *et al.*, 1987; 1988, Marletta *et al.*, 1988; Stuehr *et al.*, 1989). In addition, activated macrophages exert cytostatic or cytotoxic actions on fungal and bacterial and protozoal

pathogens by events involving NO (Granger *et al.*, 1990; Adams *et al.*, 1990, Liew *et al.*, 1990), while induction of NO synthesis is also implicated in damage to adenocarcinoma cells (O'Connor & Moncada, 1991). Whether NO is cytotoxic to a cell type may relate to the importance of iron-sulphur-centred enzymes in that target cell (Draper & Hibbs, 1988), and the current findings may thus reflect a significant presence of such cellular enzymes in the gastric mucosa.

The induction of NO synthase has also been implicated in the cardiovascular crisis and collapse seen over several hours in both animals and man in endotoxaemia (Nava *et al.*, 1991; Petros *et al.*, 1991). However, it is also clear that the physiological formation of NO by the constitutive enzyme has importance in protecting against the initial detrimental cardiovascular changes and the intestinal vascular injury seen in the acute phases of endotoxin shock (Wright *et al.*, 1992; Hutcheson *et al.*, 1990). The present results similarly suggest a dual role for NO in the regulation of gastric mucosal integrity. Thus, the physiological formation of NO plays a role in modulating both mucosal blood flow and tissue integrity (Pique *et al.*, 1989; 1992; Whittle *et al.*, 1990). The protection by exogenously generated NO observed in the current study may reflect microcirculatory interactions with the vasoconstrictor, ET-1, both on changes of vascular tone and on endothelial cell continuity. However, the inappropriate release of NO, mimicked in the present study by the use of high doses of spontaneously decomposing NO donors, can lead to extensive tissue injury. This could result from direct cytotoxicity of the NO radical or a subsequent metabolite such as the peroxynitrite radical which generates the highly reactive hydroxyl radical (Beckman *et al.*, 1990). Thus, while a reduction in the physiological formation of NO may compromise gastric integrity leading to ulceration (Whittle *et al.*, 1990), the unregulated excessive formation of NO may also be involved in the pathology of gastro-intestinal mucosal injury.

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Sympathetic innervation and α-adrenoceptor profile of blood vessels in the posterior region of the rabbit knee joint

Hamid Najafipour & [1]William R. Ferrell

Institute of Physiology, University of Glasgow, G12 8QQ

1 Experiments were performed to determine the nature of adrenoceptors mediating neurally-induced vasoconstriction of blood vessels in the posterior region of the rabbit knee joint capsule.

2 Electrical stimulation of the posterior articular nerve resulted in frequency-dependent vasoconstriction which was maximal at 10 Hz. This response was mediated predominantly by α_2-adrenoceptors as it was only slightly reduced by prazosin administration and was not only abolished but converted into a dilator response by the α_2-adrenoceptor antagonist rauwolscine. Further experiments with another specific α_1-adrenoceptor antagonist YM-12617 showed that the frequency-response curve in the presence of this antagonist did not differ significantly from control.

3 Neurally-induced vasoconstriction did not appear to have a purinergic component as it was unaffected by the P_{2x}-purinoceptor desensitiser α,β methylene ATP.

4 The rank-order of potency of α-adrenoceptor agonists given as a bolus by close intra-arterial injection was: adrenaline = UK-14304 > clonidine > phenylephrine, suggesting that the vasoconstrictor effects were mediated predominantly by postjunctional α_2-adrenoceptors.

5 The α_2-adrenoceptor antagonist rauwolscine converted the constrictor response to close intra-arterial injection of adrenaline into a dilator response. The vasoconstrictor responses to UK-14304, clonidine and phenylephrine were substantially inhibited by rauwolscine. The α_1-adrenoceptor antagonist prazosin failed to inhibit the vasoconstrictor responses to adrenaline, clonidine and UK-14304 and resulted in enhancement of their constrictor effects.

6 The enhancement of the responses to the α_1 and α_2 agonists by prazosin appeared to be specifically related to this agent as administration of YM-12617 did not show such enhancement. The dose-response curves to both clonidine and UK-14304 in the presence of YM-12617 did not differ significantly from control responses. Responses to phenylephrine were significantly reduced by YM-12617, indicating the presence of post-junctional α_1-adrenoceptors.

7 These results show almost complete reversal of the adrenoceptor profile compared to results obtained in an earlier *in vitro* study, where responses were mediated predominantly by α_1-adrenoceptors with a small population of postjunctional α_2-adrenoceptors (Ferrell & Khoshbaten, 1989). This suggests that the differing environment *in vitro* may not completely reflect the conditions prevailing *in vivo*.

Keywords: Joint; adrenoceptors; blood flow; neural stimulation

Introduction

The presence of sympathetic nerve fibres innervating articular blood vessels was first demonstrated by Cobbold & Lewis (1956a) who used an electromagnetic flowmeter to measure blood flow of the canine knee joint and observed that traction of the lumbar sympathetic chain reduced knee joint blood flow. In addition, they found that section of the articular nerve supply resulted in increased blood flow, suggesting that these vessels possess sympathetic vasoconstrictor 'tone'. In other experiments Cobbold & Lewis (1956b) found that close intra-arterial injection of noradrenaline produced vasoconstriction, indicating the presence of α-adrenoceptors. In more recent experiments in a rabbit isolated perfused knee joint preparation, it was observed that the α_1-adrenoceptor agonist phenylephrine elicited potent vasoconstriction but the α_2 agonist clonidine was ineffective and another α_2 agonist UK-14304 exerted only small constrictor effects at high doses (Ferrell & Khoshbaten, 1989). Electrical stimulation of the joint capsule also produced vasoconstriction which was substantially reduced by adding the α_1-adrenoceptor antagonist prazosin to the perfusate, but inclusion of the α_2-adrenoceptor antagonist rauwolscine did not produce additional inhibition of the constrictor response (Ferrell & Khoshbaten, 1990a). These results indicate that in this preparation, sympathetic effects are mediated mainly via α_1-adrenoceptors which predominate in resistance vessels of the rabbit knee joint. Advantages of the *in vitro* preparation described above are the reduced number of variables which require to be controlled and that this model provides an indication of the overall vascular resistance of the joint capsule. The disadvantage of the *in vitro* technique is that the environment is artificial and lacks many of the vasoactive agents which normally circulate in the blood stream and which could modify the response of adrenoceptors. Recent observations in both *in vitro* and *in vivo* models suggest that in the latter, effects mediated by postjunctional α_2-adrenoceptors are more readily detectable, whereas *in vitro* these are difficult to detect and vasonconstrictor effects are mediated principally by postjunctional prazosin-sensitive α_1-adrenoceptors (for review see McGrath *et al.*, 1989). Thus, the present study was performed in anaesthetized rabbits to examine nerve-mediated vascular responses and assess the nature of adrenoceptors which mediate vasoconstriction in the posterior region of the knee joint capsule *in vivo*.

Methods

Experiments were performed on adult New Zealand rabbits (2.2–3.5 kg) which were anaesthetized initially by injection of diazepam (1.5 mg kg^{-1} i.p.) and Hypnorm (Janssen; 0.15 ml kg^{-1} i.m.) and thereafter with a 1% halothane/O_2/N_2O mixture during the surgical procedures. On completion of surgery, the gaseous anaesthetic was discontinued and a slow continuous infusion of pentobarbitone (0.25–0.5 mg

[1] Author for correspondence.

min^{-1} depending on the weight of the animal) administered throughout the remainder of the experiment via a cannula inserted into the left jugular vein. The trachea was cannulated, with the animals breathing spontaneously throughout the experiments. Deep anaesthesia was maintained throughout as judged by the absence of a flexor withdrawl response to a pinch applied to the forelimb. Arterial blood pressure was recorded throughout the experiment by a cannula inserted into the left carotid artery. A 0.9 mm diameter fibre-optic probe connected to a laser Doppler flowmeter (Moor Instruments MBF3) provided a measure of relative changes in blood flow in the volume of tissue beneath the tip of the probe. Previous experiments have validated the use of this technique in assessing alterations in knee joint blood flow in both cats and rabbits (Ferrell *et al.*, 1990). The probe was positioned just above the surface of the postero-medial aspect of the knee joint capsule. Alterations in blood flow are expressed as percentage change from control (0%) values occurring immediately before each test procedure. The posterior articular nerve (PAN), which arises from the tibial nerve in the popliteal fossa, was dissected free and transected proximally. Bipolar bright silver stimulating electrodes were then placed on PAN and pulse trains delivered. The stimulus parameters were: width 1 ms, voltage 10 V, frequency 1–30 Hz and duration 90 s. In previous experiments we have demonstrated that these stimulus parameters give rise to frequency-dependent vasoconstrictor responses (Ferrell & Najafipour, 1992). As long as the laser Doppler probe remained in the same position, the constrictor responses remained stable and reproducible over several hours. Termination of the experiment was achieved by intravenous injection of 1 M KCl.

Drugs were administered by close intra-arterial injection via a polythene cannula (Portex 2FG) inserted into the caudal femoral artery (Barone *et al.*, 1973) and advanced until the tip was located close to the main branch (popliteal artery) just before the origin of the joint branches. All non-articular branches of the latter were ligated. The α-adrenoceptor antagonists were administered as a continuous infusion (0.25 ml min^{-1}; 10^{-4} M) via this cannula, with the duration of infusion being maintained for a minimum of 30 min before α-agonists were administered or nerve stimulation applied. The antagonists were also administered intravenously via the cannula in the external jugular vein as a bolus (1–1.5 mg kg^{-1} body weight) prior to antagonist infusion. In some animals only one antagonist was administered whilst in other prazosin was administered first followed by rauwolscine. Other drugs were administered as a bolus injection into the caudal femoral intra-arterial cannula in a volume of 0.25 ml. The adrenoceptor agonists were administered in random order. All drugs were dissolved in physiological saline. Control injections of 0.25 ml saline were found to have no effect on blood flow. The following agents were used: adrenaline HCl (Evans); phenylephrine HCl (Sigma); clonidine HCl (Sigma); phenoxybenzamine HCl (Smith Kline & French); prazosin HCl (Pfizer); UK-14304 bitartarate (5-bromo-6[2-imidazolin-2-ylamino]-quinoxaline; Pfizer); rauwolscine HCl (Roth); α,β methylene adenosine 5'-triphosphate (lithium salt; Sigma); YM-12617 ((−)-(**R**)-5-[2-[[2-(o-ethoxyphenoxy)ethyl]amino]propyl]-2-methoxybenzenesulphonamide hydrochloride; Yamanouchi Pharmaceutical).

The values expressed in the figures are means ± s.e.mean and comparisons were performed by use of Student's paired or unpaired *t* test as appropriate.

Results

Nerve stimulation

Electrical stimulation of PAN consistently produced vasoconstriction which was unaffected by pretreatment with α,β methylene ATP (Figure 1a) which is a P_{2x}-purinoceptor desensitiser (Kasakov & Burnstock, 1983). The results are summarised in Figure 2 and demonstrate that the mean values before and after injection of α,β methylene ATP were not significantly different (unpaired *t* test).

Vasoconstriction elicited by PAN stimulation (Figure 1bii) was, however, abolished by administration of phenoxybenzamine and converted into a dilator response (Figures 1bii and 2). Adrenaline (2.5 nmol), given by close intra-arterial injection, produced vasoconstriction (Figure 1bi) which was similarly altered by phenoxybenzamine (Figures 1bii and 2). In both cases the change in response was significant ($P<0.001$; unpaired *t* test).

Vasoconstriction elicited by PAN stimulation was frequency-dependent over the range 1 to 10 Hz (Figure 3). Administration of the α_1-adrenoceptor antagonist prazosin produced a significant reduction in this constrictor response at all frequencies except 30 Hz. In contrast, the α_2-adrenoceptor antagonist rauwolscine completely reversed the response and produced significant vasodilatation during nerve stimulation.

As prazosin appeared to give a smaller reduction than rauwolscine, suggesting that the constrictor response to PAN stimulation might be partially mediated by α_1-adrenoceptors, to clarify further the role of α_1-adrenoceptors, another selective α_1-adrenoceptor antagonist, YM-12617 (Honda *et al.*,

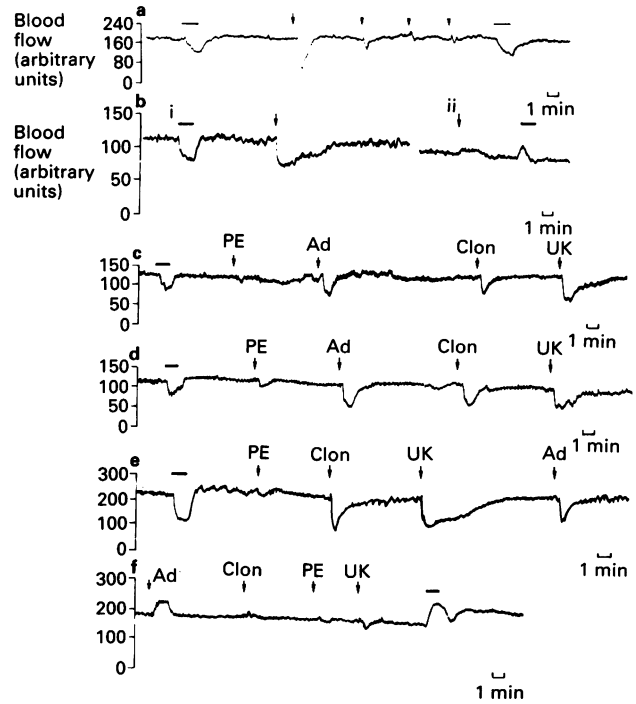


Figure 1 (a) Constrictor response to electrical stimulation of the rabbit posterior articular nerve (PAN) (10 V, 10 Hz, 1 ms width; 90 s train; denoted by black bar over trace) is unaffected by pretreatment with the P_{2x}-purinoceptor desensitiser α,β methylene ATP (each dose = 2.5 nmol in a volume of 0.25 ml injected at the arrows). (b) (i) PAN stimulation (as in a) and close intra-arterial injection of adrenaline (2.5 nmol at the arrow) produce constrictor responses; (ii) 45 min after onset of phenoxybenzamine administration, these responses are abolished and replaced by dilatations. (c) Responses to PAN stimulation (black bar; parameters as in (a)), and close intra-arterial injection of phenylephrine (PE), adrenaline (Ad), clonidine (Clon) and UK-14304 (UK) before prazosin treatment. Dose for each agent = 2.5 nmol. (d) As in (c) but 45 min after onset of prazosin administration. The responses are little affected. (e) Responses to PAN stimulation and the same agents as in (c) before the administration of rauwolscine. (f) As in (e) but 45 min after onset of rauwolscine administration. Responses to agents and nerve stimulation are substantially modified. See methods for details of administration of agonists and antagonists.

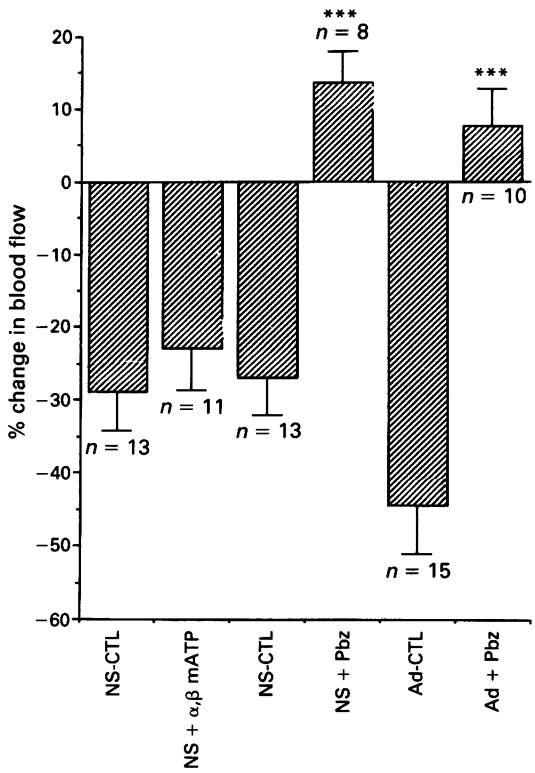


Figure 2 Vascular responses to stimulation of the posterior articular nerve (PAN) before treatment with α,β methylene ATP (NS-CTL) and after four injections of this agent (NS + αβmATP). PAN stimulation before treatment with phenoxybenzamine (NS-CTL) produced vasoconstriction but during administration of phenoxybenzamine (NS + Pbz) this converts to vasodilatation. Close intra-arterial injection of 2.5 nmol adrenaline also produces constriction prior to phenoxybenzamine (Ad-CTL) and this also becomes a dilator response during treatment with this agent (Ad + Pbz). ***$P < 0.001$.

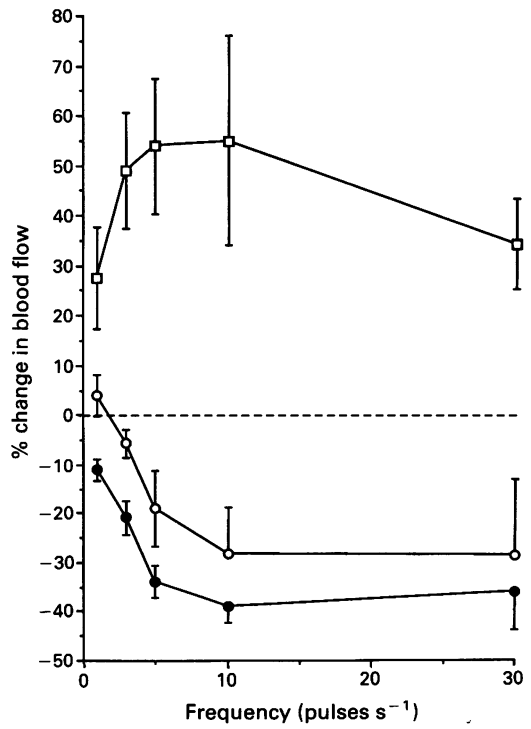


Figure 3 Frequency-response curves to electrical stimulation of the posterior articular nerve (PAN) (10 V, 1 ms, width, 1 min train) under control conditions (●; $n = 15-17$), during prazosin administration (○; $n = 4$), and during perfusion with rauwolscine (□; $n = 5$). Data shown are means and vertical lines indicate s.e.mean.

1985) was used in a smaller series of experiments. This showed that the frequency-response curve in the presence of YM-12617 only differed significantly from the control curve at the lowest frequency (Figure 4), again highlighting the importance of α_2-adrenoceptors in nerve-mediated vasoconstriction.

α-Adrenoceptor agonists and antagonists

In order to assess the nature of the adrenoceptors mediating these constrictor responses, selective α-adrenoceptor antagonists and agonists were employed. The α_1 agonist phenylephrine failed to show any effects at doses below 2.5 nmol (Figure 5d) whereas the α_2 agonists UK-14304 and clonidine both gave rise to constrictor responses at lower doses (Figure 5b and c). The dose-response curves of these agents suggest that adrenaline and UK-14304 are equipotent (Figure 5a and b), with clonidine giving rise to smaller responses which in general showed the same trend as those of UK-14304 (Figure 5c).

The effect of the α_1-adrenoceptor antagonist prazosin was investigated as illustrated in Figure 1c and d, which demonstrates that the constrictor responses to close intra-arterial injection of phenylephrine, adrenaline, UK-14304 and clonidine (all 2.5 nmol) were not attenuated by prazosin administration. These results are detailed in Figure 5 for different doses of each of the agonists, which show that in the presence of prazosin the constrictor responses to clonidine, phenylephrine and UK-14304 across a range of doses were actually significantly greater than control values. This effect appeared to be specific to prazosin, as repeating these experiments with YM-12617 at similar intravenous doses and perfusate concentrations (10^{-4} M) neither potentiated nor inhibited the actions of clonidine and UK-14304 (Figure 5b and c). The constrictor response to phenylephrine was, however, significantly reduced by YM-12617 (Figure 5d) suggesting the presence of α_1-adrenoceptors. The responses to adrenaline at doses below 2.5 nmol were reduced by both prazosin and YM-12617, again indicating the involvement of α_1-adrenoceptors at these lower doses.

The α_2-adrenoceptor antagonist rauwolscine gave rise to pronounced inhibition of the responses to all the agonists. As illustrated in Figure 1e and f the responses to PAN stimulation and close intra-arterial injection (2.5 nmol in all cases) of phenylephrine, clonidine, adrenaline and UK-14304 were

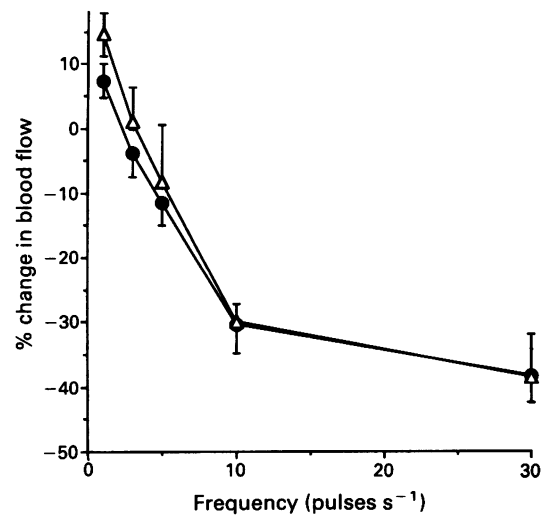


Figure 4 Frequency-response curves to electrical stimulation of the posterior articular nerve (PAN) (10 V, 1 ms, width, 1 min train) under control conditions (●; $n = 4-6$) and during administration of YM-12617 (△; $n = 4-6$). Data shown are means and vertical lines indicate s.e.mean.

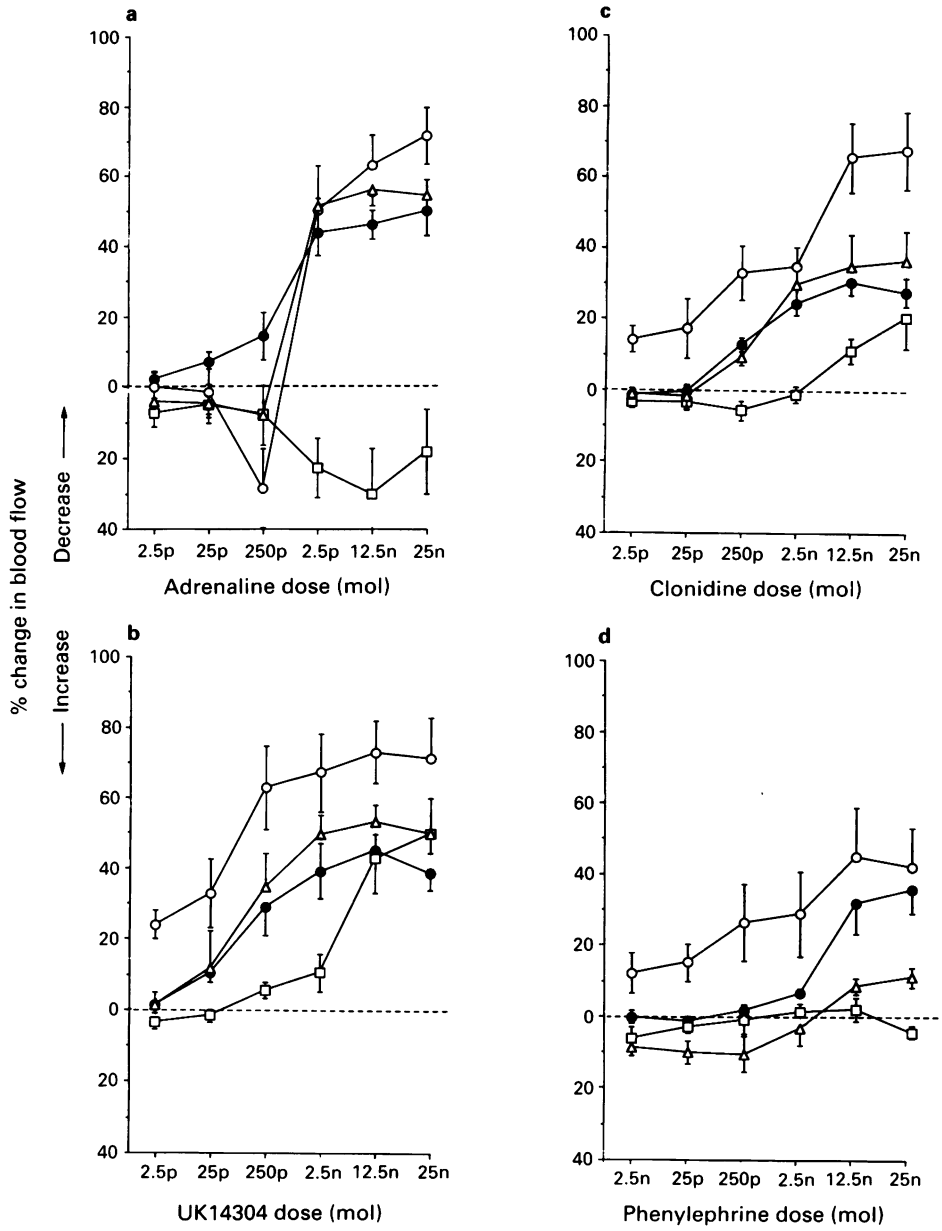


Figure 5 Dose-response curves for close intra-arterial injection (0.25 ml volume) of (a) adrenaline, (b) UK-14304, (c) clonidine and (d) phenylephrine under control conditions (●; $n = 5-10$), during administration of either prazosin (○; $n = 4$), YM-12617 (△; $n = 4-6$) or rauwolscine (□; $n = 5$). Data shown are means and vertical lines indicate s.e.mean. p = pico. n = nano.

substantially inhibited by rauwolscine. Figure 5 shows that rauwolscine shifted the dose-response curves to the right and that adrenaline now gave rise to dilator responses. In all cases rauwolscine produced significant alteration in the responses.

Administration of the α-adrenoceptor antagonists produced some reduction in arterial blood pressure and consequently of synovial blood flow. Before administration of antagonists mean arterial blood pressure (± s.e.mean) was 71 ± 7 mmHg ($n = 8$). The bolus injection of antagonist produced an initial reduction of blood pressure and joint blood flow, but these partially recovered and levelled out by 40 min at which time the test procedures were once again performed. Mean arterial blood pressure at this time for phenoxybenzamine, prazosin and rauwolscine was 54.3 ± 4.4 ($n = 8$), 60.8 ± 3.7 ($n = 7$) and 62 ± 3 ($n = 7$) mmHg, respectively, and these values did not differ significantly from each other although they did differ significantly ($P < 0.0004$) from control (unpaired t test). Administration of YM-12617 decreased blood pressure from 82.8 ± 5.6 to 77.7 ± 5.9 mmHg ($P < 0.05$; $n = 6$; paired t test)

Discussion

The results of this study have shown that electrical stimulation of the nerve supply to the posterior region of rabbit knee joint resulted in vasoconstriction which was mediated predominantly via α_2-adrenoceptors. Although purinergic co-transmission has been described in other vascular beds such as the urinary bladder (Kasakov & Burnstock, 1983), vas deferens (Sneddon & Burnstock, 1984) and colon (Hedlund *et al.*, 1983), no evidence was obtained in the present study to indicate that ATP has a role to play in mediating the actions of sympathetic vasoconstrictor fibres innervating the joint. The pronounced constrictor effect of the first injection of α, β methylene ATP (Figure 1a) suggests the presence of P_{2x}-purinoceptors in these knee joint blood vessels, although this

could also have resulted from a direct depolarizing effect of α,β methylene ATP on smooth muscle cells (Komori *et al.*, 1988). However, previous experiments in an isolated knee joint preparation showed both P_1- and P_{2x}-purinoceptors to be present, although relatively high doses of purines were required (Ferrell & Khoshbaten, 1990b). If these purinoceptors are indeed present, their functional role remains unclear.

Although phenoxybenzamine is not specific to α-adrenoceptors, the finding that rauwolscine gave rise to pronounced inhibition suggests that phenoxybenzamine was acting at these receptors. It was clear that the constrictor responses to nerve stimulation and the α_2-adrenoceptor agonists were mediated predominantly via α_2-adrenoceptors as these were substantially altered by rauwolscine but unaffected by YM-12617. The effects of prazosin are difficult to interpret as it potentiated the responses to UK-14304, clonidine and phenylephrine but not to the cathecholamine, adrenaline. The mechanism underlying the potentiation is unclear at present but was specifically related to prazosin, as the structurally different α_1-adrenoceptor antagonist YM-1267 did not produce this effect. The potentiating effect of prazosin on the above α_1 and α_2 agonists meant that its expected inhibitory effect on phenylephrine could not be detected. However, the nerve-mediated vasoconstriction (Figure 3) was significantly reduced by prazosin which could be explained by its lack of potentiation of the effect of neurally-released catecholamine, noradrenaline, leaving the inhibitory effects of prazosin on α_1-adrenoceptors unopposed. The vasoconstriction induced by phenylephrine at higher doses was reduced both with YM-12617 and rauwolscine (Figure 5d), suggesting that phenylephrine may act at both α_1 and α_2-adrenoceptors at such high doses. The conversion of the constrictor responses to nerve stimulation and adrenaline into vasodilator ones suggests that the latter are normally masked by the more pronounced constrictor responses. In the case of adrenaline, the dilator response during blockade with phenoxybenzamine (and rauwolscine) could be explained by adrenaline acting on β-adrenoceptors. This would be consistent with the finding of Cobbold & Lewis (1956a) that close intra-arterial injection of noradrenaline produced greater vasoconstriction of dog knee joint blood vessels than adrenaline, suggesting the presence of β-adrenoceptors, although this was not discussed in their paper. Although vasoconstriction induced by adrenaline was not antagonized by prazosin or YM-12617 at doses $\approx$2.5 nmol, both of these agents produced inhibition at lower doses, with rauwolscine being effective at all doses (Figure 5a). This could be interpreted as adrenaline acting at both α_1- and α_2-adrenoceptors, with the α_1 subtype having a lower activation threshold.

The mechanisms underlying the dilator response which occurred with PAN stimulation during α-adrenoceptor blockade were not investigated in the present study. However, it is possible that this effect was mediated by substance P released from nerve endings of C afferent fibres present in PAN as in previous experiments on cat knee joint blood vessels it was shown that dilator responses following the initial constrictor responses to PAN stimulation were reduced by a substance P antagonist (Khoshbaten & Ferrell, 1990).

Although the administration of all the α-adrenoceptor antagonists resulted in similar decrease in arterial blood pressure and thus altered vascular resistance in the knee joint, the strikingly different responses to PAN stimulation and α-adrenoceptor agonists occurring during rauwolscine treatment contrasts with the lack of inhibitory effect of prazosin. This suggests that the differences between these agents reflects their differing effects on α-adrenoceptors in these vessels and is not secondary to altered vascular resistance.

In conclusion, the most striking finding of this study was that α_2-adrenoceptors play a major role in mediating the vasoconstrictor responses to α-agonists and nerve stimulation, whereas in previous *in vitro* studies these responses were predominantly mediated by α_1-adrenoceptors (Ferrell & Khoshbaten, 1989; 1990a). The mechanisms underlying this change are unclear, but may reflect the differences in the experimental conditions in terms of the artificiality of the *in vitro* preparation. It has been demonstrated in the rabbit isolated distal saphenous artery that contractile responses to sympathetic nerve stimulation are substantially reduced by prazosin but a small residual component remains which is potentiated in the presence of angiotensin II (Dunn *et al.*, 1991). Thus, it is possible that circulating vasoactive substances which are present *in vivo* modify the response to nerve stimulation. However, in the present experiments the virtual absence of inhibition by YM-12617 coupled with the powerful effect of rauwolscine suggests that in this tissue the nerve-mediated constrictor responses are mediated predominantly by α_2-adrenoceptors. Evidence for α_2-adrenoceptor involvement in blood vessel contractions evoked by nerve stimulation has proved difficult to obtain in animal tissues, and when demonstrated, the effects were predominantly mediated by α_1-adrenoceptors (for review see Docherty, 1989). Only in the human saphenous vein have contractions evoked by nerve stimulation been shown to be mediated predominantly by α_2-adrenoceptors, although even in this preparation some inhibition by prazosin was observed at concentrations above 10^{-7} M (Docherty & Hyland, 1985).

It could be argued that the difference in the results obtained in the *in vitro* and *in vivo* preparations may be related to different methodologies. The isolated knee joint preparation reflects changes of the resistance vessels in the whole capsule, whereas Laser Doppler flowmetry samples a small volume of tissue under the probe and therefore is more regionally specific. In addition, laser Doppler flowmeters are designed to sample flow in the microcirculation where it has been shown that α_2-adrenoceptors predominate, whereas α_1-adrenoceptors are more common on the larger resistance arteries (Faber *et al.*, 1991). However, recent *in vivo* experiments using the laser Doppler flowmeter on the anterior knee joint capsule in anaesthetized rabbits have shown that vasoconstrictor effects to nerve stimulation are mediated predominantly via α_1-adrenoceptors, even though postjunctional α_2-adrenoceptors are also present (Khoshbaten & Ferrell, unpublished observations). This implies that there are differences in α-adrenoceptor subtypes even within different regions of the same joint. The functional significance of this finding is unclear at present. The present investigation indicates that studies using isolated tissue preparations require careful interpretation and may not accurately reflect the conditions prevailing *in vivo*.

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Adenosine A_1-receptor stimulated increases in intracellular calcium in the smooth muscle cell line, DDT_1MF-2

[1]John M. Dickenson & Stephen J. Hill

Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham, NG7 2UH

1 The effect of a range of adenosine receptor agonists on intracellular free calcium concentration ($[Ca^{2+}]_i$) has been studied in the hamster vas deferens smooth muscle cell line DDT_1MF-2.

2 Adenosine receptor agonists elicited a rapid and maintained increase in $[Ca^{2+}]_i$ in fura-2 loaded DDT_1MF-2 cells. The initial rise could be maintained in the absence of extracellular calcium, whereas the maintained or plateau phase was dependent upon the presence of extracellular calcium and appeared to be associated with calcium influx. The rank order of agonist potencies was N^6-cyclopentyladenosine > 5'-N-ethylcarboxamidoadenosine > 2-chloroadenosine > adenosine.

3 The response to 2-chloroadenosine was antagonized by the antagonists 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, K_D 0.14 nM) and 8-phenyltheophylline (K_D 112 nM).

4 Pretreatment with the 5-lipoxygenase inhibitor AA861 (20 μM) produced only a small (14 ± 2%) inhibition of the $[Ca^{2+}]_i$ response elicted by N^6-cyclopentyladenosine (300 nM), in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA. The cyclo-oxygenase inhibitor, indomethacin (2 μM) was without effect.

5 The Ca^{2+}-influx associated with the plateau phase required the continued presence of agonist on the receptor. The antagonist DPCPX (100 nM) attenuated the rise in $[Ca^{2+}]_i$ observed when extracellular Ca^{2+} was re-applied after the cells had been stimulated with N^6-cyclopentyladenosine (CPA;300 nM) in experiments initiated in nominally Ca^{2+}-free buffer.

6 Pretreatment with pertussis toxin (200 ng ml^{-1} for 4 h) inhibited the CPA (100 nM) stimulated intracellular Ca^{2+} release and Ca^{2+} influx but was without effect on the response to histamine (100 μM).

7 These data suggest that adenosine A_1-receptor activation in DDT_1MF-2 cells stimulates release of Ca^{2+} from intracellular stores and influx of extracellular Ca^{2+} through Ca^{2+} entry pathways in the plasma membrane which required the continued presence of agonist on the receptor.

Keywords: Adenosine; A_1-adenosine receptors; intracellular calcium; DDT_1MF-2 cells; smooth muscle

Introduction

Adenosine receptors are widely distributed in mammalian tissue and have been divided into two major subtypes A_1 and A_2 (for reviews see Stiles, 1986; 1992). Adenosine A_1-receptors are negatively coupled to adenylate cyclase via a pertussis toxin-sensitive G_i guanosine 5'-triphosphate (GTP)-binding protein, whereas adenosine A_2-receptors are positively coupled to adenylate cyclase via a G_s protein (Stiles, 1992). In addition, A_1-receptors are now known to regulate a variety of different effector systems. These include: (1) opening of potassium channels through pertussis toxin-sensitive G_i proteins in cardiac tissue (Kurachi *et al.*, 1986), (2) inhibition of Ca^{2+} channel opening in neurones (Olsson & Pearson, 1990), (3) activation of guanylate cyclase in smooth muscle cells (Kurtz, 1987), (4) inhibition of phospholipase A_2 activity in hamster brown adipocytes (Schimmel & Elliott, 1988), (5) stimulation of the glucose transporter (Londos *et al.*, 1981) and (6) the modulation of agonist-stimulated inositol phospholipid hydrolysis (Linden & Delahunty, 1989; Hill & Kendall, 1989).

The intracellular cross-talk between adenosine-receptor stimulation and agonist-induced inositol phospholipid hydrolysis has been widely studied in the central nervous system (CNS). For example, the activation of adenosine A_1 receptors in guinea-pig cerebral cortical slices (Hollingsworth *et al.*, 1986; Hill & Kendall, 1987) augments the histamine H_1-receptor induced accumulation of inositol phosphates, whereas in the cerebral cortex of mouse (Kendall & Hill, 1988) and man (Kendall & Firth, 1990) the histamine response is attenuated. In all three species, adenosine-receptor activation failed to stimulate inositol phospholipid hydrolysis on its own. Intracellular cross-talk between adenosine-receptors and agonists coupled to inositol phospholipid hydrolysis has also been demonstrated in cells grown in culture. Inhibitions of agonist-induced inositol phospholipid hydrolysis have been described in the rat pituitary cell line GH_3 (Delahunty *et al.*, 1988) and the human astrocytoma cell line 1321N1 (Nakahata *et al.*, 1991), whereas in FRTL-5 thyroid cells adenosine-receptor agonists enhanced α_1-adrenoceptor-stimulated inositol phospholipid hydrolysis (Okajima *et al.*, 1989). However, in addition to these modulatory effects there are several accounts of adenosine-receptor agonists stimulating inositol phospholipid directly. The adenosine A_1-receptor mediates the inositol phospholipid hydrolysis responses found in guinea-pig myometrium (Schiemann *et al.*, 1991) and the rabbit cortical collecting tubule cell line RCCT-28A (Arend *et al.*, 1989). In contrast, in the rat tumour-derived mast cell line RBL-2H3 the order of agonist potencies stimulating inositol phospholipid hydrolysis was characteristic of the adenosine A_2 receptor (Ali *et al.*, 1990). Interestingly, the adenosine A_1-receptor stimulated inositol phospholipid hydrolysis in the guinea-pig myometrium is completely blocked by cyclo-oxygenase inhibitors suggesting that inositol phospholipid hydrolysis is secondary to synthesis of prostaglandins from arachidonic acid. Measurements of adenosine-receptor induced changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) have also been obtained in the renal epithelial cell line LLC-PK_1 (Weinberg *et al.*, 1989), rat cultured mesangial cells (Olivera *et al.*, 1992), cortical collecting tubule cells (Arend *et al.*, 1988), RBL-2H3 cells (Hide & Beavan, 1991) and human tracheal epithelial cells

[1] Author for correspondence.

(Galietta *et al.*, 1992).

The DDT_1MF-2 smooth muscle cell line, derived from hamster vas deferens (Norris *et al.*, 1974) expresses both adenosine A_1- and A_2-receptors that are respectively coupled negatively and positively to adenylate cyclase (Gerwins *et al.*, 1990; Ramkumar *et al.*, 1990). Our previous studies (White *et al.*, 1992) and those of Gerwins & Fredholm (1991) have also shown that adenosine A_1-receptor activation in DDT_1MF-2 cells results in inositol phospholipid hydrolysis. In contrast, Schachter *et al.* (1992) recently showed that in DDT_1MF-2 cells adenosine A_1-receptor activation potentiated the inositol phospholipid response elicited by noradrenaline, but in the absence of noradrenaline caused only a minor stimulation of phospholipase C, which was abolished in the absence of extracellular calcium.

In DDT_1MF-2 cells we have previously shown that a single concentration of 2-chloroadenosine (10 μM) elicited a significant rise in $[Ca^{2+}]_i$ in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA (White *et al.*, 1992). In this present paper we have examined the pharmacological characteristics of the adenosine-receptor mediated increases in $[Ca^{2+}]_i$. Furthermore, we clearly demonstrate that adenosine-receptor stimulated increases in $[Ca^{2+}]_i$, measured in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA, can occur in the presence of 5-lipoxygenase and cyclo-oxygenase inhibitors. These data suggest that in DDT_1MF-2 cells the adenosine-receptor mediated increase in $[Ca^{2+}]_i$ is a consequence of direct receptor coupling to phospholipase C rather than being secondary to the products of arachidonic acid metabolism.

Methods

Cell culture

The hamster vas deferens smooth muscle cell (DDT_1MF-2) was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, U.K.). DDT_1MF-2 cells were cultured at 37°C in a humidified air/CO_2 (90:10) atmosphere in 75cm^2 flasks (Costar). The growth medium was Dulbecco's modified Eagle's medium supplemented with 2 mM-L-glutamine and 10% (v/v) foetal calf serum (FCS). Cells were passaged twice a week (1/6 split ratio) by vigorous shaking of the flask and placed into 75 cm^2 flasks and fed with fresh growth medium every 48 h. Cells for $[Ca^{2+}]_i$ determinations were grown on 24 mm × 10 mm glass coverslips in 90 mm petri dishes. All experiments were performed on confluent monolayers (passages 4–17, numbers assigned after receiving the cell line).

Measurement of intracellular free calcium

Intracellular free calcium was measured by loading confluent cell monolayers with the calcium-sensitive fluorescent dye fura-2. Individual coverslips were placed in 35 mm petri dishes with 1 ml of physiological buffer (composition, mM: NaCl 145, glucose 10, KCl 5, $MgSO_4$ 1, HEPES 10, $CaCl_2$ 2, pH 7.45) containing 10% FCS (v/v), 3 μM fura-2/AM and incubated for 30 min at 37°C. After this 'loading' period the fura-2 containing buffer was replaced with fresh buffer, that was free of fura-2 and FCS but contained 0.1% bovine serum albumin, and left at 37°C for a further 15 min. Loaded coverslips were then mounted in a specially designed holder which enabled the coverslip to be positioned across the diagonal of a polymethacrylate cuvette. Each cuvette contained 2.9 ml of physiological buffer (drugs were added to the cuvettes in 100 μl aliquots) and fluorescent measurements were made at 37°C by use of a Perkin Elmer LS 50 spectrometer. The excitation wavelengths were 340 and 380 nm, with emission at 500 nm. The slit-widths were set at 10 nm for both the excitation and emission wavelengths and the time taken to switch between 340 and 380 nm was 0.8 s. Intracellular free $[Ca^{2+}]$ was calculated every 1.9 s from the equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = (R - R_{min}) \times (S_{380,min}/S_{380,max}) \times K_D / (R_{max} - R)$$

where K_D is the affinity of fura-2 for Ca^{2+} (224 nM at 37°C) and $S_{380,min}/S_{380,max}$ is the ratio (β value) of the fluorescent values obtained at 380 nm in the absence and presence of saturating $[Ca^{2+}]_i$. The maximum and minimum R values (R_{max} and R_{min}) were determined on separate coverslips under saturating $[Ca^{2+}]_i$ (achieved by increasing the extracellular $[Ca^{2+}]$ to 20 mM followed by 10 μM ionomycin, pH 7.45) and calcium-free (achieved using 8.3 mM EGTA immediately followed by 25 μl of 1.0 M NaOH to compensate for the decrease in pH, in the presence of 10 μM ionomycin) conditions respectively. Corrections for autofluorescence were made by measuring the fluorescence produced by coverslips that had not been loaded with fura-2. Where Ca^{2+}-free conditions were required experiments were performed in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA.

Accumulation of [^{3}H]-cyclic AMP

Adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation was measured by prelabelling cell monolayers with [^{3}H]-adenine as described previously (Ruck *et al.*, 1991).

Data analysis

Agonist and antagonist concentration-response curves were fitted to a logistic equation using the non-linear regression programme GraphPAD (ISI). Data are shown as means ± s.e.mean. Statistical analysis was performed by use of Student's unpaired *t* test. A P value < 0.05 was considered as statistically significant. Rises in intracellular free $[Ca^{2+}]$ were evaluated by importing the fluorescence data into the spreadsheet AsEasyAs (TRIUS Inc). Basal $[Ca^{2+}]_i$ levels were determined by calculating the mean of the ten data points (measured every 1.9 s) before drug addition, whereas the maximum Ca^{2+} signal was deemed to be the largest Ca^{2+} response obtained immediately after drug addition.

Apparent antagonist equilibrium dissociation constants (K_D) were estimated by a modification of the null method described by Lazereno & Roberts (1987). Briefly, a concentration-response curve to 2-chloroadenosine was generated and a concentration (C; usually 10 μM) of 2-chloroadenosine was chosen which gave a response greater than 50% of the maximum agonist response. The concentration of antagonist (IC_{50}) required to reduce the response to this concentration (C) of 2-chloroadenosine by 50% was then determined. The 2-chloroadenosine concentration-response curve was fitted to a logistic equation as described above and a concentration (C′) identified which yielded a response equivalent to 50% of that produced by concentration C (in the absence of antagonist). The apparent K_D was then determined from the relationship:

$$C/C' = IC_{50}/K_D + 1$$

Chemicals

Fura-2/AM, ionomycin and pertussis toxin were from Calbiochem. Histamine, mepyramine, forskolin, adenosine, 2-chloroadenosine, 5′-N-ethylcarboxamidoadenosine (NECA), N^6-cyclopentyladenosine (CPA), indomethacin and 8-phenyltheophylline were purchased from Sigma and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) from Research Biochemicals Incorporated. AA861 (2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)1,4-benzoquinone) was a generous gift from Takeda Chemicals Industries (Osaka, Japan). Dulbecco's modified Eagle's medium and foetal calf serum (FCS) were

from Northumbria Biologicals (U.K.). All other chemicals were of analytical grade.

Results

Adenosine receptor-stimulated increases in intracellular Ca^{2+}

Adenosine receptor-stimulation in the smooth muscle cell line, DDT_1MF-2, results in an increase in intracellular Ca^{2+} which appeared to be biphasic in nature. Adenosine (100 μM, see Figure 1a) increased basal $[Ca^{2+}]_i$ from 106 ± 11 nM to 217 ± 40 nM ($n = 9$, $P<0.05$), within 20 s of application. The response was fairly well maintained and declined slowly towards basal levels in the presence of extracellular Ca^{2+} (see Figure 1a). Similar responses were obtained with 2-chloroadenosine (10 μM, 86 ± 8 nM to 250 ± 25 nM, $n = 6$, $P<0.05$), N^6-cyclopentyladenosine (1 μM, 98 ± 10 nM to 201 ± 18 nM, $n = 4$, $P<0.05$) and 5'-N-ethylcarboxamidoadenosine (10 μM, 100 ± 11 nM to 226 ± 41 nM, $n = 3$, $P<0.05$). Representative $[Ca^{2+}]_i$ profiles are shown in Figure 1. Concentration-response curves for CPA (EC_{50} = 19 ± 7 nM, $n = 5$) and NECA (EC_{50} = 770 ± 165 nM, $n = 4$) are shown in Figure 2. Concentration-response curves for adenosine and 2-chloroadenosine-stimulated calcium responses yielded EC_{50} values of 1.6 ± 0.6 μM ($n = 5$) and 1.3 ± 0.4 μM ($n = 4$), respectively. The rank order of agonist potencies i.e. CPA> NECA> 2-chloroadenosine> adenosine was indicative of the adenosine-receptor mediating increases in $[Ca^{2+}]_i$ being the A_1 subtype. These values are in close agreement with agonist potencies reported for the stimulation of [^{3}H]-inositol phosphate accumulation in DDT_2MF-2 cells (White *et al.*, 1992). The class of adenosine-receptor involved was further examined by using the selective A_1-receptor antagonist DPCPX (Bruns *et al.*, 1987 a) and partially selective A_1-receptor antagonist 8-phenyltheophylline. Inhibition concentration-response curves were obtained under antagonist equilibrium conditions (15 min preincubation) in the presence of a constant 2-chloroadenosine concentration (10 μM). The inhibition curves for DPCPX (IC_{50} = 1.3 ± 0.2 nM, $n = 3$) and 8-phenyltheophylline (IC_{50} = 1.03 ± 0.3 μM, $n = 4$) are shown in Figure 3. This particular approach enabled apparent equilibrium dissociation constants (K_D) to be calculated assuming a competitive interaction between agonist and antagonist (see Methods). The K_D values obtained were 0.14 ± 0.02 nM ($n = 3$) and 112 ± 33 nM ($n = 4$) for DPCPX and 8-phenyltheophylline, respectively. These values are again indicative of the involvement of the A_1-receptor subtype and are in agreement with antagonist K_D values for the inhibition of [^{3}H]-cyclohexyladenosine binding to the adenosine A_1-receptor in rat brain membrane (Bruns *et al.*, 1987b).

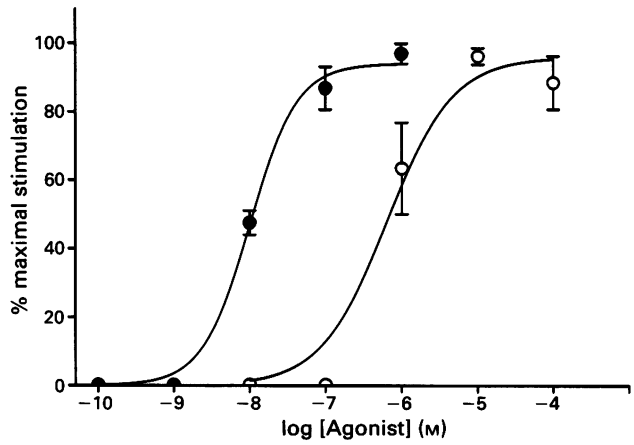


Figure 2 Concentration-response curves for adenosine-receptor agonists on increases in intracellular Ca^{2+} concentration in DDT_1MF-2 cells. Responses to N^6-cyclopentyladenosine (CPA: ●) and N-ethylcarboxamidoadenosine (NECA: ○) are expressed as a percentage of the maximal stimulation (expressed as an increase in F_{340}/F_{380} ratio minus the basal fluorescence ratio). Curves were fitted by use of a logistic equation as described under Methods. Data are means and vertical lines show s.e.mean of five (CPA) or four (NECA) experiments.

The role of arachidonic acid metabolites

To assess the involvement of arachidonic acid metabolites i.e. prostaglandins and leukotrienes in the adenosine-receptor mediated increase in $[Ca^{2+}]_i$ we performed experiments using the cyclo-oxygenase inhibitor indomethacin and the 5-lipoxygenase inhibitor AA861 (Yoshimoto *et al.*, 1982). The rise in $[Ca^{2+}]_i$ elicited by CPA (300 nM), in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA, was slightly attenuated

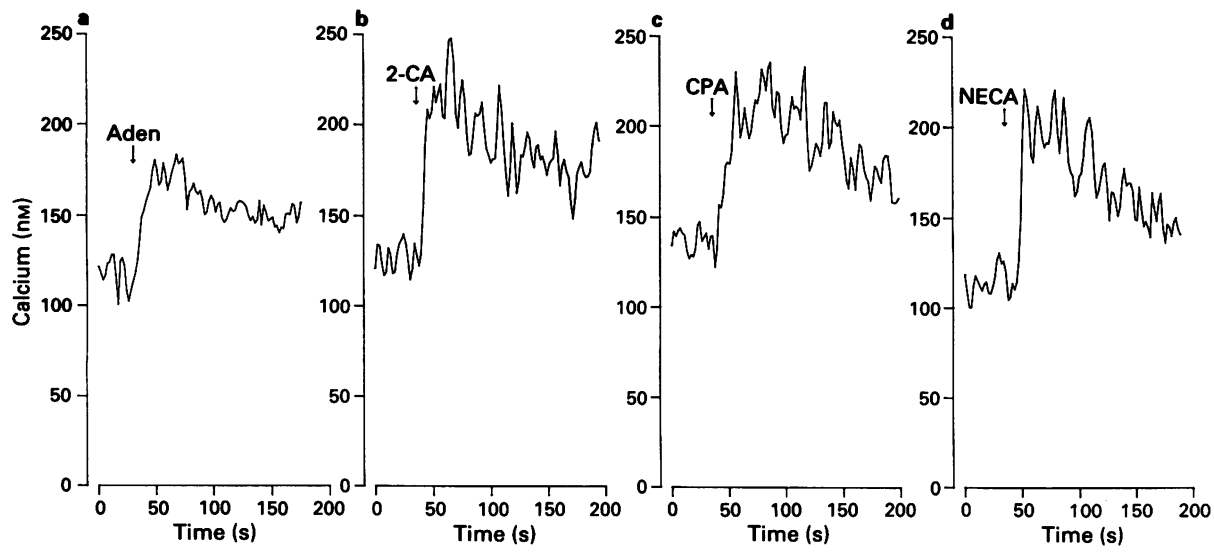


Figure 1 Effect of adenosine-receptor stimulation on intracellular Ca^{2+} concentration in fura-2 loaded DDT_1MF-2 cells. Cells were stimulated with the agonists (a) adenosine (Aden; 100 μM), (b) 2-chloroadenosine (2-CA; 10 μM), (c) N^6-cyclopentyladenosine (CPA; 1 μM) or (d) 5'-N-ethylcarboxamidoadenosine (NECA; 10 μM) in the presence of extracellular Ca^{2+} (2 mM), added where indicated.

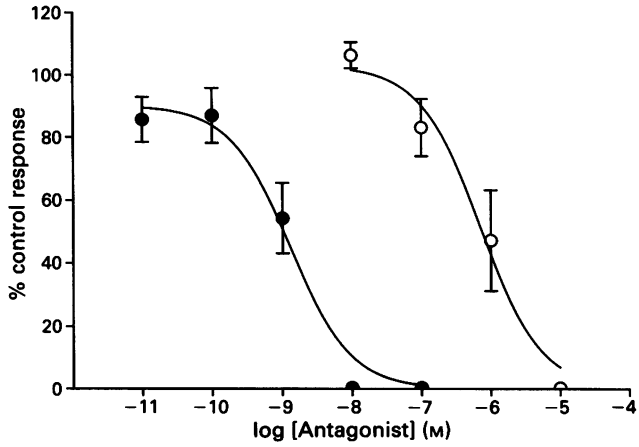


Figure 3 Concentration-response curves for adenosine-receptor antagonists on increases in intracellular Ca^{2+} concentration in DDT_1MF-2 cells. Antagonism produced by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, ●) or 8-phenyltheophylline (8-PT: ○) of the response to 10 μM 2-chloroadenosine. The data are expressed as a percentage of the response to 10 μM 2-chloroadenosine (expressed as an increase in F_{340}/F_{380} ratio minus the basal fluorescence ratio). Curves were fitted by use of a logistic equation as described under Methods. Data are means and vertical lines show s.e.mean of three (DPCPX) or four (8-PT) experiments.

by pretreatment of cells with AA861 (86 ± 2.1% of the maximum CPA response, $n = 5$, $P < 0.05$, cf Figure 4a and b). In contrast, indomethacin (2 μM) did not effect the CPA-induced rise in $[Ca^{2+}]_i$ (124 ± 18% of the maximum CPA response, $n = 4$, Figure 4c). These data suggest that adenosine-receptor stimulation, in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA, can produce a direct release of Ca^{2+} from intracellular stores which is independent of arachidonic acid metabolites.

To eliminate the possibility that the rise in $[Ca^{2+}]_i$ is a consequence of adenosine-receptor agonists augmenting the inositol phospholipid response elicited by endogenous histamine or noradrenaline (Schachter *et al.*, 1992) leading to a release of intracellular Ca^{2+}, we performed experiments in the presence of the histamine H_1-receptor antagonist mepyramine and the α-adrenoceptor antagonist phentolamine. Pretreatment of cells with mepyramine (100 nM, Figure 4d) or phentolamine (300 nM, Figure 4e) did not affect the response elicited by CPA (300 nM) in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA. In the presence of mepyramine and phentolamine the CPA response was 107 ± 7.2% ($n = 4$) and 116 ± 11% ($n = 3$) respectively of that obtained in the absence of antagonist.

Role of extracellular Ca^{2+} ions

The role of extracellular calcium in the overall response to adenosine-receptor stimulation was examined by performing experiments in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA. Figure 5a shows a profile obtained by stimulating adenosine-receptors with 300 nM CPA in the absence of extracellular Ca^{2+}. Clearly, the removal of extracellular Ca^{2+} results in a more transient response to CPA with $[Ca^{2+}]_i$ returning to basal levels approximately 150 s after stimulation (also see Figures 5b and 7c). The attenuation of the maintained phase of the response is clearly demonstrated if the percentage of the maximum response (obtained using 300 nM CPA) remaining after 100 s is compared for experiments performed in Ca^{2+}-free and Ca^{2+}-containing buffer. In nominally Ca^{2+}-free buffer containing 0.1 mM EGTA the percentage of the maximum CPA response remaining at 100 s is significantly lower (19.6 ± 4.7%, $n = 6$, $P < 0.05$) than that obtained in the presence of extracellular Ca^{2+} (56.3 ± 3.2%, $n = 6$). Furthermore, if Ca^{2+} is re-applied, after the cells have been stimulated with 300 nM CPA in the absence of extracellular Ca^{2+}, there is a rapid rise in $[Ca^{2+}]_i$ indicative of Ca^{2+} influx (see Figures 5b and 7c). To dismiss the possibility that this rise in $[Ca^{2+}]_i$ is simply a consequence of fura-2 leakage into the extracellular medium an experiment was performed (data not shown) in which

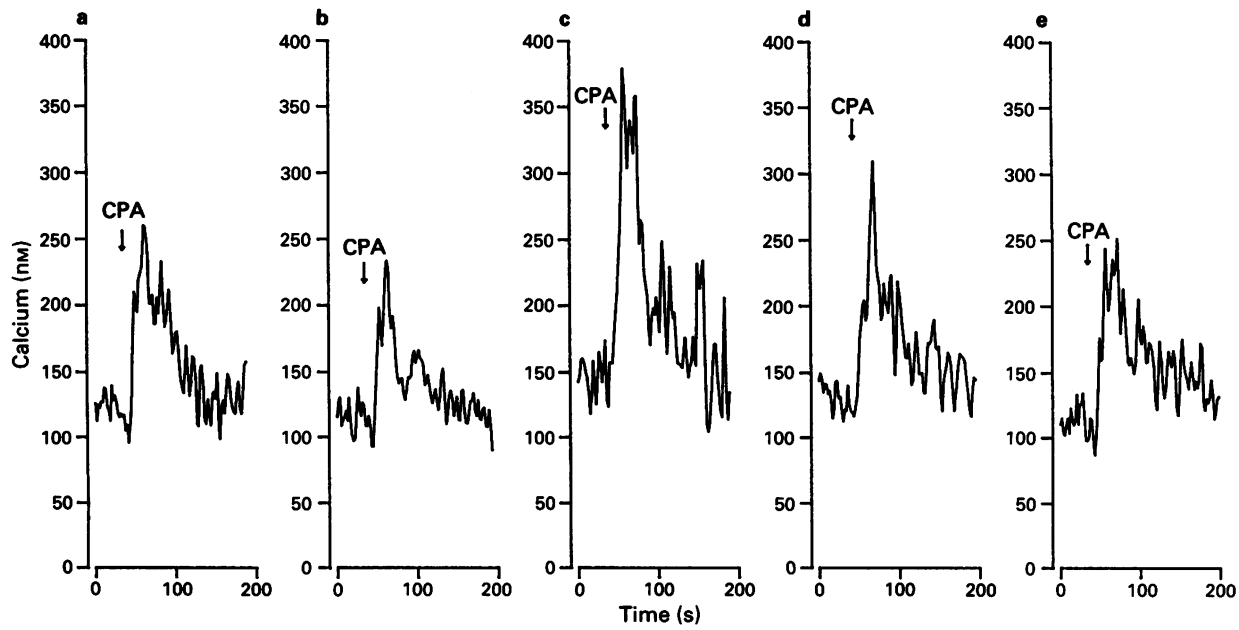


Figure 4 Effects of arachidonic acid metabolism inhibitors and antagonists of the histamine H_1-receptor and α-adrenoceptor on adenosine-receptor stimulated increases in intracellular Ca^{2+} concentration in DDT_1MF-2 cells. Cells were stimulated in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA with N^6-cyclopentyladenosine (CPA) in the absence (a) or presence of 20 μM AA861 (b), 2 μM indomethacin (c), 100 nM mepyramine (d) or 300 nM phentolamine (e). Cells were incubated with the relevant inhibitor or antagonist for 15 min before the addition of CPA. N^6-cyclopentyladenosine (CPA: 300 nM) was added where indicated. Similar results were obtained in at least three other experiments.

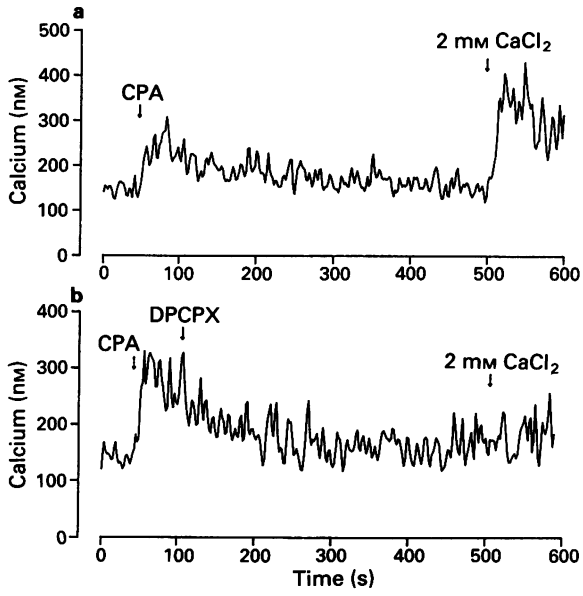


Figure 5 Effects of re-applying extracellular Ca^{2+} (2 mM) in the absence (a) and presence (b) of the A_1-receptor antagonist (8-cyclopentyl-1,3-dipropylxanthine (DPCPX), during experiments performed in nominally Ca^{2+}-free buffer and 0.1 mM EGTA.

N^6-cyclopentyladenosine (CPA; 300 nM), DPCPX (100 nM) or $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in at least five other experiments.

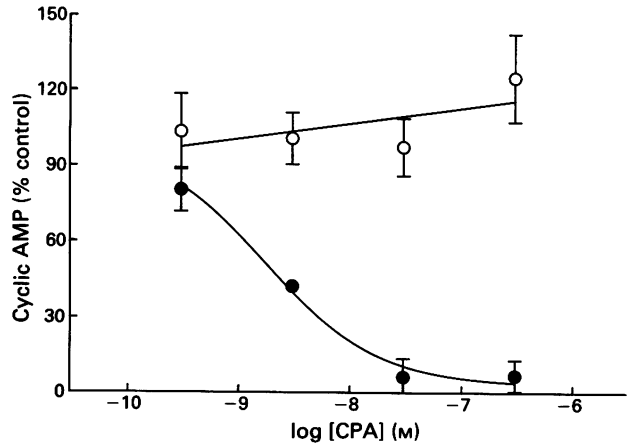


Figure 6 Effects of N^6-cyclopentyladenosine (CPA) and pertussis toxin (PTX) on forskolin-induced cyclic AMP accumulation in DDT_1MF-2 cells. Control cells (●) and cells treated with PTX (○) were incubated with 10 μM forskolin for 10 min in the presence of different concentrations of CPA. Data are expressed as a percentage of the response to 10 μM forskolin in the presence of PTX or CPA, which was taken as 100%. Data are means and vertical lines show s.e.mean of quadruplicate determinations in a single experiment. Curves were fitted by use of a logistic equation as described under Methods. Similar data were obtained in two other experiments.

CPA was replaced with vehicle. There was no observable increase in $[Ca^{2+}]_i$ during this experiment, indicating that the rise in $[Ca^{2+}]_i$ (after reapplying 2 mM $CaCl_2$) shown in Figure 5a is a result of calcium entry into the cell. However, as shown in Figure 5b, removing CPA from the receptor (with 100 nM DPCPX; applied 6 min before 2 mM $CaCl_2$) attenuates the rise in $[Ca^{2+}]_i$ observed when Ca^{2+} (2 mM) is re-applied after the cells have been stimulated with CPA (300 nM) in the absence of extracellular Ca^{2+}. These data suggest that the Ca^+ entry (influx) that occurs during the later phase of the response to CPA is dependent on the continued presence of CPA at the A_1-receptor.

Pertussis toxin sensitivity of the Ca^{2+} response

We performed experiments with pertussis toxin (PTX) to determine whether the two components of the adenosine A_1-receptor-mediated increase in $[Ca^{2+}]_i$, i.e. intracellular Ca^{2+} release and influx, are equally sensitive to inhibition by PTX. Previous studies with DDT_1MF-2 cells have demonstrated that adenosine receptor agonists are able to inhibit the formation of cyclic AMP induced by isoprenaline and that PTX pretreatment reversed these effects (Gerwins *et al.*, 1990). In our DDT_1MF-2 cells, the addition of CPA inhibited the accumulation of [^{3}H]-cyclic AMP induced by forskolin. CPA (300 nM) inhibited 93 ± 4% ($n = 3$) of the cyclic AMP accumulation induced by 10 μM forskolin, with an IC_{50} of 2.8 ± 0.5 nM ($n = 3$, see Figure 6). However, pretreatment of cells with PTX (200 ng ml^{-1} for 4 h) attenuated the ability of CPA to inhibit forskolin-induced [^{3}H]-cyclic AMP accumulation (Figure 6). PTX pretreatment (200 ng ml^{-1} for 4 h) completely abolished the CPA-induced release of intracellular Ca^{2+} and subsequent Ca^{2+} influx observed when Ca^{2+} is re-applied after the cells had been stimulated with CPA in the absence of extracellular Ca^{2+} (compare c and d in Figure 7). In contrast, PTX pretreatment did not attenuate histamine H_1-receptor stimulated intracellular Ca^{2+} release or Ca^{2+} influx (compare a and b in Figure 7). The maximum peak response to histamine (100 μM) obtained in Ca^{2+}-free buffer and upon re-addition of extracellular Ca^{2+} (2 mM) in PTX treated cells were 133 ± 2% ($n = 3$) and 96 ± 8% ($n = 3$) respectively, of those obtained with untreated cells. Furthermore, PTX pretreatment had no effect on bradykinin (100 nM) induced release of intracellular Ca^{2+} (95 ± 3% of the maximum response measured in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA; $n = 3$) or noradrenaline (100 μM) stimulated increases in $[Ca^{2+}]_i$ measured in the presence of extracellular Ca^{2+} (107 ± 5% of the maximum response; $n = 3$).

Discussion

Recent studies have shown that adenosine A_1-receptor activation in DDT_1MF-2 cells stimulates inositol phospholipid hydrolysis and calcium mobilization from intracellular stores (Gerwins & Fredholm, 1991; White *et al.*, 1992). However, other studies with DDT_1MF-2 cells (Hoiting *et al.*, 1990; Schachter & Wolfe, 1992; Schachter *et al.*, 1992) have suggested that adenosine A_1-receptor activation does not stimulate the hydrolysis of inositol phospholipids or mobilize intracellular calcium. Schachter *et al.* (1992) found that adenosine A_1-receptor activation potentiated the inositol phospholipid response elicited by noradrenaline. However, in the absence of noradrenaline adenosine A_1-receptor activation caused only a minor stimulation of phospholipase C, which was abolished in the absence of extracellular calcium. The reasons for these conflicting results remain to be established.

The data presented in this study clearly show that adenosine-receptor activation in monolayers of the hamster vas deferens derived smooth muscle cell line DDT_1MF-2 stimulates a rapid and fairly well maintained increase in $[Ca^{2+}]_i$. In the absence of extracellular Ca^{2+} ions (experiments performed in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA) the rise in $[Ca^{2+}]_i$ was transient in nature (see Figure 4a) and is probably due to the release of Ca^{2+} from intracellular stores. Furthermore, in our previous study (White *et al.*, 1992) we found that removal of extracellular Ca^{2+} had no significant effect on the initial Ca^{2+} peak elicited by 2-chloroadenosine (10 μM) compared with the response obtained in Ca^{2+}-containing buffer. This is in contrast to histamine-induced changes in $[Ca^{2+}]_i$ where the response in

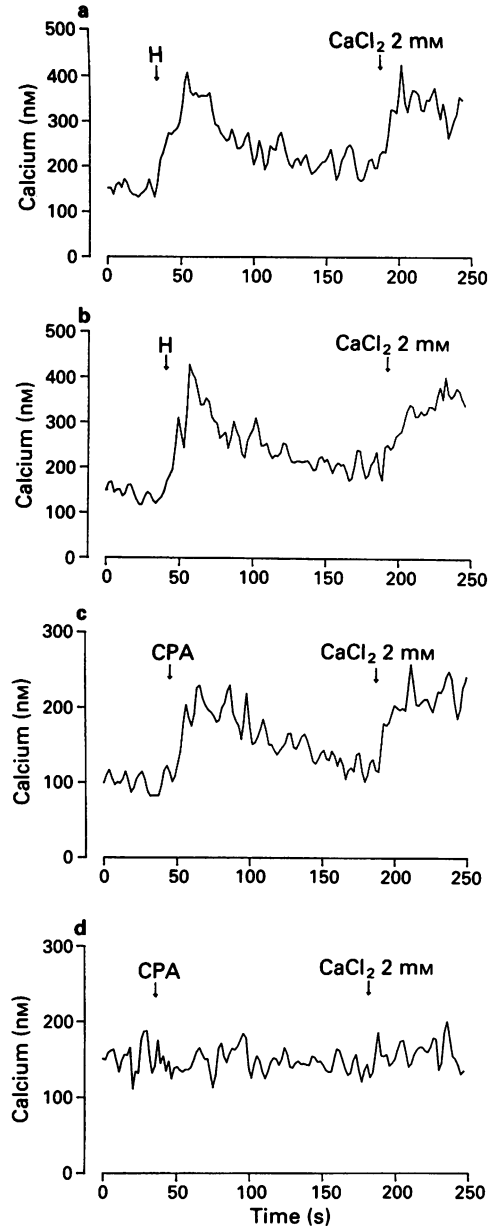


Figure 7 Effect of pertussis toxin (PTX) on histamine H_1-receptor and adenosine-receptor stimulated increases in intracellular Ca^{2+} concentration in DDT_1MF-2 cells. In all four experiments (a)–(d) the cells were initially exposed to the appropriate agonist in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA after which exogenous Ca^{2+} (2 mM) was re-applied. In experiments (b) and (d) cells were treated with PTX (200 ng ml^{-1}) for 4 h before measurement of $[Ca^{2+}]_i$. The control experiments for histamine (a) and adenosine-receptor (c) stimulation were obtained on the same experimental day.

Histamine (H; 100 μM), N^6-cyclopentyladenosine (CPA; 100 nM) or $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in two other experiments.

Ca^{2+}-free buffer is significantly lower than that obtained in Ca^{2+}-containing buffer (Dickenson & Hill, 1991; 1992).

We have shown conclusively that adenosine-receptor agonists can elicit increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (see Figures 4 and 5). However, there is some uncertainty as to whether the hydrolysis of inositol phospholipids (White *et al.*, 1992) and the mobilization of intracellular Ca^{2+} are a consequence of adenosine-receptors being directly coupled to phospholipase C via a G-protein or whether they are mediated via indirect mechanisms. For example, the inositol phospholipid response to CPA in DDT_1MF-2 cells is sensitive to inhibition by the 5-lipoxygenase inhibitor AA861 (White *et al.*, 1992). Thus, the calcium response may be secondary to the formation of leukotrienes. Alternatively, CPA may augment responses to endogenous noradrenaline or histamine (Schachter *et al.*, 1992). The data presented in Figure 4 clearly showed that in the absence of extracellular Ca^{2+} ions (Ca^{2+}-free buffer containing 0.1 mM EGTA) and in the presence of the 5-lipoxygenase inhibitor AA861 or the cyclo-oxygenase inhibitor indomethacin, CPA still elicited significant increases in $[Ca^{2+}]_i$. These data suggest that the adenosine-receptor mediated release of intracellular Ca^{2+} is not dependent upon the products of arachidonic acid metabolism. In addition, the inclusion of mepyramine or phentolamine to block histamine H_1-receptors and α-adrenoceptors respectively, did not attenuate the response to CPA, obtained in the absence of extracellular Ca^{2+} ions. These data eliminated the possibility that the rise in $[Ca^{2+}]_i$ is mediated via augmentation of histamine or noradrenaline mediated calcium responses.

The adenosine-receptor mediated increase in $[Ca^{2+}]_i$ comprises two distinct components: (1) release of Ca^{2+} from intracellular storage sites (mobilization), which is probably secondary to the production and action of inositol 1,4,5-trisphosphate (Berridge & Irvine, 1989), and (2) influx of extracellular Ca^{2+} through Ca^{2+} entry pathways in the plasma membrane. Furthermore, the influx of extracellular Ca^{2+} into the cytoplasm is dependent upon continued adenosine-receptor occupancy (receptor-mediated) since it is blocked by the A_1-receptor antagonist DPCPX (see Figure 5b). These data are similar to those obtained for the histamine H_1-receptor mediated increases in $[Ca^{2+}]_i$ in DDT_1MF-2 cells (Dickenson & Hill, 1992), although the relationship between adenosine-receptor activated Ca^{2+} influx and the refilling of intracellular Ca^{2+} stores remains to be established.

The rank order of agonist potencies (CPA>NECA>2-chloroadenosine>adenosine) is characteristic of the A_1-receptor. The involvement of the A_1 subtype was confirmed by the sensitivity of the adenosine-receptor mediated calcium response to inhibition by the A_1-selective antagonist DPCPX. The rise in $[Ca^{2+}]_i$ is inhibited by concentrations of DPCPX that are indicative of the adenosine A_1-receptor, i.e. K_D values in the order of 0.5 nM (Bruns *et al.*, 1987a).

There have been several reports in the literature of adenosine-receptor activation stimulating increases in $[Ca^{2+}]_i$. Transient and pertussis toxin (PTX) sensitive Ca^{2+} responses were demonstrated in rat mast cell line, RBL-2H3 and kidney epithelial cells (Arend *et al.*, 1988; 1989; Weinberg *et al.*, 1989; Ali *et al.*, 1990). The rank order of agonist potencies suggested that in RBL-2H3 cells the Ca^{2+} response was mediated via A_2-receptors. In contrast, the Ca^{2+} response obtained with cultured rat mesangial cells (Olivera *et al.*, 1992) was biphasic in appearance and comprised of an initial peak followed by a secondary increase which was smaller than the initial rise. In addition, the intracellular Ca^{2+} release was dependent upon extracellular Ca^{2+} since it did not occur in Ca^{2+}-free buffer. These data suggest that in other cell preparations the adenosine-receptor stimulated rise in $[Ca^{2+}]_i$ occurs either via A_2-receptors or requires the presence of extracellular Ca^{2+}. However, in DDT_1MF-2 cells the response is mediated via A_1-receptors and can occur in the absence of extracellular Ca^{2+} suggesting a coupling of the adenosine-receptor to phospholipase C.

The finding that pertussis toxin pretreatment inhibits the adenosine A_1-receptor induced rise in cytosolic Ca^{2+} (this study) and inositol phospholipid hydrolysis (White, T.E. & Hill, S.J; unpublished observations) is consistent with the involvement of a PTX sensitive G-protein in adenosine A_1-receptor-mediated events (Stiles, 1992). These data contrast with those obtained in guinea-pig uterine smooth muscle where adenosine A_1-receptor stimulation of inositol phospholipid hydrolysis is not blocked by PTX pretreatment (Schiemann *et al.*, 1991). In addition, the inositol phosphate

response in guinea-pig uterine smooth muscle is cyclo-oxygenase sensitive whereas, in DDT_1MF-2 cells indomethacin was without significant effect (White *et al.*, 1992).

Recent studies using cloned human 5-HT_{1A} receptors expressed at high concentrations in transfected HeLa cells have revealed that they are able to couple both negatively to adenylate cyclase (which is the normal effector system for 5-HT_{1A} receptors) and positively to phospholipase C (Boddeke *et al.*, 1992). In view of the high level of adenosine A_1-receptors expressed in DDT_1MF-2 cells (Gerwins *et al.*, 1990; Ramkumar *et al.*, 1990) it may be that in this particular cell line adenosine A_1-receptors can couple, via different G-proteins, both negatively to adenylate cyclase (as indicated by the ability of CPA to inhibit forskolin-induced [^{3}H]-cyclic AMP accumulation) and positively to phospholipase C (as indicated by CPA mediated inositol phospholipid hydrolysis and calcium mobilization). Further evidence for this proposal is provided by comparing the EC_{50} values obtained for CPA-induced inositol phospholipid hydrolysis (26 nM, White *et al.*, 1992) and calcium mobilization (19 nM) with the IC_{50} value for CPA inhibition of forskolin-induced [^{3}H]-cyclic AMP accumulation (2.8 nM). The difference between these EC_{50} and IC_{50} values may reflect the differing efficiences of the coupling of adenosine A_1-receptors to the two separate G-protein linked effector systems.

In summary, the present results demonstrate that in monolayers of DDT_1MF-2 cells adenosine-receptor agonists elicite increases in $[Ca^{2+}]_i$ which are mediated via the A_1-receptor subtype and are pertussis toxin sensitive. The Ca^{2+} response involves two components: (i) release of Ca^{2+} from intracellular stores, and (ii) Ca^{2+} influx into the cytoplasm which requires the continued presence of agonist on the receptor (receptor-mediated Ca^{2+} influx).

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Identification of a novel calcium antagonist binding site in rat brain by SR 33557

[1]Barry A. Kenny, Stuart Fraser & [2]Michael Spedding

Department of Pharmacology, Syntex Research Centre, Research Park, Riccarton, Edinburgh, EH14 4AP

1 In K^+-depolarized taenia preparations from guinea-pig caecum SR 33557 was a potent antagonist of Ca^{2+}-induced contractions and antagonized the effect of the calcium channel activator Bay K 8644.

2 SR 33557 displayed high affinity (pK_i 9.54 ± 0.04, *n*H 1.01) for the [^{3}H]-(±)-PN 200-110 binding site in rat cerebral cortex membranes. In the presence of 5 mM Ca^{2+} this affinity was reduced (pK_i 8.82 ± 0.01, *n*H 1.05) whilst the affinity of nitrendipine was unaffected by this concentration of Ca^{2+}.

3 Saturation binding experiments in rat cerebral cortex carried out in the absence and presence of SR 33557 (0.1–1.0 nM) indicated an apparently competitive interaction at the dihydropyridine site, in that SR 33557 reduced the K_D of [^{3}H]-(±)-PN 200-110 binding without any effect on B_{max}. In kinetic experiments, the rate of dissociation of [^{3}H]-(±)-PN 200-110 from rat cerebral cortex was unchanged in the presence of SR 33557 (5 nM).

4 D-cis-diltiazem fully reversed the inhibition [^{3}H]-nitrendipine binding to rat cerebral cortex produced by SR 33557 indicating the site of action of SR 33557 to be distinct from the dihydropyridine (DHP) binding site.

5 Saturation analysis indicated that [^{3}H]-SR 33557 (0.01–0.8 nM) labelled a single class of binding sites in rat cerebral cortex membranes with high affinity (K_D 0.12 ± 0.01, B_{max} 222 ± 20 fmol mg^{-1} protein), although kinetic data indicated the existence of negative cooperativity between the binding sites.

6 In competition studies, a variety of different calcium antagonists displayed similar affinity for [^{3}H]-SR 33557 and [^{3}H]-(±)-PN 200-110 sites. The [^{3}H]-SR 33557 site was sensitive to the inhibitory effect of divalent cations. The affinity of Cd^{2+} was 0.026 ± 0.015 mM and the rank order of affinity was $Cd^{2+} > Ca^{2+} > Mn^{2+} > Mg^{2+} > Na^+$.

7 We propose that SR 33557 labels a distinct site in rat cerebral cortex. The coupling between the SR 33557 and DHP site appears to be very close, resulting in apparently competitive interactions in some experimental protocols but can be revealed as negatively allosteric in other circumstances.

Keywords: SR 33557; ligand binding; dihydropyridines; L-type calcium channels

Introduction

It is now well established that at least three distinct sites exist on the L-type voltage sensitive calcium channel (Glossmann *et al.*, 1982; Glossmann & Ferry, 1985; Mir & Spedding, 1987) for the diverse group of agents classified as calcium antagonists (Fleckenstein, 1983). Distinct sites for dihydropyridines (DHPs), phenylalkylamines and benzothiazepines (chemically represented by the prototypical drugs nitrendipine, verapamil and diltiazem respectively) have been demonstrated in brain, skeletal muscle and cardiac tissue (Glossmann & Ferry, 1985; Qar *et al.*, 1987; Lazdunski *et al.*, 1988). Galizzi *et al.* (1986) first demonstrated a putative fourth binding site for fluspirilene, a diphenylbutylpiperidine molecule, in skeletal muscle T-tubule membranes, at a site distinct from the sites of action of other calcium antagonists. Similar high affinity binding sites for fluspirilene have also been demonstrated in cardiac sarcolemmal membranes with an interaction consistent with a distinct but allosterically coupled binding site (King *et al.*, 1989), although several studies have failed to identify this site in brain membranes (Qar *et al.*, 1987; Kenny *et al.*, 1990). Indeed, in skeletal muscle and cardiac membrane preparations at least five separate, mutually coupled binding sites for different chemical classes of calcium antagonist have now been demonstrated (for a review see Janis & Triggle, 1991). Although heterogeneous in nature, this group of compounds have all been shown to interact at distinct, high affinity, channel linked domains, each being reciprocally and allosterically coupled and residing on a 165–200 KDa polypeptide or α_1 subunit of the L channel (Striessnig *et al.*, 1987; Schmid *et al.*, 1989; Glossmann *et al.*, 1989). However, in brain membranes, only three definitive sites remain clearly established for DHPs, phenylalkylamines and benzothiazepines.

SR 33557, a novel indolizine sulfone calcium antagonist, also possesses high affinity for a site in skeletal muscle associated with the L-channel α_1 polypeptide (Schmid *et al.*, 1989). The compound is a potent antagonist of calcium-induced contractions in K^+-depolarized rat aorta and displays high affinity for DHP and phenylalkylamine sites in rat brain membranes (Nokin *et al.*, 1989). Recently, a novel high affinity site for [^{3}H]-SR 33557 has been demonstrated in rat heart sarcolemmal membranes (Chatelain *et al.*, 1991) with properties similar to those described in skeletal muscle (Schmid *et al.*, 1989; Sol-Rolland *et al.*, 1991). In this paper, we have fully characterized the interaction of SR 33557 with [^{3}H]-DHP sites in rat cerebral cortex and identified a site for [^{3}H]-SR 33557 which appears to be distinct but allosterically coupled to the DHP site. A preliminary account of some of these findings has been presented to the British Pharmacological Society (Kenny & Spedding, 1992).

Methods

K^+-depolarized smooth muscle

Strips of taenia (1–2 mm diameter, 1.5–2.0 cm relaxed length) from the caecum of female guinea-pigs (200–350 g)

[1] Author for correspondence at present address: Department of Discovery Biology, Pfizer Central Research, Sandwich, Kent, CT13 9NJ.
[2] Present address: Institut de Recherches Servier, 92150 Suresnes, Paris, France.

were set up in 30 ml isolated organ baths containing K^+-Tyrode solution which was gassed with 95% O_2:5% CO_2 and maintained at 35°C. The K^+-Tyrode solution had the following composition (mM): NaCl 97, KCl 40, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5. The preparations were washed in the Ca^{2+}-free, 40 mM K^+-Tyrode solution for 30 min, by which time they were fully relaxed. Contractile responses were measured under isotonic conditions (1 g load) with Harvard or Bioscience isotonic transducers connected to BBC Goetz SE460 potentiometric recorders. Cumulative concentration-response curves to Ca^{2+} (10–3000 μM) were obtained by increasing the Ca^{2+} concentration at 3–5 min intervals in logarithmic increments (Van Rossum, 1963). When concentration-response curves were repeated the curves were obtained at 30 min intervals. Antagonists were preincubated for 30 min. Concentration-ratios were calculated as the ratios of the concentrations of Ca^{2+} which produced a 50% maximal response (EC_{50}) in the presence and in the absence of the antagonists. In the experiments where Bay K 8644 was used the preparations were partially contracted with Ca^{2+} and Bay K 8644 added in a cumulative fashion. The increase in contraction caused by Bay K 8644 was calculated as a % increase over the tone established by the prior addition of Ca^{2+} (normally 25–45% of Ca^{2+} maximum).

[^{3}H]-dihydropyridine binding assays

Membranes were prepared from the cerebral cortex of male Sprague-Dawley rats by homogenization in ice-cold 50 mM Tris-HCl (pH 7.4 at 4°C) with a polytron (Kinematica PT 10) tissue disruptor at setting 8, by us of two 10 s bursts followed by centrifugation at 48,000 g_{av} at 4°C for 15 min. The resultant pellet was washed twice by resuspension and centrifugation and the final pellet was resuspended in 50 mM Tris-HCl (pH 7.4 at 4°C) at a protein concentration of 4–5 mg ml^{-1}. Membranes were stored in liquid nitrogen until required for assay. Aliquots of membrane homogenate (80–100 μg protein per assay) were incubated in 50 mM Tris-HCl (pH 7.4 at 25°C) with [^{3}H]-(±)-PN 200-110 in a total volume of 2.0 ml for 120 min at 25°C under subdued light. Non-specific binding was determined in the presence of 1 μM nitrendipine. Reactions were terminated by the addition of 5 ml ice-cold Tris-HCl and rapid filtration over Whatman GF/B filters, followed by two washes with ice cold buffer by use of a Brandel Cell harvester under a constant vacuum of 22 mmHg. Bound radioactivity was determined by liquid scintillation counting. In experiments with [^{3}H]-nitrendipine, incubations were carried out for 90 min and non-specific binding was determined in the presence of 1.0 μM nifedipine.

[^{3}H]-SR 33557 binding assay

Aliquots of Tris-washed membranes (prepared as above) were incubated with [^{3}H]-SR 33557 in buffer (50 mM Tris-HCl, pH 7.4 at 25°C, containing 0.1% bovine serum albumin) in a total volume of 1.0 ml. Non-specific binding was determined in the presence of 1 μM SR 33557. Incubations were carried out for 100 min under subdued light before filtration over Whatman GF/B filters as described for [^{3}H]-DHP binding. For [^{3}H]-SR 33557 experiments, filters were pretreated with 0.05% polyethyleneimine for at least 45 min. Failure to include bovine serum albumin in the assay media or pretreatment of the filters with polyethyleneimine resulted in low levels of specific binding.

Data analysis

Competition curves were generated by the use of at least ten concentrations of competing drug. Assay points were determined in duplicate and the resultant displacement isotherms were analysed by a non linear least square fitting program by a microcomputer as described by Michel & Whiting (1988). The K_i, where appropriate, was obtained from the equation of Cheng & Prussof (1973). Equilibrium saturation parameters (K_D and B_{max}) of assay points carried out in triplicate were determined by the non linear least square fitting program LIGAND (Munson & Rodbard, 1980). Protein concentration was determined by use of a Pierce BSA protein assay reagent kit with bovine serum albumin as standard.

In kinetic experiments the association rate constant (K_1) and dissociation constant (K_{-1}) were determined according to Weiland & Molinoff (1981).

Drugs

The following drugs were used: [^{3}H]-SR 33557 (2-isopropyl-1-((4- (3-N- methyl-N- (3, 4-dimethoxy-B-phenethyl) amino) propyloxy) benzenesulphonyl))-inolazine) was a gift from Sanofi (Bejar, Belgium), specific activity 83 Ci $mmol^{-1}$ (98% purity) and was stored at −18°C and protected from light. [^{3}H]-nitrendipine (86 Ci $mmol^{-1}$) and [^{3}H]-(+)-PN 200-110 (82 Ci $mmol^{-1}$) were obtained from DuPont. SR 33557 was a kind gift from Dr P. Chatelain, Sanofi (Bejar, Belgium). Lidoflazine, fluspirilene and flunarizine were obtained from Janssen (Beerse, Belgium), D-cis-diltiazem from Synthelabo (Paris, France), nifedipine, nitrendipine and Bay K 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) from Bayer AG (Leverkusen). Verapamil and D-600 (methoxyverapamil) were obtained from Sigma (U.K.). All other drugs and chemicals were obtained from Sigma (U.K.) and were of the highest purity available. All drugs were dissolved in ethanol or distilled water at a stock concentration of 1 mM and subsequent dilutions made in distilled water or assay buffer, ensuring that the concentration of assay ethanol never exceeded 0.1% vol. All inorganic ions were used as their chloride salts.

Results

Functional effects of SR 33557 in smooth muscle

SR 33557 (1–1000 nM) caused a concentration-dependent inhibition of the contractions induced by the addition of Ca^{2+} in K^+-depolarized taenia preparations from the guinea-pig caecum, with threshold antagonism of 1 nM (Figure 1a). Functional antagonism of the effects of Bay K 8644 has been shown to be a simple method for differentiating between subtypes of calcium antagonists (Spedding & Berg, 1984; Spedding, 1985b). Concentration-response curves to Bay K 8644 were constructed under constant depolarizing conditions (40 mM K^+) in preparations which are partly (25–45% of the maximal response) contracted to Ca^{2+} (0.1 mM). Under these conditions, SR 33557 1.0 nM ($n = 4$) caused a rightward shift and slight depression of the maximum response to Bay K 8644 (Figure 1b). Similarly, at concentrations of 10 and 100 nM ($n = 4$; experiments where the tissues were initially contracted with 0.3 and 3.0 mM Ca^{2+}) SR 33557 caused a rightward shift of the Bay K 8644 curve without depression of the maximum response, indicating a high affinity interaction of SR 33557 at the DHP site in K^+-depolarized smooth muscle.

Effects of SR 33557 on [^{3}H]-DHP binding parameters

The binding of [^{3}H]-(+)-PN 200-110 to rat cortical membranes at 25°C was saturable, reversible, and of high affinity. Iterative non linear analysis of the binding isotherms indicated a single class of high affinity sites with a K_D of 0.046 ± 0.006 nM and a B_{max} of 186 ± 5 fmol mg^{-1} protein. In competition experiments (Table 1) SR 33557 displayed high affinity for the [^{3}H]-(+)-PN 200-110 site (pK_i 9.54 ± 0.04, nH 0.97) and the Hill slope of unity was consistent with simple competitive interaction. In the presence of 5 mM Ca^{2+} the affinity of SR 33557 for the DHP site was reduced more than five fold (pK_i 8.82 ± 0.01, nH 1.05). The potency of nitren-

dipine was not affected by this concentration of Ca^{2+} (pK_i values of 9.48 and 9.53 respectively, $n = 2$).

Saturation binding experiments carried out with [^{3}H]-(+)-PN 200-110 in the presence and absence of SR 33557 (Figure 2) indicated that the compound caused a decrease in binding affinity (K_D) without any effect on binding capacity (B_{max}). Similar findings were obtained with the unlabelled DHP nifedipine (control values K_D 0.029 ± 0.008 nM, B_{max} 221 ± 20 fmol mg^{-1} protein, and in the presence of 5.0 nM nifedipine K_D 0.17 ± 0.02 nM, B_{max} 196 ± 6 fmol mg^{-1} protein). In both cases these results were indicative of a competitive interaction at the [^{3}H]-DHP site.

In order to characterize the interaction of SR 33557 further, the effect of the compound on [^{3}H]-(+)-PN 200-110 dissociation kinetics was examined. The concentration of SR 33557 employed in these experiments was more than 25

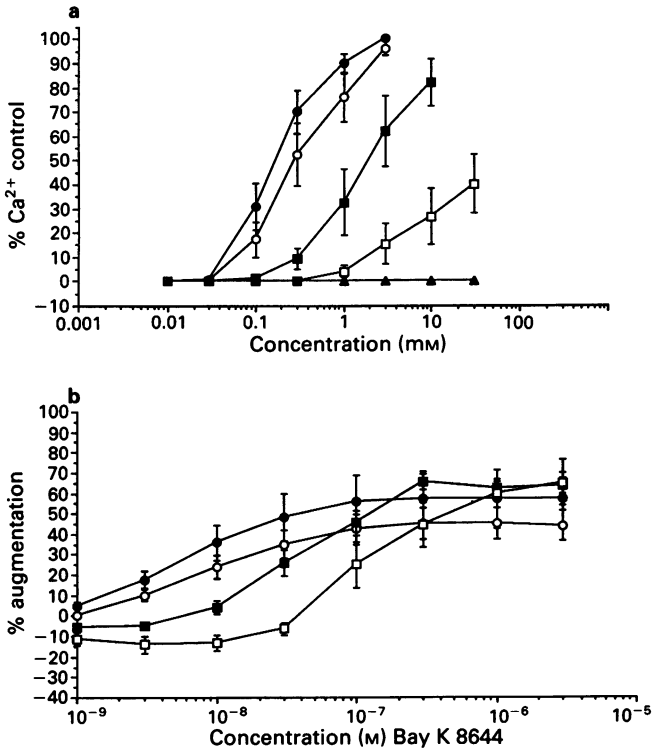


Figure 1 (a) Antagonism of the Ca^{2+}-induced contractions in K^+-depolarized taenia preparations from the guinea-pig caecum by SR 33557. Control (●) and in the presence of 1 nM (○), 10 nM (■), 100 nM (□) and 1 μM SR 33557 (▲). (b) Cumulative concentration-response curves to Bay K 8644 in K^+-depolarized taenia preparations from the guinea-pig caecum which were contracted to 24–45% of maximum with Ca^{2+} (0.1–3.0 mM). Control (●), and in the presence of SR 33557, 1 nM (○), 10 nM (■) and 1000 nM (□).

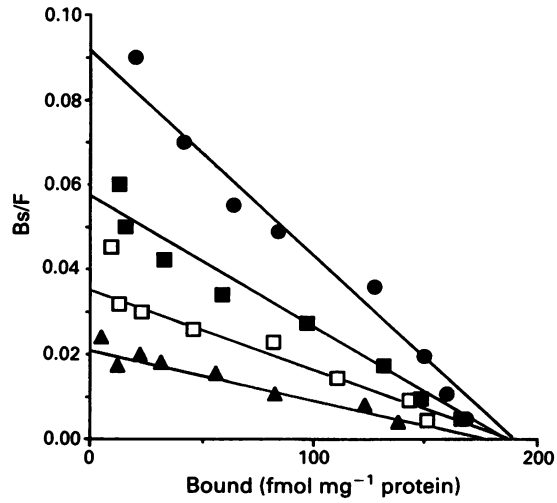


Figure 2 Scatchard transformation of [^{3}H]-(+)-PN 200-110 binding to rat cerebral cortex in the presence and absence of SR 33557. Control (●), $n = 4$, K_D 0.04 ± 0.006 nM, B_{max} 186 ± 5 fmol mg^{-1} protein; 0.1 nM SR 33557 (■) $n = 4$, K_D 0.09 ± 0.004 nM, B_{max} 192 ± 11 fmol mg^{-1} protein; 0.5 nM SR 33557 (□) $n = 4$, K_D 0.15 ± 0.02 nM, B_{max} 193 ± 16 fmol mg^{-1} protein, 1.0 nM SR 33557 (▲) $n = 4$, K_D 0.24 ± 0.03 nM, B_{max} 188 ± 12 fmol mg^{-1} protein.

Table 1 Comparative affinity values at the [^{3}H]-dihydropyridine and [^{3}H]-SR 33557 sites in rat cerebral cortex for calcium channel antagonists, activators and cations

A

	[^{3}H] SR 33557		*[^{3}H] PN 200-110*	
Compound	*pK_i*	*nH*	*pK_i or pIC_{50}*	*nH*
SR 33557	9.60 ± 0.10	0.82	9.54 ± 0.04	1.00
Nitrendipine	9.48 ± 0.14	0.90	9.67 ± 0.15	0.94
Bay K 8644	8.23 ± 0.17	0.87	8.43 ± 0.20	0.96
Fluspirilene	8.13 ± 0.17	0.87	7.69 ± 0.02	0.66*
D-600	8.09 ± 0.02	0.92	Not determined	
Verapamil	7.97 ± 0.03	0.98	7.69 ± 0.09	0.92 (*42)
D-*cis*-diltiazem	7.47 ± 0.09	0.93	7.58 ± 0.13	1.00 (*37)
Flunarizine	7.15 ± 0.14	1.06 (*92)	6.10 ± 0.20	1.28 (*92)
Lidoflazine	7.14 ± 0.11	0.82	6.44 ± 0.06	0.84 (*90)

B

Ion	*IC_{50}* (mM)
Cd^{2+}	0.026 ± 0.015
Ca^{2+}	2.09 ± 0.48
Mn^{2+}	5.01 ± 0.7
Mg^{2+}	11.06 ± 1.1
Na^{2+}	> 100

Values represent mean ± s.e.mean of 3–5 separate determinations. (A) Competition curves were generated by use of 12 concentrations of competing drug in duplicate. Experiments were performed as described in Methods with 0.03 nM [^{3}H]-(+)-PN 200-110 and 0.1 nM [^{3}H]-SR 33557. Values represent pK_i or *pIC_{50} where inhibition of binding is incomplete or the Hill slope (nH) was < 1.0 ( ) maximal inhibition at 10 μM.

(B) Affinity values (IC_{50}) for inorganic cations at the [^{3}H]-SR 33557 site. Ions were present as their chloride salts and values represent mean ± s.e.mean of 3 separate experiments.

fold greater than its K_i value for this site. Figure 3 illustrates that the rate of dissociation of [^{3}H]-(+)-PN 200-110 from the DHP binding site at equilibrium was unaffected in the presence of SR 33557. This was further evidence for an apparently competitive interaction with SR 33557.

Comparative reversal of calcium antagonism by D-cis-*diltiazem*

When [^{3}H]-nitrendipine was employed as a radioligand in rat cerebral cortex at 25°C, D-*cis*-diltiazem caused a concentration-dependent enhancement of specific binding through a positive allosteric mechanism (Figure 4). In this series of experiments we examined the ability of D-*cis*-diltiazem to reverse the inhibition of [^{3}H]-nitrendipine binding produced by nifedipine, SR 33557 and flunarizine. Figure 4 illustrates that in the presence of 0.1–2.0 nM SR 33557 ($n = 4$), D-*cis*-diltiazem fully reversed the inhibition of [^{3}H]-nitrendipine binding. This effect was dependent on the concentration of SR 33557, with diltiazem reversal curves shifted to the right with increasing concentrations of SR 33557. The observation that SR 33557 did not block the positive allosteric interaction of D-*cis*-diltiazem indicated that the interation of SR 33557 was not mediated through the DHP site. Thus, D-*cis*diltiazem was unable to exert a positive allosteric effect in the presence of the competitive dihydropyridine antagonist nifedipine (2 nM, $n = 3$) with this experimental protocol. In contrast to the full reversal of SR 33557, D-*cis*-diltiazem only partially reversed the inhibitory effects of flunarizine (Figure 4; 0.1–2.0 μM, $n = 3$). Furthermore, the extent of this reversal was similar at all flunarizine concentrations, irrespective of the amount of [^{3}H]-nitrendipine initially displaced.

[^{3}H]-SR 33557 binding to rat cerebral cortex membranes

Saturation experiments carried out with [^{3}H]-SR 33557 over the concentration range 0.01–0.8 nM indicated that the compound labelled a single population of binding sites in rat cerebral cortex with high affinity (B_{max} 222 ± 20 fmol mg^{-1} protein, K_D 0.12 ± 0.01 nM). Specific binding was saturable and reversible, and at a concentration equivalent to the K_D represented 65–70% of the total binding. However, kinetic studies of [^{3}H]-SR 33557 binding to cerebral cortical membranes illustrated that when dissociation was initiated from equilibrium by the addition of excess SR 33557 the resulting dissociation rate was rapid (K_{-1}, 0.052 ± 0.014 min^{-1}, $n = 4$) in comparison to the observed pseudo first order association rate (K_{obs} 0.013 ± 0.001 min^{-1}, $n = 4$) thus making a kinetic derivation of K_D impossible under this experimental protocol since

$$K_D = \frac{K_{-1}}{K_1}$$

where $K_1 = (K_{obs} - K_{-1})/[L]$, when [L] represents the concentration of radioligand employed. These data indicated the presence of complex binding phenomena for [^{3}H]-SR 33557, since on the basis of the data obtained above, specific binding would not be observed if true $K_{-1} >> K_{obs}$. However, when the dissociation rate of [^{3}H]-SR 33557 was monitored under conditions where dissociation was initiated from equilibrium by infinite dilution, a slow rate of dissociation was observed (0.005 ± 0.001 min^{-1}, $n = 3$; Figure 5). Furthermore, when dissociation was initiated by infinite dilution in the presence of excess unlabelled SR 33557, a fast curvilinear dissociation rate was observed. These results suggested the existence of negative co-operativity between the sites, since occupation of the sites with unlabelled ligand would reduce the overall affinity of the binding site population, thus resulting in an increased dissociation rate.

Pharmacological specificity of [^{3}H]-SR 33557 binding

Competition studies carried out with a range of calcium antagonists against [^{3}H]-(+)-PN 200-110 and [^{3}H]-SR 33557

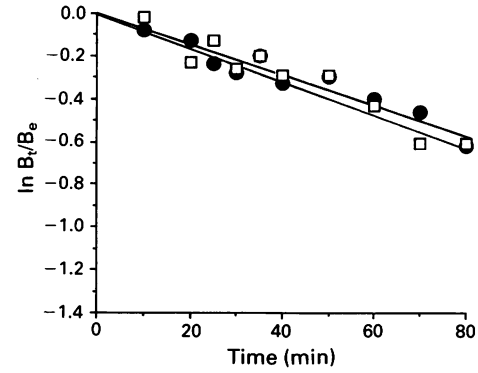


Figure 3 The effect of SR 33557 on the rate of [^{3}H]-(+)-PN 200-110 dissociation from rat cerebral cortex at 25°C initiated by 1 μM nitrendipine alone (control, ●) or in the presence of 5 nM SR 33557. Mean data (± s.e.mean) of 3 separate experiments performed in duplicate: Control, 0.007 ± 0.0009 min^{-1}, and in the presence of 5 nM SR 33557, 0.006 ± 0.001 min^{-1}.

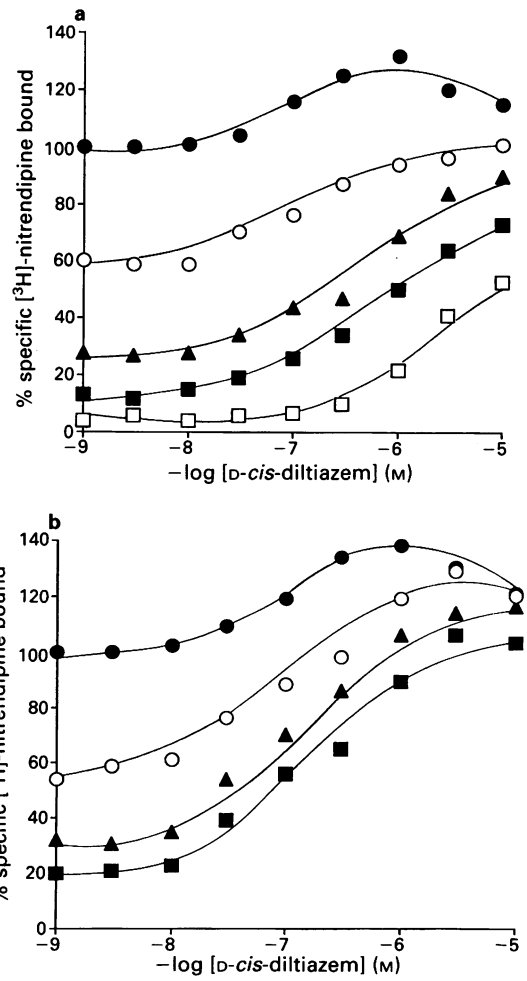


Figure 4 Comparative effect of D-*cis*-diltiazem on the inhibition of [^{3}H]-nitrendipine by (a) flunarizine and (b) SR 33557. Rat cerebral cortex membranes were incubated with 0.2 nM [^{3}H]-nitrendipine with D-*cis*-diltiazem alone (●), control and in the presence of (a) flunarizine, (○) 0.1 μM, (▲) 0.5 μM, (■) 1.0 μM and (□) 2.0 μM or (b) SR 33557 (○) 0.1 nM, (▲) 0.5 nM and (■) 2.0 nM. Representative experiments of 3 others are shown.

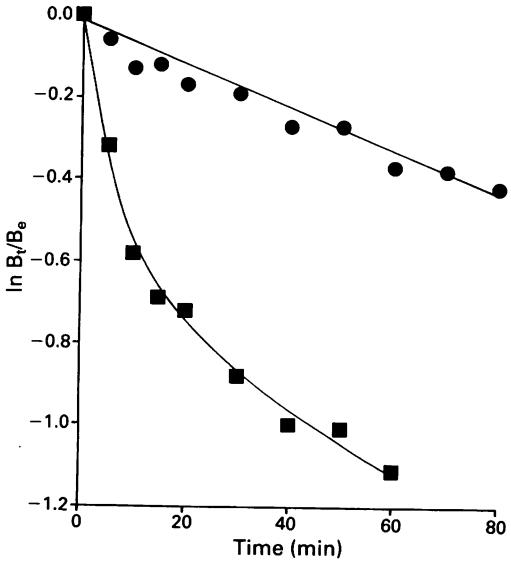


Figure 5 Dissociation of [^{3}H]-(+)-PN 200-110 from rat cerebral cortex membranes at 25°C by infinite dilution (●). Dissociation was achieved by dilution of pre-equilibrated 30 ml total and non-specific assay mixtures (containing 0.1 nM [^{3}H]-SR 33557) into 3 litres of an identical assay buffer and the specific binding of 50 ml aliquots determined at the indicated time points. A parallel experiment was performed with the dilution buffer containing 1 μM unlabelled SR 33557 (■).

binding in rat cerebral cortex are shown in Table 1a. SR 33557 displayed high and similar affinity for both sites, although the lower Hill slope at the [^{3}H]-SR 33557 site may be a reflection of the negative cooperativity confirmed in kinetic experiments. Both dihydropyridine agonists and antagonists showed high affinity for the [^{3}H]-SR 33557 site and the rank order of potency and affinity of other compounds including benzothiazepines, phenylalkylamines, and diphenylbutylpiperidines was essentially the same for both sites, although several agents such as fluspirilene, flunarizine and lidoflazine displayed higher affinity for the [^{3}H]-SR 33557 site. The allosteric interactions whereby D-*cis*-diltiazem and verapamil inhibited [^{3}H]-(+)-PN 200-110 binding incompletely was not seen at the [^{3}H]-SR 33557 site. A positive allosteric effect of diltiazem (comparative to its effects against [^{3}H]-nitrendipine) at the [^{3}H]-SR 33557 binding site was not observed, and in all cases (with the exception of flunarizine) competing agents displaced specific binding maximally.

Effect of inorganic cations

In contrast to the lack of effect on [^{3}H]-DHP binding (the presence of low concentrations of divalent cations in washed membranes being a requisite for high affinity binding), all the divalent cations tested appeared to inhibit [^{3}H]-SR 33557 binding in a concentration-dependent manner. The rank order of potency was $Cd^{2+} > Ca^{2+} > Mn^{2+} > Mg^{2>}$ (Table 1b). The monovalent cation Na^+ was inactive at concentrations up to 100 mM.

Discussion

In this paper we have demonstrated a high affinity interaction of SR 33557 to a novel site in rat cerebral cortex which appears to be distinct from, but tightly coupled to the DHP site. In functional experiments, SR 33557 was a highly potent calcium antagonist in K^+-depolarized smooth muscle and the interaction resembled those of the dihydropyridines in that Ca^{2+}-concentration curves were displaced to the right without depression of the maximum response until the solubility of the Ca^{2+} in the K^+-Tyrode solution was exceeded (10–30 mM, Spedding, 1982). Furthermore, SR 33557 was a potent antagonist of the effects of Bay K 8644. DHPs have been shown to antagonize competitively the effects of Bay K 8644, whereas verapamil and diltiazem non-competitively antagonize contractile effects and diphenylpiperidines are highly potent antagonists which depress the maximum response (Spedding & Berg, 1984; Spedding, 1985a,b). SR 33557 appeared to interact with the DHP site in functional tests and the effects did not appear to resemble the effects of verapamil, diltiazem or diphenylalkylamines. A full analysis of the interaction of SR 33557 with the DHP site was therefore performed by use of radioligand binding techniques.

Schmid *et al.* (1989) first demonstrated specific binding of [^{3}H]-SR 33557 to skeletal muscle membranes and a variety of studies in cardiac and skeletal muscle membranes have confirmed a distinct binding site associated with the α_1 subunit of the channel which appears to be allosterically coupled to other calcium antagonist sites (Chatelain *et al.*, 1991; Sol-Rolland *et al.*, 1991). On the basis of the interaction at the [^{3}H]-(+)-PN 200-110 binding site in rat cortex, the site for SR 33557 and DHPs appeared to be very tightly coupled, to the extent that the interaction of SR 33557 at the [^{3}H]-(+)-PN 200-110 site appeared to be competitive under several experimental approaches. This is consistent with a previous report in which the only suggestive evidence for an allosteric interaction was obtained with SR 33557 when the compound was found to increase the rate of [^{3}H]-DHP dissociation at 10 μM (Nokin *et al.*, 1989), a concentration inappropriate relative to its nM K_i for the DHP site. Furthermore, in competition experiments the rank order and affinity for a range of different calcium antagonists was similar to the affinities of calcium antagonists for the DHP site. The affinities obtained in the present work are also similar to those found at the [^{3}H]-SR 33557 site in skeletal muscle (Schmid *et al.*, 1989; Sol-Rolland *et al.*, 1991) and cardiac sarcolemmal membranes (Chatelain *et al.*, 1991). In cardiac and skeletal muscle membranes SR 33557 has affinities at [^{3}H]-verapamil, [^{3}H]-diltiazem and [^{3}H]-fluspirilene sites comparable to the homologous compounds indicating mutually high affinity for other calcium antagonist sites (Schmid *et al.*, 1989). In brain tissue, however, the affinity displayed by verapamil and diltiazem was similiar for both [^{3}H]-DHP and [^{3}H]-SR 33557 sites indicating that the site which [^{3}H]-SR 33557 labelled with low nM affinity did not appear to correspond to the phenylalkylamine or benzothiazepine site. The maximal inhibition of binding observed with both diltiazem and verapamil indicated a possible difference in the interaction of these compounds at the [^{3}H]-SR 33557 site in comparison to their positive and negative allosteric regulation of [^{3}H]-DHP binding. However, due to the negative cooperativity amongst [^{3}H]-SR 33557 binding sites, it was not possible to examine the interaction of other classes of calcium antagonist at this site in kinetic experiments. Indeed, we also detected the appearance of non linear Scatchard transformations of [^{3}H]-SR 33557 saturation binding when the ligand was employed over a wide (0.002– 2.0 nM) concentration range. This also precluded an examination of competing calcium antagonists in saturation shift experiments.

Nevertheless, the observation that the inhibition of [^{3}H]-nitrendipine binding by SR 33557 could be fully reversed by D-*cis*-diltiazem was evidence for a distinct site of action for SR 33557. This allosteric interaction has been used to differentiate the site of action of several calcium antagonists such as prenylamine (Murphy *et al.*, 1983), amiodarone (Nokin *et al.*, 1986) and fluspirilene (Qar *et al.*, 1987). However, in these previous studies where diltiazem has been used to reverse the inhibition of [^{3}H]-DHP binding, reversal curves have been generated only in the presence of one concentration of calcium antagonist. In this respect, whilst we found the inhibitory effect of flunarizine on [^{3}H]-nitrendipine binding to be partially reversed, the efficacy of diltiazem was

different in comparison to the reversal with SR 33557 when both drugs were employed at several different concentrations. In the case of flunarizine, the degree of reversal produced by diltiazem was similar to all concentrations, irrespective of the amount of [^{3}H]-nitrendipine binding initially displaced. In contrast, the inhibition of [^{3}H]-nitrendipine binding by SR 33557 was reversed by diltiazem in a surmountable fashion, producing a rightward shift in the diltiazem reversal curves. This latter effect was an accurate reflection of the allosteric interaction of diltiazem, as the extent of the reversal is likely to be dependent on the degree of allosteric control from other mutually coupled sites. In the case of flunarizine, the relatively weak effect of diltiazem may reflect membrane interactions unrelated to specific interactions for [^{3}H]-nitrendipine and other allosterically coupled sites. This would not be surprising given the relative weakness of flunarizine at [^{3}H]-DHP sites, its poor selectivity for L-type channel (Spedding, 1985a; Tytgat *et al.*, 1988), and extremely high concentrations present in the membrane due to its high partition coefficient (Mason *et al.*, 1991). The membrane concentration arising from aqueous concentrations around 1 μM (likely to be nearer 1 mM in the membrane) would give rise to many non-selective effects.

The site labelled in rat cerebral cortex by [^{3}H]-SR 33557 was found to be susceptible to the inhibitory effects of divalent cations. In this respect, the rank order and affinity of a variety of divalent cations closely resembled that found at other [^{3}H]-SR 33557 binding sites (Chatelain *et al.*, 1991), with Cd^{2+} displaying the highest affinity. Similar inhibitory properties of divalent cations have been demonstrated at verapamil and diltiazem sites (Galizzi *et al.*, 1985; Balwierczak & Schwartz, 1985) in contrast to their requirement for high affinity DHP binding. Thus, the decreased affinity of SR 33557 at the [^{3}H]-(+)-PN 200-110 site in rat cortical membranes probably reflects an ionic effect at the SR 33557 recognition site. Interestingly, the inhibitory effect of Cd^{2+} at the SR 33557 site is in contrast to the marked enhancement of [^{3}H]-fluspirilene binding which occurs in the presence of this ion (Garcia *et al.*, 1990) providing further evidence of ligand specific variation in the ionic regulation of calcium antagonist binding interactions.

Several lines of evidence now indicate the distribution and coupling between the various sites for calcium antagonists to exist in a 1:1 stoichometrical arrangement (King *et al.*, 1989). Whilst in the present experiments we found the number of [^{3}H]-SR 33557 and DHP sites to be similar in rat cortical membranes, we have previously suggested that specific sites for other ligands such as fluspiriline appear to be absent in this membrane preparation. Indeed, the present paper represents the first pharmacological characterization of a specific, high affinity binding site in rat cortical membranes for a calcium antagonist other than a prototypical DHP, phenylalkylamine or benzothiazepine. However, the coupling with the DHP site appears to be very tight resulting in apparently competitive interactions under several experimental approaches, although these can be revealed as being allosteric under certain defined conditions. Nevertheless, these data do support the proposal that SR 33557 binds to a distinct site associated with the L-type calcium channel.

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Selective inhibition of agonist-induced but not shear stress-dependent release of endothelial autacoids by thapsigargin

Heather Macarthur, [1]*Markus Hecker, *Rudi Busse & John R. Vane

The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ and *Department of Applied Physiology, University of Freiburg, Hermann-Herder-Strasse 7, D-7800 Freiburg, Germany

1 The effects of the Ca^{2+}-ATPase inhibitor, thapsigargin, on the shear stress-dependent and on the agonist-stimulated release of endothelium-derived relaxing factor, i.e. nitric oxide (NO), and prostacyclin (PGI_2) were studied in bovine and human cultured endothelial cells as well as in endothelium-intact arterial segments of the rabbit.

2 Preincubation with thapsigargin (1 μM for 10 min) had no effect on the shear stress-dependent release of NO from bovine aortic endothelial cells grown on beads, but abolished the release of NO induced by ADP, bradykinin, ionomycin or poly-L-lysine. Similarly, thapsigargin completely abrogated the agonist-stimulated PGI_2 release from these cells, but had no effect on the shear stress-dependent release of PGI_2.

3 The acetylcholine-induced release of NO from the luminally perfused thoracic aorta and femoral artery of the rabbit was suppressed by pretreatment with thapsigargin (1 μM). In contrast, thapsigargin did not affect the shear stress-dependent release of NO from the femoral artery.

4 Administration of thapsigargin to these vascular preparations or to cultured endothelial cells alone produced a substantial release of both NO and PGI_2. This release declined towards previous values after washout of thapsigargin.

5 In human and bovine cultured endothelial cells, thapsigargin (1–1000 nM) caused a dose-dependent sustained rise in $[Ca^{2+}]_i$, an effect that was abolished in the absence of extracellular Ca^{2+}. Stimulation of these cells with bradykinin, histamine, ADP or ionomycin after previous exposure to thapsigargin (30–1000 nM) no longer caused an increase in $[Ca^{2+}]_i$.

6 These findings demonstrate that by emptying intracellular Ca^{2+} stores, thapsigargin selectively blocks the agonist-stimulated release of both NO and PGI_2, suggesting a principal difference in the control by $[Ca^{2+}]_i$ of the release of these endothelial autacoids caused by shear stress or receptor-dependent and independent agonists.

Keywords: Vascular endothelium; nitric oxide; prostacyclin; endothelium-derived relaxing factor; thapsigargin; calcium signalling; shear stress

Introduction

In both conduit and resistance-sized arteries, even after deduction of neurogenic, metabolic as well as hormonal influences, a sizeable component of vascular tone persists which is commonly referred to as basal tone. This component of vascular tone is modulated and presumably controlled by two physical forces, the distending transmural pressure and fluid shear stress, resulting from the movement of viscous blood along the endothelial surface. While an increase in transmural pressure is balanced by viscoelastic forces of the media and adventitia, the fluid shear stress only affects the endothelium. An increase in fluid shear stress is thought to be responsible for the phenomenon of flow-induced endothelium-dependent dilatation (Davies, 1989), which is crucial for the maintenance of an adequate oxygen supply to the tissue(s).

Flow-induced endothelium-dependent dilatations were first recognized in conduit arteries (Smiesko *et al.*, 1985; Pohl *et al.*, 1986a; Melkumyants *et al.*, 1987), and since then have also been documented in resistance-sized arteries (Griffith *et al.*, 1987; Koller & Kaley, 1990a). These endothelium-dependent dilatations are generally considered to be mediated by an enhanced release of endothelium-derived relaxing factor (EDRF; for reference see Hutcheson & Griffith, 1991), the active principle of which has been identified as nitric oxide (NO; Palmer *et al.*, 1987), and additionally, in some vascular regions, by prostacylin (PGI_2; Pohl *et al.*, 1986b; Koller & Kaley, 1990b). However, little is known about the precise mechanism coupling the increase in blood flow to the enhanced formation and release of these autacoids.

It is now well established that increases in $[Ca^{2+}]_i$ of vascular endothelial cells produced by physiological agonists such as ATP, bradykinin or histamine cause the release of EDRF (from here on referred to as NO) and PGI_2 (for references see Newby & Henderson, 1990). This effect occurs via activation of specific cell surface receptors and subsequent stimulation of the phosphoinositol pathway, and can be maintained by an influx of extracellular Ca^{2+}, presumably through non-selective cation channels (Olesen *et al.*, 1988; Hallam *et al.*, 1989).

Removal of extracellular Ca^{2+} inhibits the formation of NO, but not that of PGI_2, stimulated by both shear stress and receptor-dependent and independent agonists (Griffith *et al.*, 1986; Lückhoff *et al.*, 1988; White & Martin, 1989; Hecker *et al.*, 1992). Whereas the inhibition of the agonist-stimulated release of NO usually progresses with subsequent challenges by the agonist until a full blockade is reached, the inhibition of the shear stress-dependent component is more immediate but often incomplete (Hecker *et al.*, 1992). Moreover, conflicting data have been reported on intracellular Ca^{2+} levels in cultured endothelial cells subjected to increased fluid flow (Ando *et al.*, 1988; Dull & Davies, 1991; Mo *et al.*, 1991; Geiger *et al.*, 1992; Schwartz *et al.*, 1992; Shen *et al.*, 1992). Thus, a definite role of $[Ca^{2+}]_i$ in the shear stress-induced release of NO remains to be established.

Sulfhydryl reagents, such as N-ethylmaleimide and 2,2'-dithiodipyridine, inhibit the agonist-stimulated but not the shear stress-induced formation of both NO and PGI_2 by bovine cultured aortic endothelial cells, presumably by interfering with the mobilization of $[Ca^{2+}]_i$ (Hecker *et al.*, 1992). We have now examined this potential regulatory difference further by using thapsigargin, the specific endoplasmic reti-

[1] Author for correspondence.

culum Ca^{2+}-ATPase inhibitor, and have extended our studies to both native and cultured endothelial cells from different species. Thapsigargin, a non-phorbol ester-type tumour promoter, acts by irreversibly inhibiting the uptake of Ca^{2+} into inositol-1,4,5-trisphosphate ($InsP_3$)-sensitive and insensitive Ca^{2+} stores, thus preventing their refilling during or after hormonal stimulation (Thastrup *et al.*, 1990; Dolor *et al.*, 1992). Here, we demonstrate that thapsigargin selectively inhibits the agonist-stimulated but not the shear stress-dependent release of NO and PGI_2 from cultured as well as that of NO from native endothelial cells, suggesting than an $InsP_3$-mediated mobilization of intracellular $[Ca^{2+}]_i$ is responsible for the agonist-stimulated release of NO and PGI_2, but does not play a role in the shear stress-dependent release of these autacoids from the vascular endothelium.

Methods

Cell culture

Human umbilical vein endothelial cells were isolated from umbilical cords and grown to confluence on coverslips in M-199 culture medium (Gibco BRL, Eggenstein, Germany) as described previously (Busse & Lamontagne, 1991). Bovine aortic endothelial cells were isolated and grown to confluence in Dulbecco's modified Eagle's medium (Flow Laboratories, Irvine, UK) on Cytodex-3 microcarrier beads (Pharmacia-LKB, Milton Keynes, UK) or in T75 culture flasks as described by de Nucci *et al.* (1988).

Measurement of intracellular Ca^{2+}

Confluent human endothelial cells on coverslips or bovine endothelial cells in suspension were loaded with the fluorescence Ca^{2+} indicator dyes Indo-1/AM or Fura-2/AM, respectively, and changes in $[Ca^{2+}]_i$ were monitored as described previously (Busse & Lamontagne, 1991; Hecker *et al.*, 1992).

Determination of NO release from cultured endothelial cells by bioassay

Approximately 6×10^7 bovine aortic endothelial cells on beads were packed into a jacketed chromatography column and superfused at 5 ml × min^{-1} with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs solution (composition mM: Na^+ 143.0, K^+ 5.9, Cl^- 127.7, Ca^{2+} 2.5, Mg^{2+} 1.2, HPO_4^{2-} 1.2, HCO_3^- 25.0, glucose 5.6), pH 7.4. The effluent from the column superfused a rabbit aortic ring with the endothelium removed which was equilibrated for 60 min under a resting tension of 2 g. Thereafter, the detector tissue was constricted further to 3–4 g of tension with 10 nM U46619 (11α,9α-epoxymethano-5*Z*,13*E*-prostaglandin $F_{2\alpha}$). Changes in tension were measured with a force displacement transducer (type 351 from Hugo Sachs Elektronik, March, Germany). Glyceryl trinitrate (GTN; 45 or 90 pmol) was given as a bolus over the detector tissue (o.t.) to calibrate its relaxant responses. Superoxide dismutase (10 u ml^{-1}) was infused through the column of endothelial cells (t.c.) to detect flow- and agonist-induced NO release. The agonists were first given o.t. to assess their effect on the bioassay preparation and then t.c. to stimulate NO release. In separate experiments, the cells were stimulated before and after a 10 min infusion t.c. of thapsigargin (1 μM), haemoglobin (10 μM) or N^G-nitro-L-arginine methyl ester (100 μM). The same bioassay technique was also applied to monitor the release of NO from the luminally perfused rabbit aorta.

Determination of PGI_2 release

The effluent from the endothelial cell column was collected after passing over the detector ring and analyzed by a specific radioimmunoassay for 6-keto-$PGF_{1\alpha}$ (Salmon, 1978), the stable hydrolysis product of PGI_2, and values are expressed as ng 6-keto-$PGF_{1\alpha}$ min^{-1}.

Preparation of arterial segments and measurement of NO release

Endothelium-intact segments of rabbit femoral arteries (8–12 mm in length) were cleaned of adventitial connective tissue, cannulated at both ends and mounted in a heated organ bath (37°C). The lumen of the segments and the organ bath were perfused separately (organ bath: 30 ml h^{-1}; lumen: 10.4 ml h^{-1}) with oxygenated (organ bath: 95% O_2/5% CO_2, Po_2 > 300 mmHg; lumen: 20% O_2/75% N_2/5% CO_2, Po_2 = 140 mmHg) Tyrode solution (composition mM: Na^+ 144.3, K^+ 4.0, Cl^- 138.6, Ca^{2+} 1.7, Mg^{2+} 1.0, HPO_4^{2-} 0.4, HCO_3^- 11.9, glucose 10.0), pH 7.4. The fluid shear stress at the luminal surface of the endothelium was increased by reducing the vessel diameter with endothelin-1 (60 nM) while maintaining the same luminal flow rate. Activation of soluble guanylyl cyclase was used to assay luminally released NO. The effluent from the perfused segments was collected for 15 s into a reaction mixture containing [α-^{32}P]-GTP and purified guanylyl cyclase. Labelled cyclic GMP formed was determined and enzyme activity (expressed as nmol mg^{-1} min^{-1}) calculated as described previously (Jackson *et al.*, 1991).

The same technique was also applied to determine the release of NO from human umbilical vein endothelial cells on coverslips superfused in a circular perfusion chamber (Bachofer) with warmed (37°C), HEPES-buffered (10 mM) Tyrode solution. Shear stress was enhanced by increasing the flow rate from 5.2 ml h^{-1} to 20.8 ml h^{-1}.

Materials

Indo-1/AM was purchased from Boehringer (Mannheim, Germany), Fura-2/AM and ionomycin were from Calbiochem Novabiochem Ltd (Nottingham, UK), and endothelin-1 from Peninsula Laboratories (Belmont, U.S.A.), respectively. ADP, bradykinin, haemoglobin, 6-keto-prostaglandin $F_{1\alpha}$ antiserum, N^G-nitro-L-arginine methyl ester, poly-L-lysine (degree of polymerization 254), superoxide dismutase, and thapsigargin were obtained from Sigma Chemical Co (Poole, U.K.). 6-Keto-prostaglandin $F_{1\alpha}$ was purchased from Cascade Biochem Ltd (Reading, U.K.), glyceryl trinitrate was from Lipha Pharmaceuticals Ltd (West Drayton, U.K.), and U46619 was a generous gift from Dr J. Pike (Upjohn Co, Kalamazoo, U.S.A.). [^{3}H]-6-keto-prostaglandin $F_{1\alpha}$ was obtained from Amersham International (Amersham, U.K.) and [α-^{32}P]-GTP from Amersham Buchler (Braunschweig, Germany).

Statistical analysis

Unless indicated otherwise, all data in the figures and text are expressed as mean ± s.e.mean. Statistical evaluation was performed by Student's *t* test or one-way analysis of variance (ANOVA; followed by a Bonferroni *t* test), where appropriate, with a P value < 0.05 considered statistically significant.

Results

NO release from cultured endothelial cells

Infusion of superoxide dismutase (SOD; 10 u ml^{-1}) through the column of bovine aortic endothelial cells on beads (t.c.) caused an average relaxation of 17 ± 3% ($n = 6$; Figures 1 and 2), which was abolished by infusions of the NO scavenger haemoglobin (10 μM; $n = 3$) as well as the NO biosynthesis inhibitor N^G-nitro-L-arginine methyl ester (Moore *et al.*, 1989; 100 μM; $n = 3$), demonstrating the involvement of NO. This relaxant response was defined as shear stress-

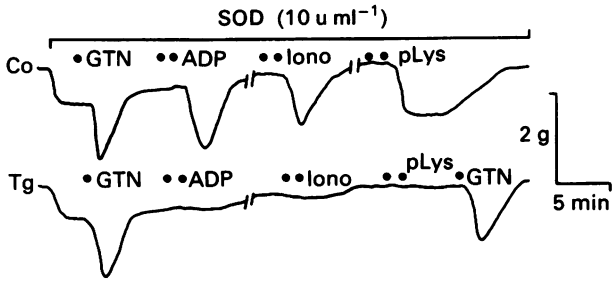


Figure 1 Effects of thapsigargin on shear stress-dependent and agonist-stimulated NO release from bovine aortic endothelial cells. The figure depicts a typical bioassay trace from 6 individual experiments with different batches of cultured endothelial cells. Shown are the relaxant responses of the detector tissue to infusion of superoxide dismutase (SOD; 10 u ml^{-1}) through the column of endothelial cells (t.c.) and bolus injections of ADP (9 nmol), ionomycin (Iono; 60 pmol) or poly-L-lysine (pLys; 550 pmol) over the tissue (o.t.; one circle) or t.c. (two circles) before (Co; top trace) and after (Tg; bottom trace) a 10 min infusion t.c. of thapsigargin (1 μM) followed by a 5 min washout period. Glyceryl trinitrate (GTN; 90 pmol) was given as a bolus injection o.t. to monitor changes in the sensitivity of the detector tissue. Note the lack of effect of thapsigargin on the relaxant response to infusion of SOD, i.e. shear stress-dependent NO release.

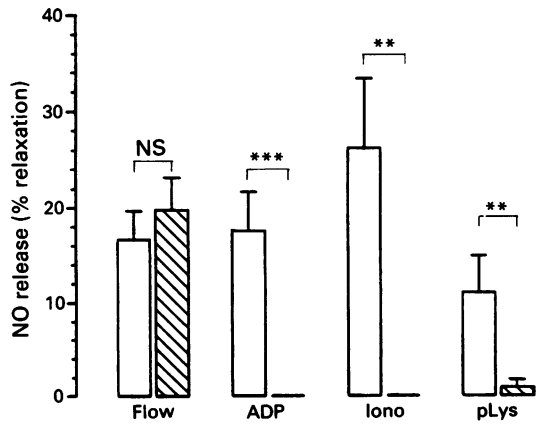


Figure 2 Selective inhibition of agonist-stimulated NO release from cultured bovine aortic endothelial cells by thapsigargin. Shown are the relaxant responses of the detector tissue (expressed as % relaxation of induced tone) to infusion t.c. of superoxide dismutase (SOD, 10 u ml^{-1}; Flow) and bolus injections t.c. of ADP (9 nmol), ionomycin (Iono; 60 pmol) or poly-L-lysine (pLys; 550 pmol) before (open columns) and after (hatched columns) a 10 min infusion t.c. of thapsigargin (1 μM) followed by a 5 min washout period ($n = 6$; $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; NS, not significant).

induced release of NO, for bovine aortic endothelial cells on beads produce only small amounts of NO under basal conditions (not shown), but at flow rates of 0.3–3 ml min^{-1} show a linear correlation between the amount of NO being released and the rate of perfusion (Buga *et al.*, 1991). The relatively high flow rate of 5 ml min^{-1} used in this study can thus be considered to produce an almost maximum shear stress-dependent release of NO from these cells. Moreover, the observed relaxations were largely attributable to the release of NO from the endothelial cell column, as PGI_2 does not relax the rabbit aorta (Moncada *et al.*, 1976). Therefore and because of the simultaneous determination of PGI_2 release, a cyclo-oxygenase inhibitor was not included in the bioassay.

The shear stress-induced relaxation of the detector tissue was further enhanced (additional 10–25% relaxation; $n = 5$) by bolus injections t.c. of ADP (9 nmol), ionomycin (60 pmol) or poly-L-lysine (550 pmol; Figures 1 and 2). Another receptor-dependent agonist, bradykinin (19 pmol), also caused an additional 14 ± 3% relaxation ($n = 3$). All of these relaxant responses were abolished by infusions t.c. of haemoglobin or N^G-nitro-L-arginine methyl ester ($n = 3$).

Infusions t.c. of thapsigargin (1 μM for 10 min) caused a sustained release of NO (additional 30 ± 8% relaxation; $n = 5$) which declined after the infusion was stopped (not shown). After removal of the thapsigargin infusion and a 5 min washout period, the shear stress-dependent release of NO remained unaffected, whereas the release of NO induced by all four agonists was completely abolished (Figures 1 and 2; $P < 0.01$ for bradykinin; $n = 3$). This also occurred when the thapsigargin infusion was continued throughout the experiment ($n = 3$) or when the Ca^{2+}-ATPase inhibitor was infused for 5 min followed by a 10 min washout period ($n = 3$). Control responses of the bioassay tissue to glyceryl trinitrate were not affected by treatment of the endothelial cell column with thapsigargin (from 37 ± 4 to 30 ± 5% relaxation; $n = 5$).

Thapsigargin administration (infusion at 1 μM for 5 min followed by a 15 min washout period) also inhibited the bradykinin-induced (30 nM) but not the shear stress-dependent release of NO from superfused human endothelial cells grown on coverslips (79 ± 5% inhibition; $n = 3$).

NO release from endothelium-intact arterial segments

In perfused femoral artery segments from the rabbit, infusion of endothelin-1 (60 nM) elicited a decrease in vessel diameter from 1651 ± 53 mm to 1156 ± 53 mm ($n = 3$; $P < 0.01$) accompanied by a distinct release of NO (Figure 3). As the segments were perfused at a constant flow rate (10.4 ml h^{-1}), the endothelin-induced decrease in vessel diameter caused an increase in shear stress at the luminal surface of the endothelium (from 0.12 to 0.55 dyn cm^{-2}, Busse *et al.*, 1991). Acetylcholine (1 μM) also significantly augmented the flow-induced release of NO (Figure 3), an effect that was strongly inhibited by pretreatment of the arterial segments with thapsigargin (infusion at 1 μM for 5 min followed by a 15 min washout period). In contrast, administration of thapsigargin did not affect the decrease in vessel diameter elicited by endothelin-1 (from 1680 ± 79 mm to 1176 ± 67 mm; $n = 3$) or the shear stress-dependent release of NO (Figure 3).

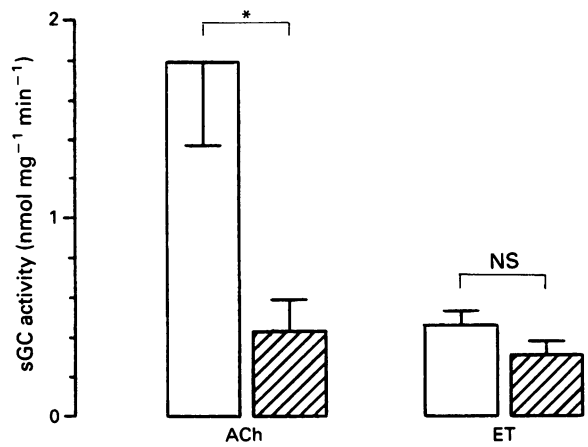


Figure 3 Effects of thapsigargin on the release of NO from isolated, luminally perfused rabbit femoral artery segments. The figure shows the net release of NO from the arterial segments (expressed as increase in specific guanylyl cyclase activity over the level induced by perfusion of the segments alone) elicited by infusions of acetylcholine (ACh; 1 μM) or endothelin-1 (ET; 60 nM) before (open columns) and after (hatched columns) a 5 min infusion of thapsigargin (1 μM) followed by a 15 min washout period ($n \geq 3$; $^{*}P < 0.05$ vs. control; NS, not significant).

Thapsigarin also suppressed the acetylcholine-stimulated release of NO from the luminally perfused, endothelium-intact rabbit aorta. Bolus injections of acetylcholine (55 nmol) stimulated the release of NO, as detected by relaxation of the endothelium-free rabbit aortic ring (15 ± 1% relaxation; $n = 3$), and this effect was abolished by pretreatment of the aorta with thapsigargin (infusion at 1 μM for 5 min followed by a 10 min washout period; $P < 0.01$). Thapsigargin alone elicited a relaxant response similar to that of acetylcholine which declined upon removal of the infusion ($n = 3$).

Moreover, thapsigargin blunted the relaxant response to acetylcholine in femoral artery segments preconstricted with noradrenaline (62% inhibition; not shown), and caused both a significant endothelium-dependent relaxation (88% increase in vessel diameter; not shown) and a substantial release of NO when administered alone (increase in soluble guanylate cyclase activity over basal levels: 1.43 ± 0.10 nmol mg^{-1} min^{-1}; $n = 3$). Both vessel diameter (from 88 to 33% increase) and NO release declined towards previous levels 15 min after washout of the Ca^{2+}-ATPase inhibitor ($n = 3$).

Release of PGI_2 from cultured endothelial cells

As determined by radioimmunoassay for 6-keto-$PGF_{1\alpha}$, the column of bovine aortic endothelial cells released 1.04 ± 0.27 ng PGI_2 min^{-1} ($n = 5$). Bolus injections of ADP, ionomycin, poly-L-lysine or bradykinin all increased the release of PGI_2 by 3 to 14 fold (Figure 4). Pretreatment of the cells with thapsigargin (1 μM for 10 min followed by a 5 min washout period) had no effect on the shear stress-dependent PGI_2 release, but abolished the release of PGI_2 induced by all four agonists (Figure 4; bradykinin: from 13.9 ± 5.5 to 1.09 ± 0.16 ng min^{-1}; $P < 0.05$; $n = 3$). Administration of thapsigargin alone caused an increase in PGI_2 release to 5.04 ± 1.99 ng min^{-1} which declined towards basal levels after removal of the thapsigargin infusion and subsequent superfusion of the cells with thapsigargin-free buffer (Figure 4).

Changes in $[Ca^{2+}]_i$ in cultured endothelial cells

Human endothelial cells grown on coverslips were loaded with Indo-1/AM. Addition of thapsigargin caused a sustained rise in $[Ca^{2+}]_i$ (Figures 5 and 6). This effect was dose-dependent with a maximum increase in $[Ca^{2+}]_i$ at approximately 100 nM thapsigargin, and a threshold concentration of 1–10 nM thapsigargin ($n = 8$). With high concentrations of thapsigargin (100–300 nM), a maximum increase in $[Ca^{2+}]_i$ was attained after 2–3 min, and sustained for more than 10 min. Replacement of the thapsigargin-containing solution by thapsigargin-free buffer had no significant effect on the elevated level of $[Ca^{2+}]_i$ (not shown), but removal of extracellular Ca^{2+} rapidly brought the thapsigargin-elevated $[Ca^{2+}]_i$ signal back to resting values (Figure 6). Stimulation of the cells with bradykinin (3 nM; $n = 4$) or histamine (1 μM; $n = 3$), after previous exposure to thapsigargin (30–300 nM for 10 min), did not result in a further increase in $[Ca^{2+}]_i$ (Figure 6).

In Fura-2/AM-loaded bovine endothelial cells in suspension, thapsigargin (1 μM) produced a rapid increase in $[Ca^{2+}]_i$ from 70–100 nM to 800–1200 nM which decreased slowly to approximately 500 nM over 15 min ($n = 4$). In the absence of extracellular Ca^{2+}, thapsigargin (1 μM) elicited only a small transient increase in $[Ca^{2+}]_i$ ($n = 4$). Addition of ADP (1 μM) or ionomycin (30 nM) to the cell suspension after 10 min exposure to thapsigargin (1 μM) did not produce any further increase in $[Ca^{2+}]_i$ ($n = 4$). This also occurred when the cells were exposed to thapsigargin for 20–60 min followed by a 5 min washout period before the measurement of $[Ca^{2+}]_i$. Resting intracellular Ca^{2+} levels in cells pretreated with thapsigargin, however, were significantly elevated (200–400 nM; $n = 4$) compared to control cells.

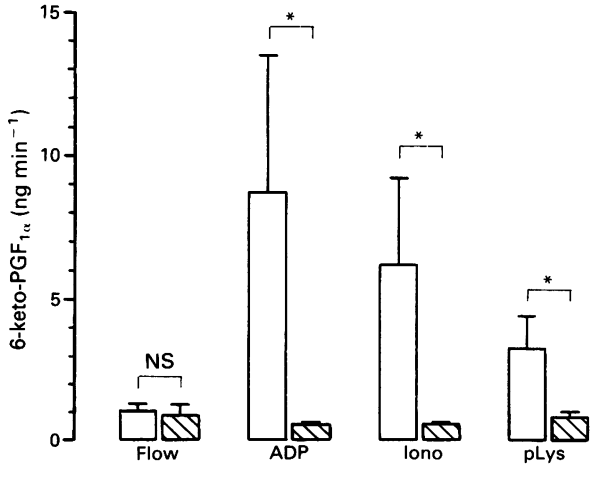


Figure 4 Selective inhibition of agonist-stimulated prostacyclin (PGI_2) release from cultured bovine aortic endothelial cells by thapsigargin. The figure depicts the release of PGI_2 (expressed as ng 6-keto-$PGF_{1\alpha}$ min^{-1}) in the absence (Flow) or presence of bolus injections t.c. of ADP (9 nmol), ionomycin (Iono; 60 pmol) or poly-L-lysine (pLys; 550 pmol) before (open columns) and after (hatched columns) a 10 min infusion t.c. of thapsigargin (1 μM) followed by a 5 min washout period ($n = 5$; $^*P < 0.05$ vs. control; NS, not significant).

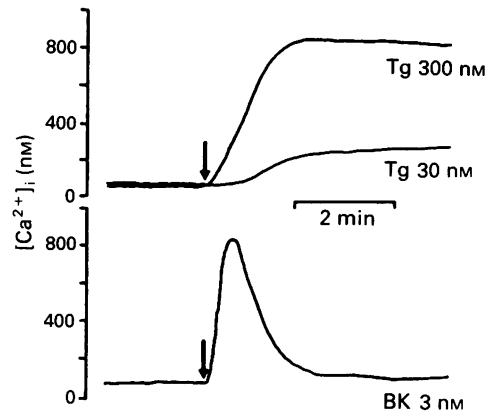


Figure 5 Changes in $[Ca^{2+}]_i$ in human cultured umbilical vein endothelial cells elicited by thapsigargin. The figure shows 2 typical traces obtained with 30 and 300 nM thapsigargin (Tg; top panel) as well as a control response to bradykinin (BK; 3 nM; bottom panel) from 8 individual experiments with different batches of endothelial cells. Arrows indicate the addition of thapsigargin or bradykinin.

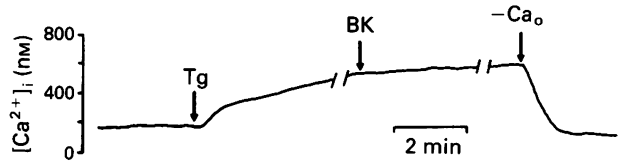


Figure 6 Inhibition of the bradykinin-induced mobilization of $[Ca^{2+}]_i$ by thapsigargin and effect of removal of extracellular Ca^{2+}. Shown is a typical trace from 8 individual experiments with different batches of human endothelial cells. Arrows indicate the addition of thapsigargin (Tg; 30 nM), bradykinin (BK; 3 nM) or Ca^{2+}-free buffer ($-Ca_o$).

Discussion

The data of the present investigation support the conclusion that a principle regulatory difference exists between the shear

stress- and agonist-induced release of both NO and PGI_2 from cultured and native endothelial cells which can be unmasked by the Ca^{2+}-ATPase inhibitor, thapsigargin. By emptying intracellular Ca^{2+} stores, including the $InsP_3$-sensitive Ca^{2+} pool, thapsigargin abolished the agonist-stimulated but did not affect the shear stress-induced release of these autacoids. This effect of the Ca^{2+}-ATPase inhibitor confirms previous findings with 2,2'-dithiodipyridine and N-ethylmaleimide, two sulphydryl reagents which, presumably by a mechanism closely related to that of thapsigargin, selectively inhibit the agonist-stimulated, but not the flow-dependent release of NO and PGI_2 from cultured bovine aortic endothelial cells (Hecker *et al.*, 1992).

Moreover, the sustained elevation of $[Ca^{2+}]_i$ in the presence of thapsigargin caused a substantial release of NO and PGI_2 from both cultured and native endothelial cells. This effect was very similar to that of thimerosal (Hecker *et al.*, 1992), a sulphydryl reagent which also seems to interfere with the uptake of Ca^{2+} by the endoplasmic reticulum (Hecker *et al.*, 1989). The molecular mechanism by which thapsigargin and thimerosal discharge intracellular Ca^{2+} pools, however, seems to be different, for in human platelets thimerosal inhibits the endoplasmic reticulum Ca^{2+}-ATPase by oxidizing one or several sulphydryl groups of this Ca^{2+} pump which are essential for its catalytic activity (Hecker *et al.*, 1989).

The agonist-stimulated release of NO and PGI_2 from endothelial cells is initiated by an increase in $[Ca^{2+}]_i$ via the phosphoinositol pathway (for references see Newby & Henderson, 1990), and is sustained by an influx of extracellular Ca^{2+}, presumably through non-selective cation channels (Olesen *et al.*, 1988; Hallam *et al.*, 1989). Although the regulation of Ca^{2+} entry into endothelial cells is still poorly understood, the most plausible hypothesis is the capacitance model proposed by Putney (1990). Therein, emptying of the $InsP_3$-sensitive intracellular Ca^{2+} store(s) initiates an influx of extracellular Ca^{2+} which is terminated upon refilling of the pool. Indeed, this process seems to be operating in endothelial cells (Dolor *et al.*, 1992), even though it is not clear whether extracellular Ca^{2+} refills endothelial Ca^{2+} store(s) directly (Buchan & Martin, 1991) or indirectly via entry into the cytoplasm and subsequent uptake by the endoplasmic reticulum (Lückhoff & Busse, 1990; Dolor *et al.*, 1992). The sensing mechanism for the filling state of these stores is also still a matter of investigation. However, it seems likely that extracellular Ca^{2+} enters the endothelial cell through non-selective cation channels (Olesen *et al.*, 1988; Hallam *et al.*, 1989), the activation of which is triggered both by an elevation in cytoplasmic Ca^{2+} and formation of inositol-1,3,4,5-tetrakisphosphate (Lückhoff & Clapham, 1992).

Thapsigargin depletes intracellular Ca^{2+} stores, and thus greatly reduces (Dolor *et al.*, 1992) or abolishes (this study) the subsequent mobilization of $[Ca^{2+}]_i$ induced by receptor-dependent agonists such as ADP, bradykinin or histamine in human and bovine endothelial cells. In contrast to the rapid transient rise in $[Ca^{2+}]_i$ initiated by these agonists, thapsigargin stimulated a slowly developing, prolonged elevation of $[Ca^{2+}]_i$ that required the presence of extracellular Ca^{2+}, and was clearly concentration-dependent. This increase in $[Ca^{2+}]_i$ attained maximum levels 2–3 min after the addition of thapsigargin (see also Dolor *et al.*, 1992), and $[Ca^{2+}]_i$ remained significantly elevated above resting levels for periods of up to 60 min, even when the thapsigargin-containing solution was replaced by thapsigargin-free buffer. Thus, as in many other cell types, thapsigargin appears to be a fast acting, irreversible inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase also in endothelial cells.

Interestingly, thapsigargin also suppressed the release of NO and PGI_2 induced by the receptor-independent agonists ionomycin and poly-L-lysine, both of which seem to activate a transmembrane Ca^{2+} influx into endothelial cells (Hecker *et al.*, 1992). The complete inhibition by thapsigargin of both the ionomycin-induced rise in $[Ca^{2+}]_i$ and the release of NO and PGI_2 indicates that the ionophore at low nanomolar concentrations releases Ca^{2+} primarily from intracellular Ca^{2+} stores which can be refilled by extracellular Ca^{2+} but were continuously depleted by thapsigargin.

Although the precise mechanism of action of poly-L-lysine has yet to be established, it seems likely that the polycation partially neutralizes the negatively charged endothelial cell membrane (Thomas *et al.*, 1989), thereby possibly modulating the activity of Ca^{2+}-permeable cation channels. One explanation for the inhibitory effect of the Ca^{2+}-ATPase inhibitor may be, therefore, that poly-L-lysine activates the same set of Ca^{2+}-permeable cation channels that are required for the refilling of endothelial Ca^{2+} stores and were already discharged by thapsigargin.

In contrast to the agonist-stimulated release of NO and PGI_2, pretreatment of both cultured and native endothelial cells with thapsigargin did not affect the shear stress-induced release of these autacoids. Thus, a mobilization of $[Ca^{2+}]_i$ from $InsP_3$-sensitive Ca^{2+} store(s) does not seem to be involved in this response. Indirect support for this conclusion comes from findings that removal of extracellular Ca^{2+} has a greater inhibitory effect on the shear stress-dependent release of NO than does the blockade of the $InsP_3$-mediated mobilization of $[Ca^{2+}]_i$ (Griffith *et al.*, 1986). In contrast, removal of extracellular Ca^{2+} progressively and completely inhibits the release of NO elicited by receptor-dependent agonists, further supporting the notion that the inhibitory effect of thapsigargin is brought about by emptying specific intracellular Ca^{2+} pools (Hecker *et al.*, 1992).

Unlike NO formation, PGI_2 biosynthesis depends critically on the release of Ca^{2+} from intracellular stores (Lückhoff *et al.*, 1988; White & Martin, 1989). Whether this is true for both agonist-stimulated and shear stress-dependent PGI_2 release, however, remains to be established. Our observation that thapsigargin did not affect the shear stress-induced release of PGI_2 argues against the latter hypothesis.

The shear stress-induced release of NO from cultured endothelial cells depends to some extent on the presence of extracellular Ca^{2+} (Hecker *et al.*, 1992). In contrast, it is difficult to evaluate whether the infrequently observed elevation of $[Ca^{2+}]_i$ in response to increased flow (Ando *et al.*, 1988; Geiger *et al.*, 1992; Schwarz *et al.*, 1992; Shen *et al.*, 1992) is simply an epiphenomenon or an integral part of the shear stress-induced signal transduction mechanism. The lack of effect of thapsigargin on the shear stress-dependent release of NO and PGI_2 strongly supports the notion that shear stress does not mobilize Ca^{2+} from intracellular Ca^{2+} stores. This does not exclude the possibility, however, that shear stress activates a set of non-selective Ca^{2+}-permeable cation channels which are different from those required for the refilling of intracellular Ca^{2+} stores (Schwarz *et al.*, 1992). As a consequence, shear stress may elevate endothelial $[Ca^{2+}]_i$ and hence stimulate NO and PGI_2 biosynthesis by a thapsigargin-independent mechanism.

Another explanation for the different sensitivity of agonist- and shear stress-induced NO release is that NO synthase in both human (Hecker *et al.*, unpublished observation) and bovine endothelial cells is membrane-bound (Förstermann *et al.*, 1991). Perturbation of the plasma membrane of endothelial cells exposed to shear stress may, therefore, lead to a $[Ca^{2+}]_i$-independent activation of this enzyme. The agonist-triggered, $InsP_3$-mediated elevation of $[Ca^{2+}]_i$ could then further augment the formation of NO in a Ca^{2+}/calmodulin-dependent manner. Alternatively, endothelial cells may contain both a Ca^{2+}-independent and a Ca^{2+}/calmodulin-dependent constitutive NO synthase (Mülsch *et al.*, 1989), the activity of which could be regulated differently by shear stress and/or receptor-dependent as well as independent agonists.

In conclusion, by emptying and preventing the refilling of intracellular Ca^{2+} stores, thapsigargin selectively inhibits the agonist-stimulated but not the shear stress-induced release of NO and PGI_2 from cultured as well as native endothelial cells. Thus, a principal difference exists in the control by $[Ca^{2+}]_i$ of these two types of stimulation of endothelial auta-

coid release, a finding which may help to unravel the signal transduction mechanism(s) mediating flow-induced endothelium-dependent dilatations *in vivo*.

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Chloride anion concentration as a determinant of renal vascular responsiveness to vasoconstrictor agents

[1]Caroline P. Quilley, Yu-Shi R. Lin & John C. McGiff

Department of Pharmacology, New York Medical College, Valhalla, New York 10595, U.S.A.

1 The role of chloride concentration in modulating vasoconstrictor responses of the rat isolated kidney, perfused with Krebs-Henseleit solution, to angiotensin II (AII), arginine vasopressin (AVP) and phenylephrine (PE) was investigated.

2 Reduction of perfusate chloride from a high (117 mM) to low (87 mM) concentration, by substitution of sodium chloride with a mixture of sodium salts of propionate, acetate and methanesulphonate, reduced responsiveness to all three vasoconstrictors, the change for AII being most pronounced.

3 For AII, reduced vasoactivity with low chloride was evident both in terms of the threshold dose and on the linear part of the dose-response curve but not for the maximum response. This attenuating effect of low chloride on the vasoconstrictor response to AII was reversed when perfusion with high chloride was reinstituted. Continuous perfusion with high chloride progressively increased the vasoconstrictor effect of low doses of AII for successive dose-response curves.

4 In addition to reducing responses on the linear part of the dose-response curve for both AVP and PE, low chloride also reduced the maximum vasoconstrictor response to PE, whereas the threshold dose for the two agonists was unchanged. In contrast to the enhanced pressor response to AII, during continuous perfusion with high chloride, tachyphylaxis occurred with AVP and PE.

5 The ability of chloride to modify renal responsiveness to vasoconstrictor agents may contribute to the increase in renal vascular resistance and decrease in glomerular filtration rate (GFR) which occurs during infusion of hyperchloremic solutions into the renal artery and explain the need for chloride as the anion accompanying sodium in salt-sensitive hypertensive models.

Keywords: Chloride; renal vasculature; vasoactivity; perfused kidney; angiotensin II; arginine vasopressin; phenylephrine

Introduction

Close renal arterial infusion of high chloride elevates renal vascular resistance (RVR) and lowers glomerular filtration rate (GFR) in dog (Wilcox, 1983) and rat (Bullivant *et al.*, 1989). In deoxycorticosteroid acetate (DOCA)-salt and in Dahl salt-sensitive hypertensive rats, separate roles for sodium and chloride in blood pressure elevation have been proposed (Boegehold & Kotchen, 1989). Chloride may contribute to the hypertension through its ability to increase RVR whereas sodium plays a role in increased cardiac output (Passmore & Jiminez, 1990). One possible mechanism for the *in vivo* increase in RVR after salt loading is that chloride increases responsiveness of the renal vasculature to pressor agents.

These studies were conducted to test whether physiologically relevant changes in chloride concentration in the vascular lumen modified renal responsiveness to vasoconstrictor agents. We used the rat isolated kidney to evaluate pressor responses to angiotensin II (AII), arginine vasopressin (AVP) and phenylephrine (PE) during perfusion with Krebs-Henseleit buffer having a high (117 mM) or low (87 mM) chloride concentration. The concentration of sodium was kept constant. This preparation excludes systemic hormonal and neuronal influences, such as those evoked by volume expansion, and enables chloride concentrations to be changed readily.

Dose-dependent vasoconstrictor responses to all three agonists, particularly AII, were modulated by changing chloride concentration, being less during perfusion with low chloride compared to responsiveness in the presence of high chloride. The reduction in the pressor effect of AII was readily reversed upon restoring the chloride concentration to 117 mM. A preliminary account of these findings has been published (Quilley *et al.*, 1992).

Methods

Isolated perfused kidney

Experiments were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. The right kidney was prepared for perfusion *in situ* by a modification of the method described previously (Quilley & McGiff, 1990). Briefly, male Sprague Dawley rats (325–400 g, Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) were anaesthetized with pentobarbitone (65 mg kg^{-1}, i.p., Butler, Co., Columbus, OH, U.S.A.). Heparin, 0.1 ml (1000 u ml^{-1}, Elkins-Sinn Inc., Cherry Hill, NJ, U.S.A.) and 0.4 ml mannitol (10% w/v) were injected via the penile vein. The renal vasculature was isolated by ligation of proximal vessels and the adrenal artery. The lower abdominal vena cava was catheterized and the ureter was cut to allow free exit of urine. The renal artery was cannulated with a blunted 18 gauge needle via the mesenteric artery so that flow to the kidney was uninterrupted. The vena cava above the kidney was then ligated and the rat killed by intracardiac injection of 1 M KCl. The kidney was perfused with warmed (37°C) Krebs-Henseleit buffer gassed with 95% O_2:5% CO_2 by means of a membrane lung. Perfusion pressure was monitored with a pressure transducer (Gould, Oxnard, CA, U.S.A.) and transcribed on a polygraph (Grass, Quincy, MA, U.S.A.). Perfusate flow rate was frequently

[1] Author for correspondence.

adjusted to maintain basal perfusion pressure at approximately 90 mmHg.

Experimental protocol

Following an equilibration period of 10–15 min with high chloride (117 mM), dose-response curves to either AII (2–100 ng), AVP (1–150 ng) or PE (0.1–30 ng) were constructed. Agonists were given in random order according to a Latin Square design. Only one agonist was tested per kidney. Injections were made at intervals of at least 5 min and perfusion pressure was allowed to stabilize before the next dose. Once a steady pressure was obtained, perfusate flow rate was adjusted to obtain a basal pressure of 90 mmHg. The time to complete each dose-response curve was approximately 50 min. One group of kidneys was then equilibrated, for 10 min with Krebs-Henseleit buffer containing low chloride (87 mM) in which NaCl was substituted with a mixture of sodium salts: acetate, propionate and methanesulphonate (10 mM each). The dose-response curve was then repeated. The second group of kidneys was perfused throughout with high chloride to serve as a time-control. In experiments with AII, the effect of reducing chloride to 87–97 mM by substitution of NaCl with only one of the three anions and the response during continued perfusion with high chloride plus 30 mM of the mixture of anions were also tested. Recovery of AII sensitivity following perfusion with low chloride was examined by repeating the AII dose-response curve during reperfusion with Krebs-Henseleit buffer containing high chloride.

Drugs and solutions

All reagents were of analytical grade. The Krebs-Henseleit stock was prepared at 10 times the final concentration as follows (mM): NaCl 63.1, KCl 4.4, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and $NaHPO_4$ 1.2 and stored at −20°C. Aliquots of the stock solution were reconstituted to final volume and the following added (mM): dextrose 5.5, $NaHCO_3$ 25. Sodium salts of acetate, propionate and methanesulphonate were substituted, singly and collectively for NaCl to maintain a constant sodium concentration of 143 mM and chloride concentrations of 87 to 117 mM. pH was not changed by these substitutions. As an additional control for the effects of the anions *per se* the mixture of anions was added to the 117 mM chloride solution. Unavoidably in this group, sodium concentration was increased by 30 mM. Stock solutions of AII, AVP and PE (Sigma Chemical Co., St. Louis, MO, U.S.A.) were dissolved in 0.9% w/v NaCl, 100 μg ml^{-1}, stored at −20°C and diluted just before use.

Statistical comparison

All values in figures and text are expressed as mean ± s.e. mean of n kidneys. Statistical comparisons were computed by analysis of covariance with the SAS/PC computer programme (SAS Instrument, Cary, NC, U.S.A.). A P value of <0.05 was considered significant. *Post hoc* comparison for specific between group differences was determined by the least significant differences test with an α value of 0.1 as only half of the possible comparisons were of interest.

Results

Effect of chloride concentration and time of perfusion on basal resistance

RVR tended to decline with time, as indicated by the increased perfusate flow rate in the face of constant perfusion pressure, regardless of whether kidneys were perfused with high followed by low chloride solution, or with a high chloride solution throughout. For example, in the experiments with AII, the initial flow rates after the equilibration period were 17.4 ± 2.0 and 15.9 ± 2.3 ml min^{-1} for the two experimental groups and had increased, by the start of the second-dose response curve, to 20.4 ± 2.0 and 21.4 ± 1.6 ml min^{-1} for kidneys perfused with high and low chloride, respectively. Thereafter, flow rate showed little change. Values at the beginning and end of the third dose-response curves to AII were 20.2 ± 2.6 and 20.2 ± 2.7 ml min^{-1} in the high chloride group compared to 20.4 ± 2.0 and 18.1 ± 2.4 ml min^{-1} in the high-low-high chloride group, respectively. The same trend, in which perfusate flow rate increased during the first h of experiment, but stabilized for the second and third h of perfusion, was seen when AVP or PE were used as agonists, irrespective of the chloride concentration used (data not shown). Thus, there were no differences in flow rates at equivalent times of perfusion among the six experimental groups.

Effect of changes in chloride concentration on renal vascular response to angiotensin II

Figure 1 is a representative trace showing the reduction in the dose-dependent vasoconstrictor responses to bolus injections of AII when the chloride concentration of the Krebs-Henseleit solution perfusing the kidney was changed from 117 mM to 97 mM by substitution of NaCl with Na acetate. This diminished response was readily reversed upon reperfusion with high chloride. Similar results were obtained when propionate or methanesulphonate were used as the substituting anions (data not shown). As an additional control, when a mixture of anions was added at the start of the second dose-response curve without a reduction in NaCl, the vasoconstrictor effect of AII was not modified, e.g. vasoconstrictor responses to 20 ng AII during perfusion with high chloride or high chloride plus anions were 86 ± 11 and 78 ± 6 mmHg, respectively ($n = 6$ per group).

The cumulative data, comparing renal vasoconstrictor responses to AII, during perfusion with either high or low chloride, are shown in Figure 2. During pretreatment with Krebs-Henseleit buffer containing 117 mM chloride, increases in perfusion pressure in response to randomly administered

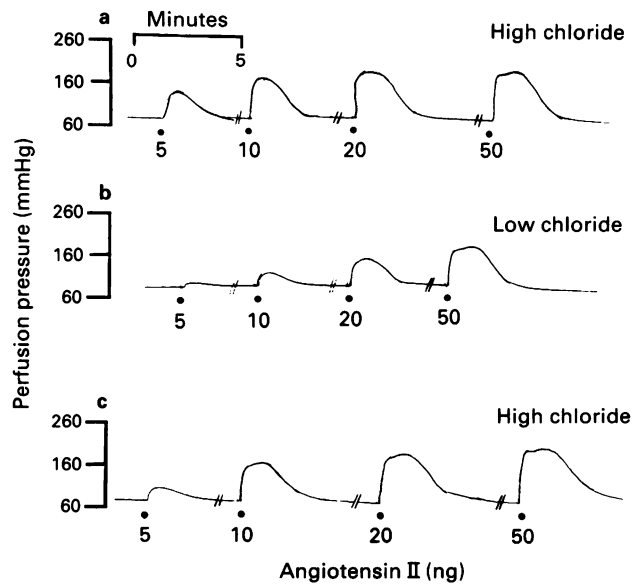


Figure 1 Representative trace showing dose-dependent changes in perfusion pressure of the rat isolated kidney following bolus injections of antiotensin II during perfusion with Krebs-Henseleit buffer gassed with 95% O_2, 5% CO_2, 37°C containing high chloride (117 mM, a) low chloride (97 mM, b), when 20 mM NaCl was substituted with sodium acetate and, during reperfusion with high chloride (c).

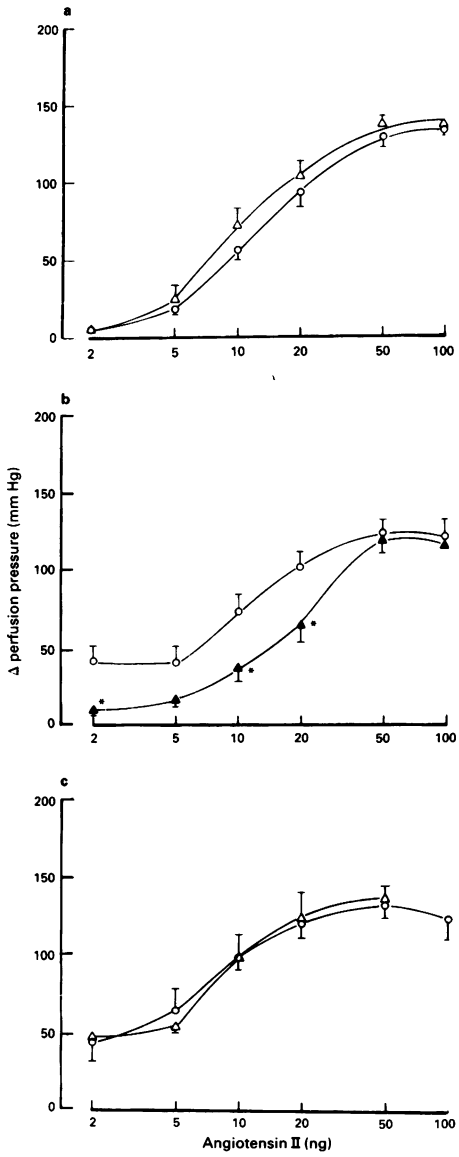


Figure 2 Mean dose-response curves to angiotensin II for experimental (triangles, $n = 7$) and time-control (circles, $n = 7$) kidneys, during initial perfusion with high chloride (117 mM) (a), and subsequently with low chloride (▲, 87 mM) (b) followed by reperfusion with high chloride (c). Values are mean ± s.e.mean (vertical bars).

doses of AII, 2–150 ng, were the same for the two experimental groups (Figure 2a). When the chloride concentration was changed to 87 mM by replacement of NaCl with sodium and assorted anion salts, vasoconstrictor responses to 2, 10 and 20 ng AII were significantly reduced compared to the 117 mM chloride time-control, whereas the maximum response was not diminished (Figure 2b). This attentuation was reversed when chloride concentration was returned to 117 mM (Figure 2c). In kidneys continuously perfused with high chloride, pressor responses to doses of AII between 2 and 20 ng increased progressively with repetition of the dose-response curves, whereas the maximum responses at 50 to 100 ng were unchanged. For example, at 10 ng AII the increases, expressed as percentages of the pretreatment dose-response curve maximum, were 42 ± 3, 58 ± 10 and 75 ± 9 for successive curves. Thus, low chloride diminished AII vasoactivity, whereas during continuous exposure to high chloride there was a progressive, time-dependent increase in the sensitivity of the renal vasculature to AII.

Effect of chloride concentration on renal vasoconstrictor responsiveness to arginine vasopressin and phenylephrine

Dose-response curves to either AVP or PE, tested in separate time-control and experimental kidneys during the initial perfusion with 117 mM chloride, were similar (Figures 3a and 4a). In contrast to AII, tachyphylaxis to both vasoconstrictors was evident with continued perfusion at a chloride concentration of 117 mM (Figures 3b and 4b) as responses on the linear part of the dose-response curve were diminished when compared to the initial values. The threshold doses and the maximum responses were unchanged. However, when perfused at 87 mM chloride, responsiveness to AVP at the intermediate doses was reduced further, being lower than the time-control kidneys perfused with high chloride, whereas the threshold dose and maximum responsiveness were not different from the high chloride group (Figure 3b). Like AVP, the threshold dose of PE was unchanged upon exposure to low chloride and pressor responses to intermediate doses were diminished. In contrast to AII and AVP, however, the maximum response to PE was also reduced (Figure 4b).

In summary, lowering the luminal chloride concentration reduced renal vascular responsiveness on the linear part of the dose-response curve to all three agonists, the effect on AII being most pronounced. Additional differences in the pattern of the changes in responsiveness with high and low chloride concentrations were evident as (1) the threshold dose of AII was altered by continuous perfusion with high chloride yet that of AVP and PE was unchanged; (2) responses to AVP and PE declined during continuous perfusion with high chloride whereas the pressor effect of AII was

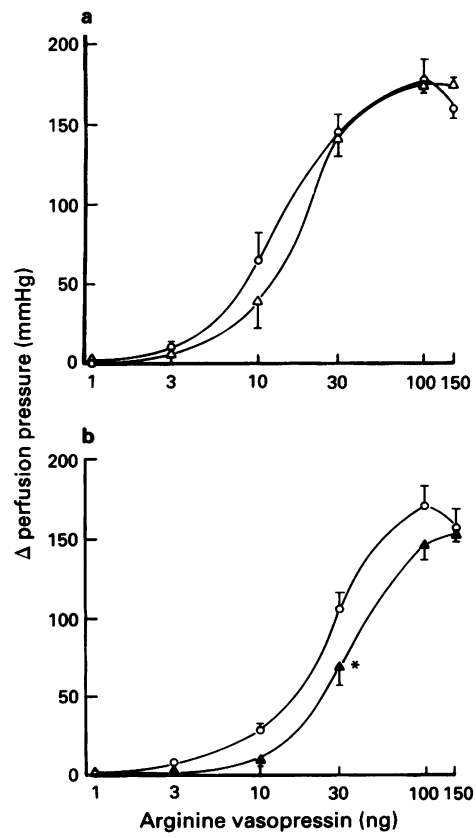


Figure 3 Mean perfusion pressure responses of arginine vasopressin-treated rat kidneys perfused with Krebs-Henseleit buffer. Dose-response curves of experimental (triangles, $n = 6$) and time-control (circles, $n = 5$) during initial perfusion of both groups with high chloride (117 mM) (a), and subsequently in the presence of either high or low chloride (▲, 87 mM) (b). Values are mean ± s.e.mean (vertical bars).

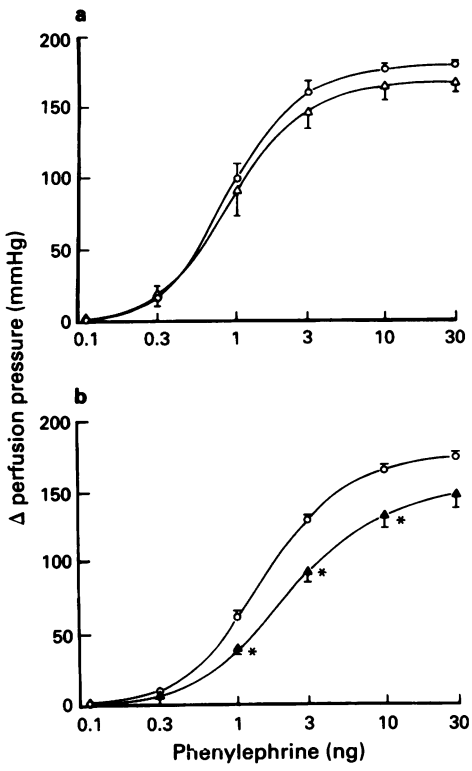


Figure 4 Changes in mean perfusion pressure of experimental (triangles, $n = 7$) and time-control (circles, $n = 7$) kidneys in response to bolus injections of phenylephrine during pretreatment with high chloride (117 mM) (a) and subsequent perfusion with low chloride (▲, 87 mM) (b). Values are mean ± s.e.mean (vertical bars).

increased with time, and (3) maximum responses to only PE and not to AII or AVP were reduced by low chloride. Thus, although the pattern of the dose-response relationships for the three agonists were affected differently by chloride, the major effect was a diminished pressor responsiveness at lower chloride concentrations.

Discussion

The diminished pressor responses of the kidney to three different agonists during perfusion with low chloride indicates that the sensitivity of the renal vasculature to vasoconstrictor agents and chloride anion availability are interrelated. It is unlikely that the reduced vasoactivity is due to an effect of the replacing anions as the addition of the assorted anions in the presence of high chloride did not alter responses to AII. Hitherto, the role of chloride in regulating vasoactivity of the responsiveness of blood vessels to AII or AVP have not been tested. However, based on the few studies that have addressed the role of anions in the regulation of vasoactivity, a general trend has emerged: anions which are more permeant than chloride; bromide, iodide, nitrate and thiocyanate, potentiate contractility whereas less permeant anions; sulphate, thiosulphate, phosphates and organic anions, depress reactivity.

Accordingly, Wahlström & Svennerholm (1973) using the rat portal vein, established a clear inverse relationship between noradrenaline sensitivity and chloride concentration when nitrate or bromide were substituted. Conversely, isoethionate and benzenesulphonate attenuated the response. However, other studies have yielded inconsistent results depending on the vascular tissue studied, contractile agent used, anion selected and time after substitution (for review see Kreye & Ziegler, 1982).

The commonality of the effect of lowering chloride concentration on vasoconstrictor responsiveness to the three different agonists suggests that the chloride anion plays a fundamental role in stimulus-contraction coupling. An effect at the receptor level, analogous to regulation of receptor affinity for α-adrenoceptor agonists (Limbird & Sweatt, 1985), AII (Griendling & Alexander, 1990) and AVP (Thibonnier *et al.*, 1987) by cation sensitive binding sites, is one possible mechanism, particularly as chloride modulates guanine nucleotide regulatory G proteins (Higashijima *et al.*, 1987).

Alternatively, the role of chloride in regulating membrane potential and ion conductance may be important to this altered sensitivity (Wahlström, 1973). Although the contribution of chloride to establishing resting membrane potential is controversial (Kreye & Ziegler, 1982), intracellular chloride levels are much higher than can be accounted for by passive ion distribution. Furthermore, increased chloride efflux from vascular smooth muscle is associated with noradrenaline vasoconstriction (Wahlström, 1973) and AII and AVP increase transport of chloride in non-vascular smooth muscle (Okuda *et al.*, 1986). The recent report that chloride channel blockers depress responses of the rat aorta and portal vein to potassium or noradrenaline underscores the importance of chloride transport and anion permeability to contraction of vascular smooth muscle (Zhang *et al.*, 1989).

Changes in ion conductance may in turn be linked to regulation of intracellular pH and changes in calcium disposition. An imbalance between HCO_3-Cl exchange in either direction can affect vascular tone (Karniski, 1990) and thus could account for the non-specific effect of alterations in chloride on vasoactivity. Similarly, the integral role of calcium in smooth muscle contraction as well as in intracellular signalling mechanisms (Khalil *et al.*, 1990) and the capacity for chloride to increase intracellular levels of free calcium (Okuda *et al.*, 1986) could also underlie the generalized depression of the vascular responsiveness of the rat kidney to the three agonists when chloride was lowered.

Selective differences in the relative importance of chloride-sensitive mechanisms which transduce the contractile response of renal vasculature to specific agonists may explain the distinctions between the effect of changes in chloride concentration on AII, AVP and PE dose-response relationships. Specifically, the capacity of the different agonists to stimulate arachidonic acid release and thereby elicit increased prostaglandin release, which is in turn subject to modulation by the prevailing chloride concentration, is a distinct possibility. Thus, in mesangial cells, low chloride increases release of prostaglandin E_2 (Okuda *et al.*, 1989) whereas, high chloride stimulates thromboxane (Wilcox *et al.*, 1985). Thus, changes in chloride concentration can affect release of both pressor and depressor prostanoids and alterations in their ratios may participate in the effect of chloride on vasoactivity.

The relationship between chloride and sensitivity of the renal vasculature to vasoconstrictors may have physiological significance as manipulation of chloride intake can result in changes in plasma chloride concentrations over the range used in this study (Abboud *et al.*, 1979; Kurtz & Morris, 1983; Passmore *et al.*, 1985). In addition, these findings may provide a link between increased chloride reabsorption in the early distal tubule in salt-sensitive hypertension and blood pressure elevation (Kirchner, 1990; Roman & Kaldunski, 1991) and, conversely, between the defect in chloride reabsorption in the loop of Henle and the hypotension in Bartter's syndrome (Gill & Bartter, 1978). This effect of chloride on vasoactivity may explain the requirement for chloride as the anion accompanying sodium in salt-sensitive (Boegehold & Kotchen, 1989) and AII-dependent (Passmore & Jimenez, 1991; Sato *et al.*, 1991) models of hypertension. Finally, the results of the present study support the

hypothesis that the ability of high chloride to increase RVR and lower GFR is due, at least in part, to the ability of chloride to modify responsiveness of the kidney to pressor agents.

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Antiarrhythmic agents act differently on the activation phase of the ACh-response in guinea-pig atrial myocytes

[1]Norio Inomata, Tomochika Ohno, Takafumi Ishihara & *Norio Akaike

Laboratory of Experimental Pharmacology, Suntory Institute for Biomedical Research, Shimamoto-cho, Mishima-gun, Osaka 618, and *Department of Neurophysiology, Tohoku University School of Medicine, Sendai 980, Japan

1 Anti-acetylcholine effects of pilsicainide, flecainide, disopyramide and propafenone on the acetylcholine (ACh)-induced K^+ current ($I_{K.ACh}$) were examined in dissociated guinea-pig atrial myocytes under whole-cell voltage clamp by the use of the 'concentration-clamp' technique.

2 The $I_{K.ACh}$ was activated with a latency of about 100 ms after 1 μM ACh application and desensitized to a steady-state level. The latent period and the time to peak response were shortened with increasing ACh concentration.

3 The values of half-maximal inhibition (IC_{50}) on the peak and steady state responses were 25 and 25 μM for pilsicainide, 1.7 and 2.0 μM for disopyramide, 19 and 2.0 μM for flecainide and 0.7 and 0.2 μM for propafenone, respectively.

4 Pilsicainide and disopyramide increased the latent period and the time to peak of $I_{K.ACh}$ in a concentration-dependent manner. Flecainide and propafenone did not change the latent period, but shortened the time to peak and hastened the decay of $I_{K.ACh}$ in a voltage-independent manner.

5 The results suggest that the mechanisms underlying the anti-acetylcholine effect of antiarrhythmic drugs are different among these drugs: i.e., pilsicainide and disopyramide mainly block the muscarinic ACh receptors while flecainide and propafenone inhibit the K^+ channel itself as open channel blockers.

Keywords: Dissociated atrial myocytes; concentration-clamp; anti-acetylcholine effect; acetylcholine; K^+ channels; antiarrhythmic drugs; pilsicainide; flecainide; disopyramide; propafenone

Introduction

Some antiarrhythmic drugs of class I are known to antagonize actions of acetylcholine (Mirro *et al.*, 1980; Endou *et al.*, 1991), and to block the cardiac voltage-operated K^+ currents in addition to depressing directly the fast inward Na^+ currents in cardiac myocytes (Imaizumi & Giles, 1987; Follmer & Colatsky, 1990). The muscarinic acetylcholine (ACh)-receptor-operated K^+ current ($I_{K.ACh}$) in atrial myocytes is regulated by guanosine 5'-triphosphate (GTP) binding proteins (Breitwieser & Szabo, 1985; Pfaffinger *et al.*, 1985; Kurachi *et al.*, 1986a,b; Yatani *et al.*, 1987). By use of GTP- or GTPγS-loaded atrial myocytes of guinea-pig, it has also been shown that disopyramide and a high-concentration of pilsicainide (N-(2,6-dimethylphenyl)-8-pyrrolizidinylacetamide hydrochloride hemihydrate) mainly blocks the muscarinic ACh receptors, while flecainide and quinidine inhibit the GTP binding proteins and/or the muscarinic K^+ channels (Nakajima *et al.*, 1989; Inomata *et al.*, 1991). The anti-acetylcholine action of antiarrhythmic agents causes tachycardia, an acceleration of A-V conduction and a shortening of refractory periods (Birkhead & Vaughan Williams, 1977). Therefore, it seems to be very important to examine more strictly the mechanism of the anti-acetylcholine effects of antiarrhythmic drugs from a clinical point of view.

It has been shown that activation phase of the ACh-response in the atrium has a latent period (Pott & Pusch, 1979; Osterrieder *et al.*, 1981; Inomata *et al.*, 1989), which is concentration-dependent and is shortened considerably with an increase in the ACh concentration (Inomata *et al.*, 1989). Therefore, the anti-acetylcholine mechanism of antiarrhythmic drugs could be also examined by analysing their effects on the activation phase of $I_{K.ACh}$.

In the present study, therefore, we clarified the characteristics of anti-acetylcholine effects of antiarrhythimc agents such as pilsicainide, flecainide, propafenone and disopyramide, which are slow- or intermediate-kinetic Na^+ channel blockers, on the activation phase of $I_{K.ACh}$.

Methods

Single atrial cells were dissociated from male guinea-pigs, weighing 300–500 g, by an enzymatic dispersion procedure similar to that described previously (Inomata *et al.*, 1989). To record the ACh-receptor mediated K^+ current, experiments were done on internally perfused single atrial cells by a suction-pipette technique (whole-cell mode) (Akaike *et al.*, 1978). A viable, rod-shaped cell was sucked into the tip of a suction-pipette (inner diameter of the tip, 2~3 μm) and perfused intracellularly with a solution composed of (in mM): K-aspartate 130, KCl 20, KH_2PO_4 1.0, Na_2ATP 3.0, $MgCl_2$ 1.0, EGTA 5.0 and GTP 0.1 (pH 7.3). External solution containing ACh and/or drugs was exchanged by a 'concentration-clamp' method (Akaike *et al.*, 1986; Inomata *et al.*, 1989). Briefly, the cell-attached tip of the suction pipette was inserted into a plastic tube through a circular hole of 500 μm in diameter. The lower end of this tube could be exposed directly to the external test solution by vertical movement of a stage holding the dishes containing test solutions. The external solution was as follows (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, NaH_2PO_4 0.33, $NaHCO_3$ 11.9, glucose 5.5 and HEPES 5.0 (pH 7.4). A negative pressure of −3 cmHg applied to the upper end of the plastic tube was controlled by an electromagnetic valve, driven by a stimulator (Dia-Medical System, DPS-160B). The speed of solution exchange by the concentration-clamp technique was estimated from the time constant of the liquid junction potential when the external K^+ concentration was changed from 5.4 to 35.4 mM. In the cell-attached condition of a dissociated atrial cell, the holding current shifted to a new value with a time constant of about 4 ms when the external K^+ concentration was increased. The membrane current was measured by means of an Ag-AgCl wire mounted on the

[1] Author for correspondence.

suction-pipette holder. The reference electrode was an Ag-AgCl wire in a Ringer plug on a solution exchange plastic tube. Both electrodes were led to a voltage-clamp circuit, and the membrane potential was controlled by a patch-clamp amplifier (List-Medical, EPC-7). To examine the muscarinic ACh-induced K^+ outward current, cells were held at a membrane potential of -50 mV. Experiments were carried out at room temperature (26 ± 1°C). The voltage and current traces were monitored on a storage oscilloscope (National, VP-5730A) and were simultaneously stored on a X-Y plotter (Hewlett Packard, 7475A) and a digital video cassette recorder (Victor, BR-S3600) for later analysis.

The relative amplitude of $I_{K.ACh}$ in the presence of various drugs was calculated as follows: the difference between 1 μM ACh-induced current and the current level in the absence of ACh was taken as 1.0. ANOVA test and Dunnette's multiple-test were used for statistical analysis of the data. A value of $P<0.05$ was considered significant.

Drugs used in the present study were acetylcholine (Sigma), pilsicainide hydrochloride, flecainide acetate, disopyramide and propafenone hydrochloride (Suntory).

Results

Effects of antiarrhythmic agents on the peak and steady-state level of $I_{K.ACh}$

The application of 1 μM ACh to the dissociated atrial cells at a holding potential (V_H) of -50 mV induced a submaximal outward current due to activation of the muscarinic linked K^+ channels (Kurachi *et al.*, 1986b; Inomata *et al.*, 1989; 1991). $I_{K.ACh}$ was activated with a latency of about 100 ms after 1 μM ACh application (Pott & Pusch, 1979; Osterrieder *et al.*, 1981; Inomata *et al.*, 1989) and desensitized to a steady-state level within several tenths of second in the continuous presence of ACh (Inomata *et al.*, 1991).

All of the antiarrhythmic agents, which were applied simultaneously with 1 μM ACh, inhibited the $I_{K.ACh}$ in a concentration-dependent manner, though propafenone and flecainide were less effective in reducing the peak current component compared to their reduction of the steady-state response (Figure 1). At the peak phase of $I_{K.ACh}$, the concentrations of test drugs that produced a half-maximal inhibition (IC_{50}) estimated from the concentration-response curves shown in Figure 2 were 25 μM for pilsicainide, 1.7 μM for disopyramide, 19 μM for flecainide and 0.7 μM for propafenone. In addition, the IC_{50} values for inhibition of the steady-state current component were 25 μM for pilsicainide, 2.0 μM for disopyramide, 2.0 μM for flecainide and 0.2 μM for propafenone. In the presence of pilsicainide or disopyramide, the blockade of the peak and steady-state components of $I_{K.ACh}$ occurred in a similar concentration-dependent manner whereas flecainide or propafenone reduced more markedly the ACh-response at the steady-state phase than at the peak (Figure 2).

Effects of antiarrhythmic agents on the activation phase of $I_{K.ACh}$

The activation phase of $I_{K.ACh}$ has a latent period, which is shortened with an increase in the ACh concentration. In addition, the time to peak of $I_{K.ACh}$ is also reduced with increasing ACh concentration (Inomata *et al.*, 1989). When the antiarrhythmic drugs were applied simultaneously with 1 μM ACh, the latent period was prolonged by pilsicainide or disopyramide in a concentration-dependent manner regardless of whether the peak or steady-state current responses were assessed (Figures 3 and 4). In addition, the time to peak also increased in a concentration-dependent manner: i.e., from 1076 ± 72 ms in control to 2491 ± 479 ms by 30 μM pilsicainide ($n=5$, $P<0.01$), and from 1571 ± 136 ms to

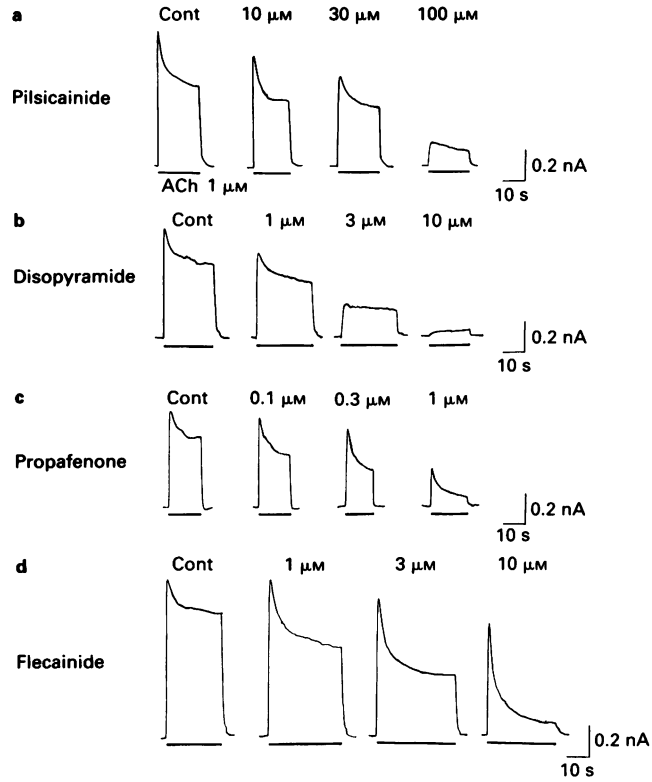


Figure 1 Effects of antiarrhythmic drugs on acetycholine (ACh)-induced outward K^+ current ($I_{K.ACh}$) in dissociated guinea-pig atrial myocytes. The cells were held at a V_H of -50 mV. Each column (a-d) shows the original current traces induced by 1 μM ACh in the absence (left) and the presence of each antiarryhthmic agent at the concentrations indicated above each current trace. ACh was applied during the period indicated by the bar under each current trace.

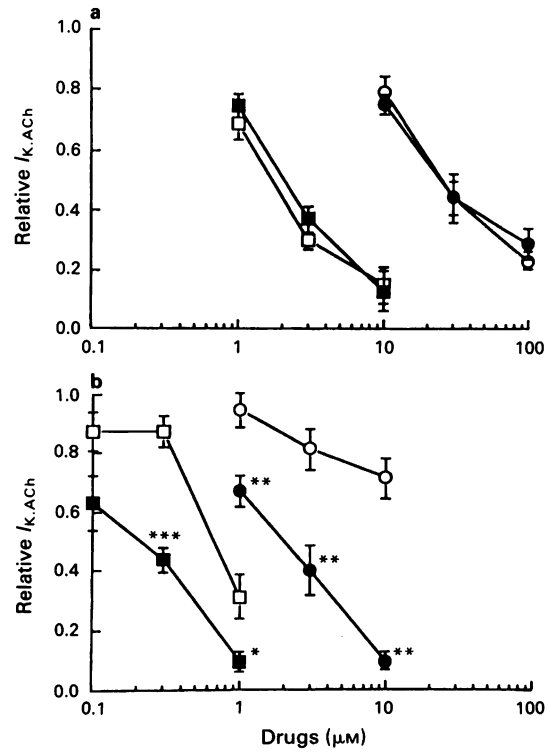


Figure 2 Effects of antiarrhythmic drugs on the peak (open symbol) and steady-state (closed symbol) phases of $I_{K.ACh}$ induced by 1 μM acetylcholine (ACh). (a) Effects of pilsicainide (circles) and disopyramide (squares). (b) Effects of flecainide (circles) and propafeneon (squares). Each symbol represents the mean of 4–8 experiments; vertical bars show s.e. mean. $*P<0.05$; $**P<0.01$; $***P<0.001$, versus value at the peak phase.

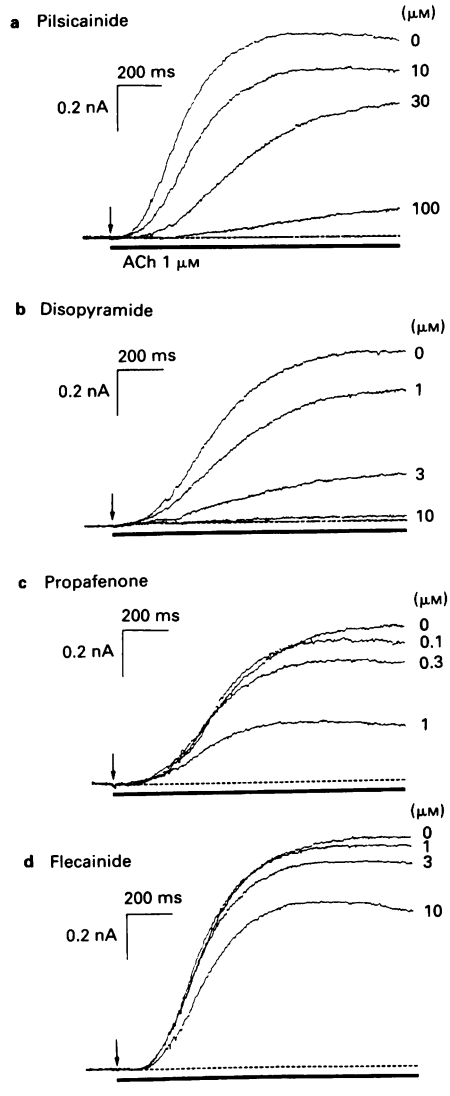


Figure 3 Effects of antiarrhythmic drugs on the activation phase of $I_{K.ACh}$. The current traces were superimposed over a much reduced sweep period. The cells were held at a V_H of -50 mV. Each drug was applied simultaneously with 1 μM ACh. The latent period is the period between the ACh application point (indicated by the arrow) and the starting point of each $I_{K.ACh}$ activation. The starting point of ACh application was determined as described in the Methods. Note that the latent period and the time to peak were increased by pilsicainide (a) and disopyramide (b). On the other hand, the latent period was not changed and the time to peak was shortened and, moreover, the decay phase of current was hastened by propafenone (c) and flecainide (d).

2845 ± 380 ms ($n = 5$, $P < 0.01$) by 3 μM disopyramide. On the other hand, both propafenone and flecainide shortened the time to peak in a concentration-dependent manner without affecting the latent period. The time to peak was decreased from 1364 ± 71 ms in control to 1115 ± 89 ms ($n = 6$, $P < 0.05$) by 0.3 μM propafenone and from 1535 ± 334 to 1327 ± 226 ($n = 5$) by 3 μM flecainide. Moreover, the decay phase of $I_{K.ACh}$ was hastened in the presence of propafenone or flecainide (Figure 3c and d).

Voltage-dependency of the $I_{K.ACh}$ blockade by propafenone and flecainide

Propafenone, 0.3 μM was applied simultaneously with 1 μM ACh at various membrane potentials between -100 and -60 mV (Figure 5a). The current-voltage (*I-V*) relationships for the peak and steady-state components of $I_{K.ACh}$ are shown in Figure 5b. In both cases of the inward and outward $I_{K.ACh}$, the transient peak component of $I_{K.ACh}$ was hardly affected, but the decay phase was hastened and the steady-state component was markedly decreased in the presence of propafenone. Flecainide, 3 μM, also reduced the steady-state current component but not in the peak current response at various membrane potentials (data not shown).

Discussion

The present study demonstrated that pilsicainide or disopyramide blocked the peak and steady-state components of $I_{K.ACh}$ in a concentration-dependent manner and that these agents increased the latent period and the time to peak. On the other hand, flecainide and propafenone markedly reduced the steady-state component more than the peak component, and decreased the time to peak without any effect on the latent period. Moreover, propafenone and flecainide hastened the decay phase of the $I_{K.ACh}$.

It is known that the muscarinic ACh-receptor of atrial myocytes activates GTP binding proteins which directly regulate the K^+ channel (Breitwieser & Szabo, 1985; Pfaffinger *et al.*, 1985; Kurachi *et al.*, 1986a,b; Yatani *et al.*, 1987). Regarding the anti-acetylcholine effects of pilsicainide and disopyramide in steady-state phase of $I_{K.ACh}$, we have reported that the IC_{50} of the drugs on intracellular GTPγS-induced $I_{K.ACh}$, a receptor-uncoupled current, is greater than that of ACh-induced $I_{K.ACh}$, a receptor-operated one (Inomata *et al.*, 1991). However, in the case of flecainide, IC_{50} values of the drug on both GTPγS-induced and ACh-induced $I_{K.ACh}$ were

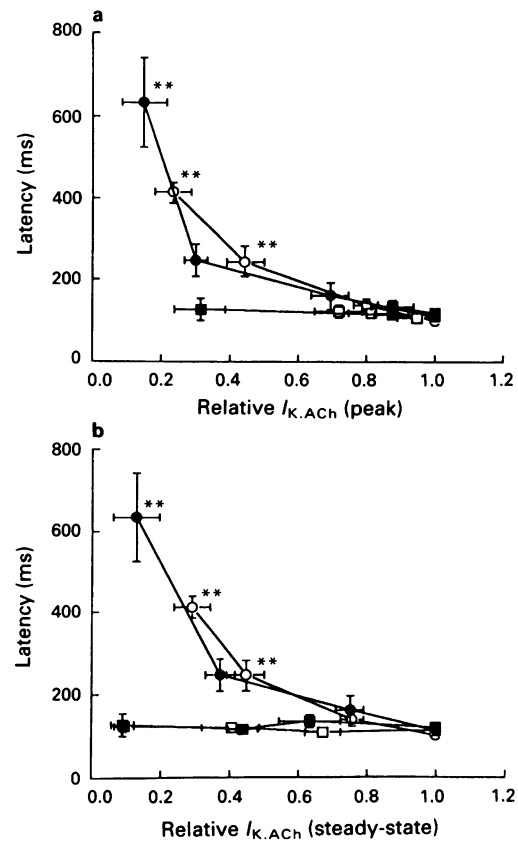


Figure 4 Effects of pilsicainide (○), disopyramide (●), flecainide (□), and propafenone (■) on the relationship between the latent period and peak $I_{K.ACh}$ (a) or steady-state $I_{K.ACh}$ (b). Each symbol represents the mean of 3–8 experiments; vertical bars show s.e.mean. ***$P < 0.01$, versus the latent period in the absence of drugs.

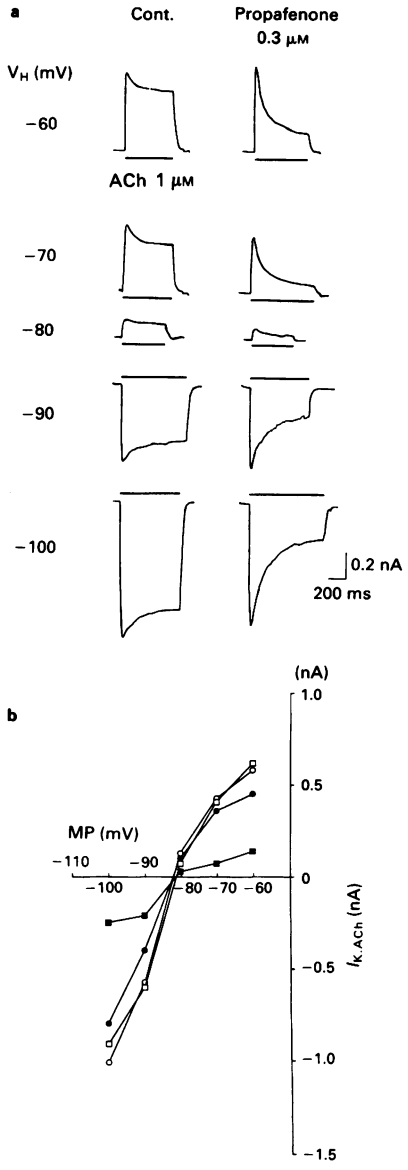


Figure 5 Voltage-dependency of blockade of $I_{K.ACh}$ by propafenone. (a) $I_{K.ACh}$ at various membrane potentials in the absence or presence of 0.3 μM propafenone. (b) Relationship between the peak (open symbol) or steady-state (closed symbol) components of $I_{K.ACh}$ and membrane potential in the absence (○, ●) or presence (□, ■) of 0.3 μM propafenone. Note that the degree of blockage of $I_{K.ACh}$ by propafenone occurred similarly at various membrane potentials.

almost of the same order (Inomata *et al.*, 1991). Propafenone also blocked both the steady-state phase of GTPγS-induced and ACh-induced $I_{K.ACh}$ in an almost identical concentration-dependent manner with IC_{50} values of 0.3 and 0.2 μM, respectively. Moreover, the latent period and the time to peak of $I_{K.ACh}$ were prolonged with decreasing ACh concentration (Inomata *et al.*, 1989). Therefore, the results indicate that pilsicainide or disopyramide, which increases the latency and the time to peak of $I_{K.ACh}$ in the present experiments, mainly blocks $I_{K.ACh}$ at the muscarinic receptor site and this action results in the reduction of both the peak and steady-state $I_{K.ACh}$. On the other hand, the action of drugs that shorten the time to peak and hasten the decay phase could be accounted for by a model in which the drug blocks the open-state of ionic channels (Yeh & Narahasi, 1977). Therefore, it seems plausible that flecainide and propafenone reduce the $I_{K.ACh}$ by blocking directly the muscarinic K^+ channels as open channel blockers. It has been reported that in the squid giant axon, tetraethylammonium (TEA) ions applied intracellularly block delayed rectifier K^+ channels only when the channel is opened by a depolarizing pulse, and that internal TEA blocks outward K^+ current greater than inward K^+ current in a voltage-dependent manner (Armstrong, 1975). Furthermore, extracellularly applied TEA also blocks K^+ currents and its blocking action is little affected by membrane potential (Hille, 1984). The blocking action of extracellularly applied propafenone or flecainide was similar in potency for both outward and inward $I_{K.ACh}$. However, it remains unknown whether these agents block $I_{K.ACh}$ voltage-dependently from the inside of the atrial myocyte membrane.

It has already been reported that flecainide at the clinically effective serum concentrations of 0.25 to 1.3 μM blocks not only a fast inward Na^+ current but also a delayed rectifier K^+ current in cat ventricular myocytes (Follmer & Colatsky, 1990). Moreover, propafenone at low concentrations of 0.3 to 0.5 μM also prolongs the duration of the guinea-pig ventricular action potential (Honjo *et al.*, 1989). It is of much interest that the blocking actions of flecainide and propafenone on these K^+ channels, which induce the prolongation of action potentials, are similar to the blocking potencies of the muscarinic ACh-receptor-operated K^+ channels. In contrast, pilsicainide did not change the duration of the action potential in papillary muscles (Hattori *et al.*, 1986) and blocked the fast Na^+ current more markedly than the slow Ca^{2+} current at effective serum concentrations in the coronary ligated arrhythmia model of dogs (Inomata *et al.*, 1987).

In conclusion, it is predicted that in addition to directly depressing the fast Na^+ currents in cardiac myocytes, disopyramide inhibits the muscarinic ACh receptors, while propafenone and flecainide block the muscarinic receptor linked K^+ channels. However, pilsicainide, at the effective serum concentrations ranging from 0.25 μM to 8.0 μM in the cainine arrhythmia models (Hashimoto *et al.*, 1985), may hardly block either the muscarinic ACh receptors or their associated K^+ channels.

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Ionic effects on amiloride block of the mechanosensitive channel in *Xenopus* oocytes

John W. Lane, Don W. McBride Jr. & [1]Owen P. Hamill

Section of Neurobiology and Behavior, Cornell University, Ithaca, New York 14853, U.S.A.

1 Patch clamp techniques were used to measure the ionic dependence of amiloride block of single mechanosensitive (MS) channels in frog (*Xenopus laevis*) oocytes.

2 The primary aim was to determine whether the difference in potency of amiloride block of MS channels in frog oocytes ($IC_{50} = 0.5$ mM) and chick auditory hair cells ($IC_{50} = 50$ μM) was due to the different ionic recording solutions.

3 Amiloride block of the oocyte MS channel does not vary significantly with complete substitution of external Na^+ (i.e. 100 mM) with K^+ in Ca^{2+}-free recording solution (in both Na^+ and K^+ the $IC_{50} = 0.5$ mM).

4 A physiological concentration (1.8 mM) of external Ca^{2+} blocks the oocyte MS channel and reduces the potency of amiloride block ($IC_{50} = 1.1$ mM) without altering the voltage-dependence or the Hill coefficient ($n = 1.8$) of amiloride block. The reduction in potency can be explained by surface charge screening by Ca^{2+} which reduces the effective amiloride surface concentration.

5 The present results indicate that factors other than ionic recording conditions must underlie the difference in potency of amiloride block of MS channels in oocytes and auditory hair cells.

Keywords: Ionic dependence; amiloride block; mechanosensitive channels; *Xenopus* oocytes; patch clamp

Introduction

Amiloride blocks mechanosensitivity in a variety of sensory and nonsensory cell types (Jorgensen & Ohmori, 1988; Lane *et al.*, 1991). Recently, it has been demonstrated that a series of amiloride analogues display the same order of potency in blocking mechanosensitive (MS) channels in frog oocytes (Lane *et al.*, 1992) and auditory hair cells (Ruesch *et al.*, 1991). This common pharmacology may indicate that mechanosensitive (MS) channels expressed in these functionally distinct cell types share a common 'amiloride receptor' and are structurally related molecules. Such a relationship could be important for biochemical studies since the oocyte MS channel is expressed with a much higher copy number ($> 10^6$ per oocyte) compared with that in the hair cell (10–100 per hair cell see Howard *et al.*, 1988). For this reason the oocyte may prove a more convenient preparation in attempts to purify the MS channel protein.

Although the amiloride receptor on MS channels in oocytes and hair cells appears qualitatively similar there is a quantitative difference in that amiloride and amiloride analogues are approximately 10 times less potent in blocking oocyte compared with hair cell MS channels (c.f., Lane *et al.*, 1991; 1992; Jorgensen & Ohmori, 1988; Ruesch *et al.*, 1991). However, this difference in absolute potency may be due to differences in the ionic recording conditions rather than structural differences in the receptor. Specifically, amiloride block in the oocyte was characterized in the absence of extracellular Ca^{2+} (Lane *et al.*, 1991; 1992), while the block in the hair cell was measured in the presence of normal (2 mM) Ca^{2+} (Jorgensen & Ohmori, 1988; Ruesch *et al.*, 1991). Since it has been demonstrated previously for other amiloride-sensitive ion pathways that extracellular Ca^{2+} may be an essential requirement for amiloride block (Cuthbert & Wong, 1972; Benos *et al.*, 1979; but see Desmedt *et al.*, 1991), we have examined the effect of extracellular Ca^{2+} on the amiloride block of the oocyte MS channel. Our study indicates that a physiological concentration of Ca^{2+} (1.8 mM) reduces rather than increases amiloride blocking potency. We also demonstrate that amiloride block of the MS channel is relatively insensitive to whether K^+ or Na^+ is the predominant charge carrier through the MS channel.

Methods

The methods used here to study amiloride block of MS channels in *Xenopus* oocytes were similar to those described previously (Lane *et al.*, 1991; 1992). Frogs were anaesthetized by being placed for approximately 20 min in a beaker containing 300 mg ethyl 3-aminobenzoate methanesulphonic acid (Aldrich) in 200 ml of distilled water. Sterile surgical procedures were used to remove the oocytes. Standard patch clamp techniques (Hamill *et al.*, 1981) were used to record single MS channel currents from *Xenopus laevis* oocytes on cell-attached patches (Methfessel *et al.*, 1986). Single MS channels were activated by brief mouth applied suction (10–50 mmHg). Different external recording (i.e. pipette) solutions were used. The control solution (Lane *et al.*, 1992) we refer to as '100 mM K^+, 0 mM Ca^{2+}' contained (in mM in all cases): KCl 100, EGTA 10 (KOH) and HEPES 10 (KOH), pH 7.2. The solution referred to as '100 mM Na^+, 0 mM Ca^{2+}' contained (mM): NaCl 100, EGTA 10 (NaOH) and HEPES 10 (NaOH). The solution referred to as '100 mM Na^+, 1.8 mM Ca^{2+}' contained: NaCl 100, $CaCl_2$ 1.8 and HEPES 10 (NaOH). Amiloride was dissolved in the pipette solution in concentrations ranging from 0.25–2 mM. The bath solution for all recordings contained (in mM): NaCl 115, $CaCl_2$ 1.8, KCl 1.5, HEPES 10 (NaOH), pH 7.2. All recordings were carried out at room temperature 19–22°C. Single MS channel currents were initially recorded on a digital video recorder (Neuro-corder, DR484, Neuro Data Instrument Corp. NY). Amplitudes were determined during subsequent playback directly from a storage oscilloscope screen, by centering the baseline current (i.e. closed channel state) on a grid line and reading the open channel current amplitude from a line judged to pass through the average of the open

[1] Author for correspondence.

channel noise. A minimum of 5 channel events per patch and 2–4 patches were used for any one condition (i.e. as defined by patch potential, amiloride concentration and ionic composition). This convenient technique is sufficiently accurate to discriminate amplitude changes as small as 0.1 pA (e.g. see Figure 2c).

Results

In our initial characterization of amiloride block of the oocyte MS channel (Lane *et al.*, 1991) we used zero Ca^{2+} and substituted Na^+ with 100 mM K^+ in the external recording solution in order to (1) remove the complication of Ca^{2+} block of the MS channel (Taglietti & Toselli, 1988; Yang & Sachs, 1989; Crawford *et al.*, 1991) and (2) measure MS channel current-voltage relations under near symmetrical transmembrane cationic conditions. In contrast, Jorgensen & Ohmori (1988) measured amiloride block with normal avian Ringer containing (in mM): NaCl 155, $CaCl_2$ 2.5, $MgCl_2$ 1.0, glucose 17, HEPES 10 (KOH). Since in other amiloride-sensitive pathways both Na^+ and Ca^{2+} have been shown to influence amiloride blocking potency, we tested their effects on amiloride block of the oocyte MS channel.

Figure 1 compares single MS channel current-voltage (I–V) relations measured in 100 mM external Na^+ with either zero or 1.8 mM Ca^{2+}. The inclusion of 1.8 mM Ca^{2+} in Na^+ pipette solution caused a significant reduction in inward MS channel currents (see single channel currents in Figure 1 insert) and gave a slope conductance measured at − 100 mV of 26 pS compared with 86 pS measured in Ca^{2+}-free Na^+ solution (Figure 1). This blocking effect of Ca^{2+} on the MS channel has been modelled previously (Taglietti & Toselli, 1988; Yang & Sachs, 1989), in terms of a 2 barrier single site permeation model in which Ca^{2+} inhibits current because of its higher affinity for a channel ion binding site compared with Na^+ and K^+.

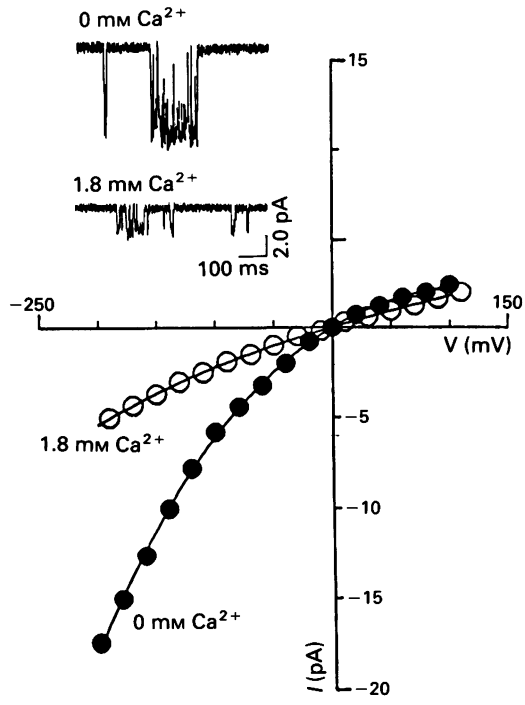


Figure 1 External Ca^{2+} block of the mechanosensitive (MS) channel. Single MS channel current-voltage relations measured from two different cell-attached patches in which the pipette solution contained either (in mM): NaCl 100, $CaCl_2$ 1.8, HEPES 10 (NaOH) (○) or NaCl 100, EGTA 10 (NaOH), HEPES 10 (NaOH) (●). The solid curves were fitted to the data by eye. The single MS channel current recordings shown in the insert were recorded at a patch potential of − 100 mV in each solution.

Figure 2 shows the I–V relations as a function of amiloride concentration and the corresponding Hill plots at − 100 mV for three different ionic solutions. The general character of the amiloride block was not changed in that the voltage-dependence was unaltered. However, the presence of Ca^{2+} caused a two fold reduction in amiloride potency. One possible explanation for this reduction is that Ca^{2+} screens surface negative charges and in this way reduces the effective amiloride surface concentration. For example, according to Hille *et al.* (1975), solutions with no divalent ions give a shift of − 18 mV in the surface potential of frog myelinated nerve relative to solutions with 2 mM Ca^{2+}. Figure 2a represents data from the initial characterization (Lane *et al.*, 1991) and are presented to facilitate comparison. Figure 2b shows data recorded when Na^+ was substituted for K^+. Consistent with previous studies of MS channels in both oocytes (Taglietti & Toselli, 1988) and hair cells (Corey & Hudspeth, 1979), we found that the MS channel discriminates weakly between Na^+ and K^+. The slope conductance measured at − 100 mV in the Na^+ solution was 86 pS compared with 91 pS K^+ solution. Figure 2c shows I–V relations obtained when 1.8 mM Ca^{2+} was included in the Na^+ solution. Although Ca^{2+} itself blocks the MS inward current by a factor of 4 or more at hyperpolarized potentials, amiloride causes an additional block which is qualitatively similar to that seen in Figure 2a and b. The solid curves fitted to the I–Vs in Figure 2a–c are the predicted relationships based on an extension of a model introduced previously (Lane *et al.*, 1991; 1992). The original model assumed a voltage-dependent conformational change of the MS channel followed by co-operative, voltage-independent binding of two blocking molecules. We have found that the reduction in amiloride potency caused by Ca^{2+} can be adequately explained by surface charge screening effects. In the extended model both the amiloride concentration and channel conformation are weighted by a Boltzmann term containing the shift in surface potential and this extended model is represented mathematically by Equation 1.

$$I_B = I_O \frac{1}{1 + ([B]/K_1 + [B]^2/(K_1K_2))(K_C/(1 + K_C))}$$

$$[B] = [B]_O f \qquad (1)$$

$$K_C = K_{OC} \exp\left\{-\frac{zF}{RT}\delta V\right\}/f$$

$$f = \exp\left\{-\frac{zF}{RT}\psi\right\}$$

In this equation, I_B and I_O are the MS currents in the presence and absence blocker, respectively. $[B]_O$ is the blocker concentration in the bulk aqueous phase, $[B]$ is the surface concentration of the blocker, K_1 and K_2 are the voltage independent dissociation constants for the first and second ligand binding, respectively. K_C and K_{OC} are the equilibrium constants at V and at V = 0, respectively, for the conformational change and δ describes the voltage-dependence of this change. f is the Boltzmann factor for the surface potential effect. ψ represents the change in the surface potential due to Ca^{2+} screening (see Hille, 1992) and was considered to be zero in the absence of Ca^{2+} and allowed to vary to give the best fit in the presence of Ca^{2+}. z, F, R, T and V have their usual meaning. By use of this model the difference in amiloride blocking potency measured in the presence and absence of Ca^{2+} could be explained by assuming the surface potential was increased by 23 mV when Ca^{2+} was present with no accompanying change in the rate constants K_1 (1.25 mM) and K_2 (160 μM). Analysis of the Hill plots for the K^+, Na^+ and Na^+ plus Ca^{2+} solutions gave IC_{50}s of 0.5 mM, 0.5 mM and 1.1 mM, respectively, and Hill coefficients of 2.0, 1.8 and 1.8, respectively.

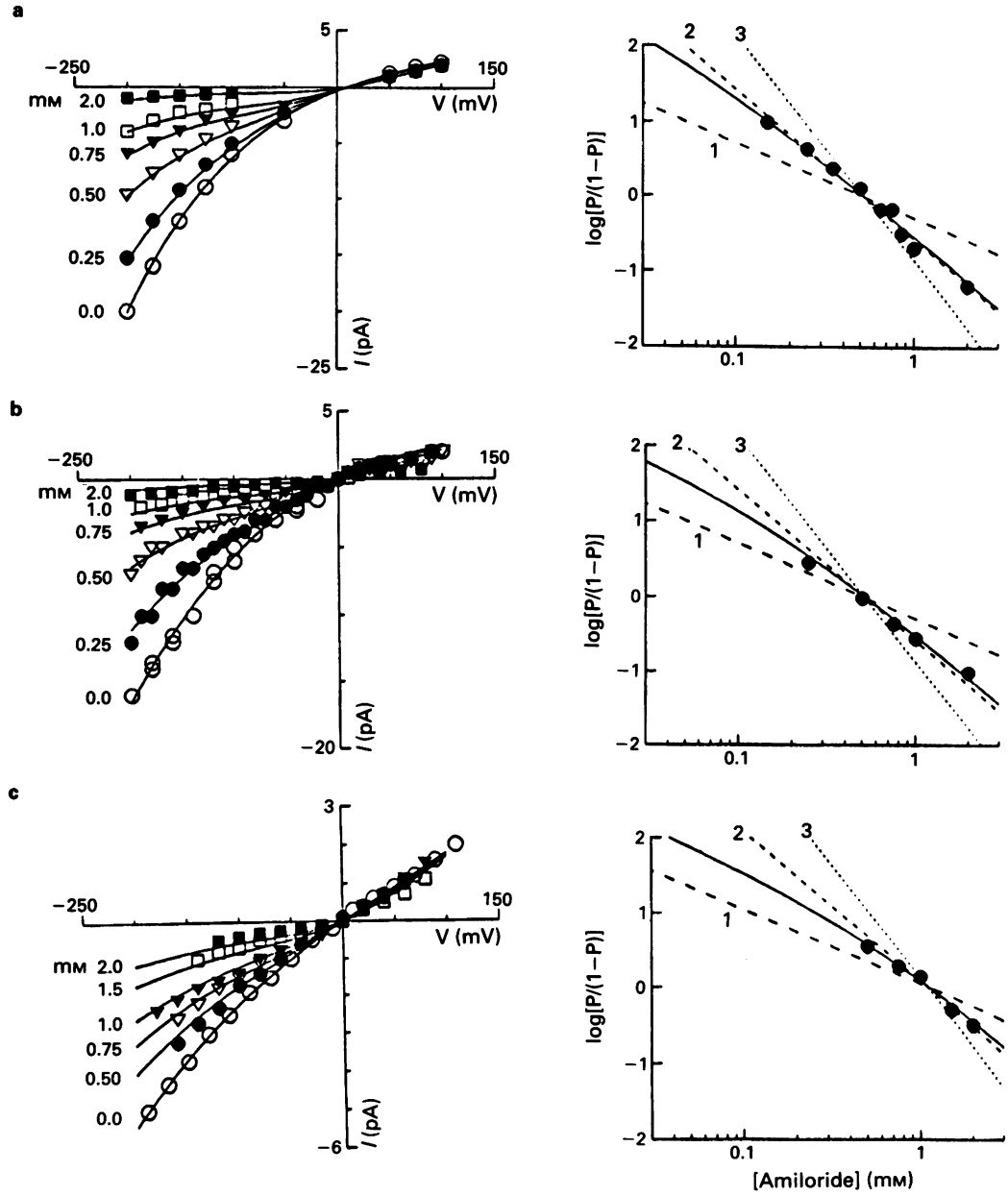


Figure 2 Single mechanosensitive (MS) channel current-voltage relations and Hill plots indicating the voltage- and concentration-dependence of amiloride block recorded under various ionic conditions. All measurements were from cell-attached patches and represent pooled data for each drug concentration from 2–4 patches. In all panels the solid lines fitted to the data were derived from the model described by equation 1. For all fits, $\delta = 0.65$ and $K_{OC} = 0.1$. The amiloride block in (a) (in 100 mM K^+ and 0 mM Ca^{2+}) produced values of $K_1 = 5$ mM (the binding of the first molecule) and $K_2 = 50$ μM (the binding of the second molecule) and for (b) (100 mM Na^+ and 0 mM Ca^{2+}), K_1 and $K_2 = 1.25$ mM and 160 μM, respectively, and (c) (100 mM Na^+ and 1.8 mM Ca^{2+}) K_1 and $K_2 = 1.25$ mM and 160 μM, respectively. For (a) and (b) the surface potential change was assumed to be zero. While for (c), K_1 and K_2 were set at the same values as in (b), and the best fit for the surface potential change gave a value of −23 mV. Hill plots calculated from MS channel currents measured at − 100 mV gave Hill coefficients of 2 (a), 1.8 (b) and 1.8 (c). $P = I/I_O$ where I and I_O are the single MS channel currents in the presence and absence of blocker, respectively. The broken lines show the slopes predicted for Hill coefficients (n) of 1, 2 and 3.

The above experiments indicate that the presence of Na^+ or Ca^{2+} does not potentiate the amiloride block of the oocyte MS channel. Furthermore, we found no significant change in blocking potency (i.e. $IC_{50} = 1.2$ mM) when 1 mM Mg^{2+} was included with Ca^{2+} in the Na^+ Ringer (i.e. 1.8 Ca^{2+}, 100 Na^+, 1 Mg^{2+}).

Discussion

This study indicates that differences in external ionic recording conditions are unable to explain the 10 fold difference in potency obtained for amiloride block of the oocyte (Lane *et al.*, 1991) compared with the hair cell MS channel (Jorgensen & Ohmori, 1988). Neither the presence of high (100 mM) external K^+nor the absence of Na^+, Ca^{2+} and Mg^{2+} in the original oocyte recording solution (Lane *et al.*, 1991) can explain the lower potency of amiloride block in this preparation. In fact the presence of a physiological Ca^{2+} concentration reduces the potency of amiloride block of the oocyte MS channel. If Ca^{2+} has a similar effect on amiloride block of the hair cell, then this drug may be an even more potent blocker *in situ* than previously found (i.e. $IC_{50} < 50$ μM). Ca^{2+} concentration in the endolymph that normally baths

the hair cell transducing apparatus has been measured as 65 μM (see Crawford *et al.*, 1991), which is much lower than the 2 mM used in artificial endolymph solution (Jorgensen & Ohmori, 1988; Ruesch *et al.*, 1991).

Although Ca^{2+} and amiloride block MS channels they do so by apparently different mechanisms. Ca^{2+} block can be modelled in terms of a permeation mechanism in which Ca^{2+} displays a higher affinity for an ion binding site compared with Na^+ and K^+ and in this way inhibits ion conduction through the channel (Taglietti & Toselli, 1988; Yang & Sachs, 1989). In contrast, the voltage- and concentration-dependence of amiloride block is more consistent with a voltage-dependent conformational change of the MS channel followed by co-operative, voltage-independent binding of two amiloride molecules (Lane *et al.*, 1991). We have shown that the effect of Ca^{2+} of reducing amiloride blocking potency can be modelled as surface charge screening effect. For example, it was possible to fit data obtained in the presence of Ca^{2+} by use of identical amiloride binding constants to those used in the absence of Ca^{2+} by including a surface potential Boltzmann coefficient (see Figure 2b and c). Despite the adequate fit we cannot exclude alternative explanations such as direct effects of Ca^{2+} on either amiloride binding reactions or on the proposed voltage-dependent conformational change of the channel.

The higher potency of amiloride block of hair cell compared with oocyte MS channels may reflect structural differences in the 'amiloride receptors' on the MS channels. However, the finding that the two MS channels display the same order of sensitivity to block by a series of amiloride analogues (Ruesch *et al.*, 1991; Lane *et al.*, 1992) indicates they are more similar to one another than to other non-MS amiloride-sensitive ion pathways, such as the epithelial Na^+ channel, the Na^+-H^+exchanger and the Na^+-Ca^{2+} exchanger (see Lane *et al.*, 1992; Hamill *et al.*, 1992). It may be that physical or structural features, not directly associated with the amiloride binding site, limit amiloride's access to an otherwise identical amiloride receptor on the two MS channel molecules.

This study indicates further differences between amiloride block of the epithelial Na^+ and the MS cation channel. In the case of the epithelial Na^+ channel the absolute potency of amiloride block has been shown to vary with changes in either extracellular Na^+ and Ca^{2+} ions (Cuthbert & Wong, 1972; Benos *et al.*, 1979; Garty & Benos, 1988, but see Desmedt *et al.*, 1991). In contrast, this study indicates that amiloride block of the oocyte MS channel is relatively insensitive to whether Na^+ or Ca^{2+} is present or absent in the extracellular solution.

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Activation of the Na^+/K^+-pump in rat peritoneal mast cells following histamine release: a possible role in cell recovery

[1]T. Knudsen, I. Ferjan & T. Johansen

Department of Pharmacology, Institute of Medical Biology, Odense University, J.B. Winsløwsvej 19, DK-5000 Odense C, Denmark

1 The activity of the Na^+/K^+-pump in rat peritoneal mast cells was measured at various time intervals after induction of cellular histamine release by compound 48/80 or by the antigen-antibody reaction. The Na^+/K^+-pump activity was assessed as the ouabain-sensitive potassium uptake of the cells using $^{86}Rb^+$ as a tracer for potassium ($K^+(^{86}Rb^+)$-uptake).

2 Stimulation of the cells with compound 48/80 induced a time and concentration dependent increase of the Na^+/K^+-pump activity. The pump activity was maximal 2 min after stimulation of the cells. Then, the activity gradually decreased and reached a level not significantly different from the controls after 2 h of incubation.

3 When the cells were stimulated by the antigen-antibody reaction, there was also a rapid (within 5 min) stimulation of the Na^+/K^+-pump. In contrast to the stimulation with compound 48/80, the pump activity returned to the control level after 60 min of incubation with antigen.

4 The ouabain-resistant potassium uptake of the cells was increased after stimulation of the cells, regardless of the secretagogue used. This probably reflects the increased surface area of the cells present after the histamine release.

5 On the basis of the present results, we suggest a role for the Na^+/K^+-pump in the recovery process of the mast cell following histamine release.

Keywords: Mast cells; Na^+/K^+-pump; ouabain; cell survival

Introduction

Activation of rat peritoneal mast cells immunologically or by non-immunological secretagogues induces exocytosis, by which the cells release their granular contents of histamine and other inflammatory mediators (Bernstein & Lawrence, 1990). The secretory process is associated with extensive structural changes in the cell morphology (Holm Nielsen *et al.*, 1981). However, the mast cells recover after the exocytosis and gradually return to their resting morphology and normal biochemical and functional characteristics. The recovery process has been demonstrated by experiments performed both *in vivo* (Padawer, 1970; Krüger & Lagunoff, 1981) and *in vitro* (Holm-Nielsen *et al.*, 1981; Burwen, 1982; Weill & Renoux, 1982; Slutsky *et al.*, 1987; Levi-Schaffer & Riesel, 1989).

Recently, it has been shown that the plasma membrane of the rat peritoneal mast cell contains a Na^+/K^+-pump (Knudsen & Johansen, 1989a). The pump activity is inhibited by calcium (Knudsen & Johansen, 1989a,b) and other divalent cations (Knudsen *et al.*, 1990). Although the pump has a large reserve capacity (Knudsen & Johnsen, 1990), increased pump activity does not seem to be needed for the process of histamine release. In contrast, inhibition of the pump activity by the digitalis glycoside, ouabain, enhances the release of histamine from rat peritoneal mast cells when stimulated immunologically (Frossard *et al.*, 1983) or by the non-immunological secretagogue, compound 48/80 (Amellal *et al.*, 1984; Johansen *et al.*, 1990). We, therefore, speculated that the Na^+/K^+-pump might be involved in the recovery process of the mast cells following the exocytotic release of their granular contents. We have measured the activity of the Na^+/K^+-pump at various time intervals after activation of the secretory process by the non-immunological secretagogue compound 48/80 or by the antigen-antibody reaction. The activity of the Na^+/K^+-pump was assayed as the ouabain-sensitive potassium uptake into the cells. For practical reasons $^{86}Rb^+$ was used as a tracer for potassium due to its longer half-life (18.6 days) compared with that of $^{42}K^+$ (12 h). This is possible because it has been shown that rubidium and potassium are treated almost identically by the Na^+/K^+-pump (Skou, 1960). In the following the cellular potassium uptake is referred to as the $K^+(^{86}Rb^+)$-uptake in order to emphasize the use of $^{86}Rb^+$.

Methods

Isolation of mast cells

Male Sprague-Dawley rats, 255–490 g, were used for the experiments with compound 48/80 and male Wistar rats, 330–360 g, for the experiments with antigen. In our experience, the latter were much easier to sensitize. The rats were killed by decapitation under light ether anaesthesia. The mast cells were isolated by differential centrifugation of mixed peritoneal cells in a self-generating gradient of Percoll as previously described (Knudsen & Johansen, 1989a). Following the isolation procedure, the cells were suspended in an appropriate volume of Krebs-Ringer solution.

An automatic cell counter was used to determine the number of cells, and inspection of a stained (toluidine blue) smear was used to assess the fraction of mast cells, which was 97.0 ± 1.8% (mean ± s.d., $n = 34$).

Sensitization of the rats

Male Wistar rats were sensitized to egg albumin by injecting 1 ml of *Pertussis* vaccine with 5 mg egg albumin subcutaneously on the first day followed by booster doses of 5 mg on the second and the third day. On the second day, the booster dose was given together with 0.5 ml of Freund's incomplete

[1] Author for correspondence.

adjuvant. The rates were used 3–5 weeks after the first injection.

Incubation procedure

Mast cell suspensions pooled from 2–8 rats were divided into samples of equal numbers of cells (between 9.2×10^4 and 2.9×10^5 cells per sample) in a volume of 0.3 ml and preincubated at 37°C as indicated in the legends to the figures. This was followed by the addition of 100 µl of a Krebs-Ringer solution containing either compound 48/80 or egg albumin and the cells were preincubated for a second period as indicated in the legends to figures. After preincubation, 100 µl of a Krebs-Ringer solution contained either trace amounts of $^{86}Rb^+$ (for determination of cellular $K^+(^{86}Rb^+)$-uptake) or no $^{86}Rb^+$ (for determination of cellular histamine release) was added to the cell samples to give a final volume of 0.5 ml during the incubation. After 3–10 min (see legends to figures), the incubation was terminated by addition of ice-chilled Krebs-Ringer solution 9.5 ml ($K^+(^{86}Rb^+)$-uptake) or 1.75 ml (histamine release) to the cell suspensions.

When egg albumin was used as secretagogue, the preincubation took place in the presence of phosphatidylserine (final concentration 0.05 mg ml^{-1}), which is known to enhance immunologically induced histamine release from rat peritoneal mast cells (Goth *et al.*, 1971). Three control experiments showed that phosphatidylserine (0.05 mg ml^{-1}) only slightly affected the Na^+/K^+-pump activity. After 60 min of preincubation of the cells, the pump activity (in pmol per 10^6 cells min^{-1}) was 126 ± 41 in the presence and 101 ± 23 (mean ± s.e.mean) in the absence of phosphatidylserine. After 5 min of preincubation (sodium loaded cells), the pump activity was 589 ± 91 pmol per 10^6 cells min^{-1} in the absence and 725 ± 120 pmol per 10^6 min^{-1} in the presence of phosphatidylserine.

Measurement of the $^{86}Rb^+$-uptake of the mast cells

The concentration of $^{86}Rb^+$ during the incubation of the cells varied from 1 to 175 µM, and the specific activity was 11.7 MBq per mmol of K^+. The radioactive concentration of $^{86}Rb^+$ during the incubation of the cells was always 1.5 µCi ml^{-1}. In order to remove the extracellular radioactivity after termination of the incubation, the cell suspensions were washed twice by centrifugation at 600 *g* for 15 min at 4°C. Cell lysis was performed by adding 1 ml of an aqueous solution of NaOH (final concentration 47.6 mM) to the cell suspension, and the whole sample was then transferred to counting vials and mixed with 4 ml of scintillation liquid. All samples were run in duplicate. $^{86}Rb^+$ was assayed by liquid scintillation counting with an efficiency of about 95% and no corrections were made for this. The radioactive concentration of $^{86}Rb^+$ in relation to the sum of the concentrations of K^+ and Rb^+ in the extracellular medium was used to calculate the cellular $K^+(^{86}Rb^+)$-uptake. Samples containing Krebs-Ringer solution and no cells were run in parallel in order to determine the radioactivity not associated with the cells. This was always 10% or less of the total radioactivity associated with the cells, and a correction was applied for this. The calculated values for the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake, which was taken to be equivalent to the activity of the Na^+/K^+-pump, was determined as the difference between the calculated uptake of $K^+(^{86}Rb^+)$ into cells incubated in absence and presence of ouabain (1 mM).

Measurement of cellular histamine release

The concentrations of compound 48/80 during the incubation varied between 0.1–1.0 µg ml^{-1} (see legends to figures). The concentration of egg albumin during incubation was always 0.5 mg ml^{-1}. After termination of the incubation, the cells were centrifuged for 10 min at 220 *g*. The supernatant was collected for determination of the amount of histamine released, and the cell pellet was suspended in 250 µl of a physiological saline solution and boiled for 3 min. After addition of 1.75 ml of Krebs-Ringer solution and centrifugation for 10 min at 220 *g* the supernatant was collected for determination of the residual histamine. Histamine was determined by the fluorimetric method (Shore *et al.*, 1959) omitting the extraction procedure. The histamine release was expressed as a percentage of the total histamine content of the cells. The spontaneous histamine release in the absence of compound 48/80 and egg albumin was always subtracted and did not exceed 10%. Since the presence of ouabain during the incubation did not affect the amount of released histamine significantly, the given values for the histamine release are those obtained in the absence of ouabain. All samples were run in parallel. None of the agents used interfered with the histamine assay.

Solutions

The Krebs-Ringer solution had the following composition (mM): NaCl 132.3, KCl 4.75, $CaCl_2$ 1.0, $MgSO_4$ 1.2, Tris-HCl 12.5. All solutions contained bovine serum albumin, 1 mg ml^{-1}, and glucose, 1 mg ml^{-1}. The pH was 7.4 (room temperature).

Materials

Bovine serum albumin, compound 48/80 and phosphatidylserine were supplied by Sigma Chemical Company (St. Louis, U.S.A.), glucose by Merck (Darmstadt, Germany), Percoll by Pharmacia Fine Chemicals (Sweden), Scintillation liquid (Ecoscint) by BN Plastics (Helsinge, Denmark), $^{86}Rb^+$ (specific activity 1–12 MCi mg^{-1} Rb^+) by Amersham (Buckinghamshire, U.K.), ouabain by Mecobenzon (Denmark), *Pertussis* vaccine and Freund's incomplete adjuvant by Statens Serum Institut (Denmark) and egg albumin by Mallickrodt Chemical Work (St. Louis, U.S.A.). All other chemicals were of analytical grade. Ouabain was prepared as a 5 mM stock in Krebs-Ringer solution. Compound 48/80 was prepared as a 1 mg ml^{-1} stock solution in 0.9% saline. Phosphatidylserine was prepared as a 0.5 mg ml^{-1} stock in Krebs-Ringer solution. $CuCl_2$ was prepared as a 5 mM stock in Krebs-Ringer solution without albumin and glucose.

Presentation of results

All results are shown as mean ± s.e.mean of five experiments. The Mann-Whitney U-test was used for the statistical analysis of the data. $P < 0.05$ was used as threshold level of significance.

Results

Exposure of mast cells to compound 48/80 caused a large increase in the activity of the Na^+/K^+-pump (Figure 1a). After 20 s preincubation of the cells with compound 48/80, the pump activity was 1033 ± 66 pmol per 10^6 cells min^{-1} and this was significantly different from the pump activity of mast cells not exposed to compound 48/80 (218 ± 58 pmol per 10^6 cells min^{-1}, $P < 0.01$). The pump activity was maximally increased 2 min after the addition of compound 48/80 (1558 ± 88 pmol per 10^6 cells min^{-1} in the presence and 206 ± 27 pmol per 10^6 cells min^{-1} in the absence of compound 48/80, $P < 0.01$). Then it gradually declined and after 2 h attained a value of 315 ± 68 pmol per 10^6 cells min^{-1}, which was not significantly different from the control value (114 ± 53 pmol per 10^6 cells min^{-1} in the absence of compound 48/80, $P > 0.05$) (Figure 1b). Histamine release induced by compound 48/80 was 69.8% ± 3.9% after 20 s preincubation and this was not changed significantly by prolonged incubation of the cells with compound 48/80.

The ouabain-resistant $K^+(^{86}Rb^+)$-uptake was also increas-

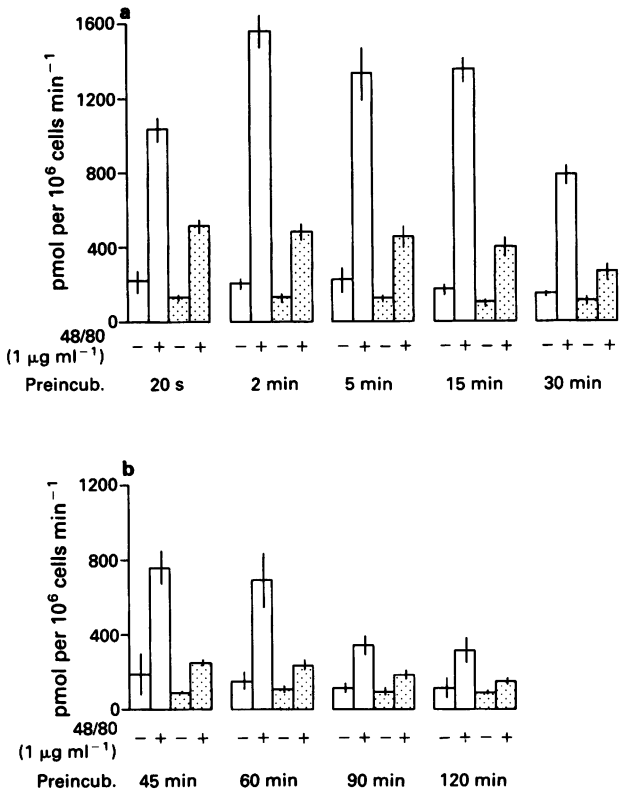


Figure 1 Time dependent stimulation of cellular $K^+(^{86}Rb^+)$-uptake after compound 48/80-induced histamine release. The cells were preincubated for 60 min in a Krebs-Ringer solution containing calcium 1 mM, magnesium 1.2 mM and potassium 4.75 mM. Then, 100 μl of compound 48/80 was added giving a final concentration of 1 μg ml^{-1} and the cells were preincubated for a second period of 20 s–30 min (a) or 45–120 min (b) before the addition of $K^+(^{86}Rb^+)$-solution containing either no ouabain or ouabain 1 mM (final concentration). For the determination of the cellular histamine release, $^{86}Rb^+$ was omitted. The incubation with $K^+(^{86}Rb^+)$ lasted for 3 min in the absence and for 10 min in the presence of ouabain. Control samples without compound 48/80 were run in parallel. The abscissa scale shows the time of the second preincubation of the cells, and the absence or presence of compound 48/80 is indicated. The ordinate scale shows the ouabain-sensitive (open columns) and the ouabain-resistant $K^+(^{86}Rb^+)$-uptake (stippled columns).

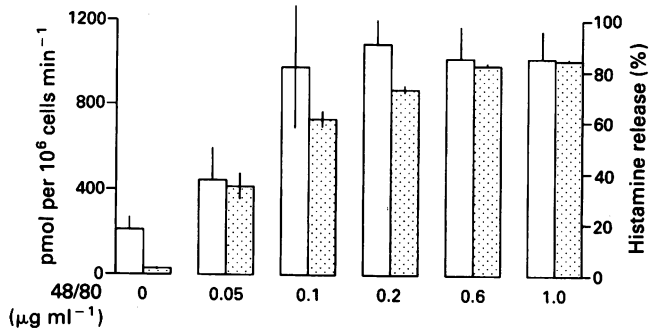


Figure 2 Dose-dependent stimulation by compound 48/80 of cellular histamine secretion and of the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake. The cells were preincubated for 60 min in a Krebs-Ringer solution containing calcium 1 mM, magnesium 1.2 mM and potassium 4.75 mM. Then 100 μl of compound 48/80 giving final concentrations from 0 to 1 μg ml^{-1} were added for another 5 min before addition of $K^+(^{86}Rb^+)$-solution. $K^+(^{86}Rb^+)$-solution containing either no ouabain or ouabain 1 mM (final concentration) was used. The cells were incubated for 3 min in the absence and for 10 min in the presence of ouabain. Samples without $^{86}Rb^+$ were run in parallel for determination of celullar histamine release. The abscissa scale shows the concentrations of compound 48/80 used, while the ordinates show the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake (left hand scale, open columns) and the cellular histamine release (right hand scale, stippled columns).

ed by compound 48/80 (Figure 1). The increase was maximal after 20 s preincubation of the cells with compound 48/80 (516 ± 38 pmol per 10^6 cells min^{-1}), and this value was statistically different from the control value from cells not exposed to compound 48/80 (131 ± 16 pmol per 10^6 cells min^{-1}, $P<0.01$). There was a slow decrease of the ouabain-resistant $K^+(^{86}Rb^+)$-uptake towards the control value from untreated cells in the following 2 h, and then the values were 150 ± 18 pmol per 10^6 cells min^{-1} in the presence and 91 ± 11 pmol per 10^6 cells min^{-1} in the absence of compound 48/80 ($P<0.05$) (Figure 1b).

The effect of compound 48/80 on the pump activity was dependent on the concentration of compound 48/80 present in the cell suspension and the increase in the pump activity was parallel to a dose-dependent increase in histamine release (Figure 2). The maximal pump activity caused by compound 48/80, 0.2 μg ml^{-1}, was 1091 ± 109 pmol per 10^6 cells min^{-1}, and the control value in the absence of compound 48/80 was 214 ± 56 pmol per 10^6 cells min^{-1}. There was a gradual increase in the ouabain-resistant $K^+(^{86}Rb^+)$-uptake from 156 ± 32 pmol per 10^6 cells min^{-1} in the absence of compound 48/80 to 412 ± 121 pmol per 10^6 cells min^{-1} in the presence of compound 48/80, 1.0 μg ml^{-1}. The histamine release also gradually increased from 2.4 ± 0.2% in the absence of compound 48/80 to 84.5 ± 0.3% in the presence of compound 48/80, 1 μg ml^{-1}.

$CuCl_2$ 100 μM, inhibited both the release of histamine induced by compound 48/80 and the increased pump activity following histamine release (Figure 3). The histamine release was 72.2% ± 2.1% in the absence and 4.7% ± 1.1% in the presence of $CuCl_2$ ($P<0.01$), while the pump activity (the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake in pmol per 10^6 cells min^{-1}) was 1274 ± 73 and 139 ± 24 in the absence and presence, respectively, of $CuCl_2$ ($P<0.01$).

A minor increase in the spontaneous histamine release was observed in presence of $CuCl_2$ from 3.3% ± 0.3% to 7.0% ± 0.5% ($P<0.01$). $CuCl_2$ had no significant effect on the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake into mast cells not exposed to compound 48/80. In the absence and presence of $CuCl_2$ the values were 98 ± 17 and 120 ± 16 pmol per 10^6 cells min^{-1}, respectively. In the absence of compound 48/80, the ouabain-resistant $K^+(^{86}Rb^+)$-uptake was reduced from 87 ± 7 pmol per 10^6 cells min^{-1} to 33 ± 4 pmol per 10^6 cells min^{-1} by the addition of $CuCl_2$ ($P<0.01$). In the presence of compound 48/80, the ouabain-resistant $K^+(^{86}Rb^+)$-uptake was 434 ± 20 pmol per 10^6 cells min^{-1} in the absence and 41 ± 6 pmol per 10^6 cells min^{-1} in the presence of $CuCl_2$ ($P<0.01$).

A control experiment showed that $CuCl_2$, 100 μM, did not inhibit the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake in a calcium-free medium, which is known to enhance the uptake (Knudsen & Johansen, 1989a). After 60 min of preincubation of the cells at 37°C, the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake was 280 pmol per 10^6 cells min^{-1} in the absence and 378 pmol per 10^6 cells min^{-1} in the presence of $CuCl_2$. In the same control experiment, the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake was 1069 pmol per 10^6 cells min^{-1} in the absence and 1146 pmol per 10^6 cells min^{-1} in the presence of $CuCl_2$ in cells which had been preloaded with sodium by allowing only 5 min of preincubation of the cells at 37°C before measurement of the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake.

Exposure of sensitized mast cells to antigen (egg albumin) caused a large increase in the activity of the Na^+/K^+-pump compared to that of the unstimulated cells (Figure 4). After 5 min incubation of the cells with antigen the pump activity was (in pmol per 10^6 cells min^{-1}) 906 ± 101, and in the absence of antigen it was 164 ± 51 ($P<0.01$). Histamine

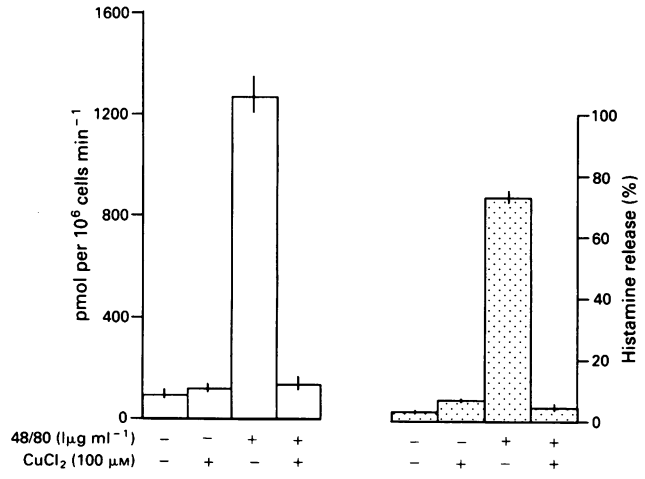


Figure 3 Effect of $CuCl_2$ on compound 48/80 induced histamine release and on the poststimulatory ouabain-sensitive $K^+(^{86}Rb^+)$-uptake. The cells were preincubated for 60 min in a Krebs-Ringer solution containing calcium 1 mM, magnesium 1.2 mM and potassium 4.75 mM in the presence of $CuCl_2$ 100 μM. Control samples without $CuCl_2$ were run in parallel. Following preincubation, 100 μl of compound 48/80 (final concentration 1 μg ml^{-1}) or 100 μl of Krebs-Ringer was added for 5 min. This was followed by addition of $K^+(^{86}Rb^+)$ solution containing either no ouabain or ouabain 1 mM (final concentration). The cells were incubated for 3 min in the absence and for 10 min in the presence of ouabain. For determination of the cellular histamine release, Krebs-Ringer solution without $^{86}Rb^+$ was added instead of the $K^+(^{86}Rb^+)$-solution. At the bottom of the figure the various combinations of compound 48/80 and $CuCl_2$ are shown. The ouabain-sensitive $K^+(^{86}Rb^+)$-uptake is shown by open columns (left hand scale) and the cellular histamine release by stippled columns (right hand scale).

release was 38.6 ± 2.0% and the spontaneous release in absence of antigen was 4.0 ± 0.4%. These values for secretion were not changed significantly by an increase in the time of incubation of the cells with antigen from 5 to 60 min. There was no significant difference in the pump activity when comparing the values from samples incubated for 60 min with and without antigen (Figure 4). The pump activities in presence and absence of antigen were (in pmol per 10^6 cells min^{-1}) 224 ± 23 and 256 ± 71, respectively.

While in the presence of antigen the ouabain-resistant $K^+(^{86}Rb^+)$-uptake was significantly larger after 5 min incubation (188 ± 23 pmol per 10^6 cells min^{-1}) than after 60 min incubation (100 ± 13 pmol per 10^6 cells min^{-1}) ($P<0.05$), there was no significant difference in the absence of antigen. After 5 min incubation without antigen the ouabain-resistant $K^+(^{86}Rb^+)$-uptake was 108 ± 10 pmol per 10^6 cells min^{-1}.

$CuCl_2$, 100 μM, inhibited both the release of histamine induced by antigen and the increased pump activity following histamine release (Figure 5). Histamine release was 44.0 ± 6.0% in the absence of $CuCl_2$ and 4.9 ± 1.2% in its presence ($P<0.01$) and the pump activity (the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake in pmol per 10^6 cells min^{-1}) was decreased from 1067 ± 117 to 167 ± 28 by the addition of $CuCl_2$ ($P<0.01$).

A minor increase in the spontaneous histamine release was observed in presence of $CuCl_2$, from 4.5 ± 0.4% to 7.9 ± 0.4% ($P<0.01$). $CuCl_2$ had no significant effect on the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake into mast cells not exposed to antigen. In the absence and presence of $CuCl_2$ the values were 128 ± 15 and 158 ± 21, respectively. In the absence of antigen the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake was reduced from 79 ± 9 pmol per 10^6 cells min^{-1} to 29 ± 5 pmol per 10^6 cells min^{-1} by the addition of $CuCl_2$ ($P<0.01$). In the presence of antigen, addition of $CuCl_2$ decreased the ouabain-resistant $K^+(^{86}Rb^+)$-uptake from 231 ± 22 pmol per 10^6 cells min^{-1} to 32 ± 9 pmol per 10^6 cells min^{-1} ($P<0.01$).

In order to compare the Na^+/K^+-pump activity in Sprague-Dawley rats and Wistar rats, four control experiments

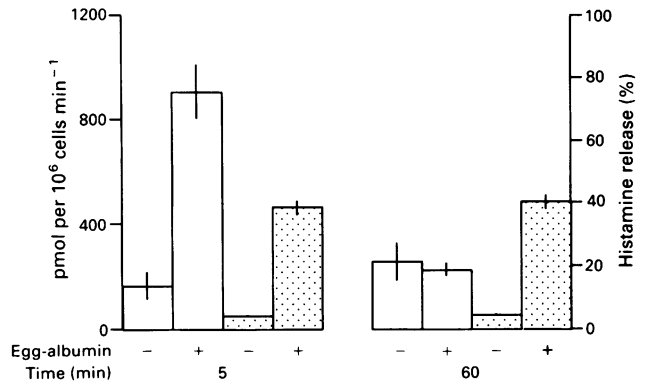


Figure 4 Effect of antigenic stimulation on the cellular histamine release and the poststimulatory ouabain-sensitive $K^+(^{86}Rb^+)$-uptake. The cells were preincubated for 60 min in a Krebs-Ringer solution containing calcium 1 mM, magnesium 1.2 mM and potassium 4.75 mM in the presence of phosphatidylserine 0.05 mg ml^{-1} (final concentration). Following preincubation 100 μl of Krebs-Ringer solution containing egg albumin (final concentration 0.5 mg ml^{-1}) was added, and the cells were preincubated for a second period of 5 or 60 min. Control samples without egg albumin were run in parallel. After the second preincubation period, $K^+(^{86}Rb^+)$-solution containing either no ouabain or ouabain 1 mM (final concentration) was added and the cells were incubated in presence of this solution for 10 min in the presence or for 3 min in absence of ouabain. For determination of the cellular histamine release, the cells were incubated in Krebs-Ringer without $^{86}Rb^+$. At the bottom of the figure the absence or presence of egg albumin and the time of the second preincubation period is indicated. Open columns show the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake (left hand scale) and stippled columns show the cellular histamine release (right hand scale).

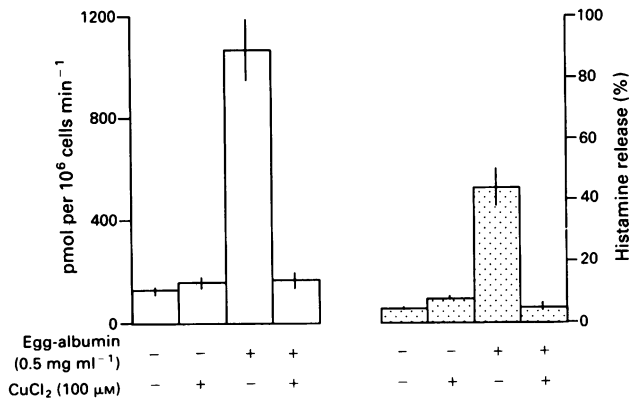


Figure 5 Effect of $CuCl_2$ on antigen induced histamine release and the poststimulatory ouabain-sensitive $K^+(^{86}Rb^+)$-uptake. The cells were preincubated for 60 min in a Krebs-Ringer solution containing calcium 1 mM, magnesium 1.2 mM and potassium 4.75 mM in the presence of phosphatidylserine 0.05 mg ml^{-1} and $CuCl_2$ 100 μM (final concentrations). Control samples without $CuCl_2$ were run in parallel. Following the preincubation, 100 μl of a Krebs-Ringer solution containing egg albumin (final concentration 0.5 mg ml^{-1}) or 100 μl of Krebs-Ringer solution was added for 5 min. This was followed by addition of $K^+(^{86}Rb^+)$-solution containing either no ouabain or ouabain 1 mM (final concentration). The cells were incubated for 3 min in the absence and for 10 min in the presence of ouabain. For determination of the cellular histamine release, Krebs-Ringer solution without $^{86}Rb^+$ was added instead of the $K^+(^{86}Rb^+)$-solution. At the bottom of the figure the various combinations of egg albumin and $CuCl_2$ are indicated. Open columns show the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake (left hand scale), while stippled columns show the cellular histamine release (right hand scale).

were performed in which Wistar mast cells were stimulated with compound 48/80. The data from these experiments were compared with the results obtained in similar experiments with Sprague-Dawley mast cells (data from Figure 2). Compound 48/80, 0.5 $\mu g\,ml^{-1}$, stimulated the Sprague-Dawley mast cells to release 34.7 ± 4.8% histamine and the pump activity 5 min after the addition of compound 48/80 was 444 ± 149 pmol per 10^6 cells min^{-1}. A similar degree of histamine release (29.2 ± 5.1%) from Wistar mast cells was obtained by exposure to compound 48/80, 0.2 $\mu g\,ml^{-1}$. In these cells, the pump activity 5 min after the addition of the secretagogue was 580 ± 112 pmol per 10^6 cells min^{-1}. The difference in pump activity between Sprague-Dawley and Wistar mast cells was not significant ($P > 0.1$).

Discussion

The Na^+/K^+-pump activity of resting intact mast cells was 100–200 pmol per 10^6 cells min^{-1} when the cells were incubated in a calcium containing medium, and similar values have been observed previously (Knudsen & Johansen, 1989a,b). Two min after activation of the secretory response with compound 48/80, the pump activity was increased to a maximal value of 10 times that of the unstimulated, resting cells. Histamine release induced by compound 48/80 is completed within 12 s (Bloom & Chakravarty, 1970; Bertelsen & Johansen, 1991). Immediately after completion of the release process, the pump activity was increased to a value of 7 times the resting value. The time relation between the release of histamine as well as the parallel dose-dependency of the release process and the stimulation of the pump activity (Figure 2) indicate a close association of histamine release with activation of the Na^+/K^+-pump. It is not possible to detect whether the pump activation occurs during the release process, since measurement of the cellular uptake of $^{86}Rb^+$ requires at least 3 min incubation of the cells with the radioactive tracer. However, the long-lasting increase of the pump activity may indicate that increased pump activity is a consequence of the release process rather than being one of the biochemical reactions necessary for the exocytosis to occur. Similarly, it is likely that antigen-induced histamine release initiates an increased pump-activity. Antigen-induced histamine release is completed within 1 min (Johansen & Chakravarty, 1975). Five min after addition of the antigen to the sensitized mast cells the pump activity was about 6 times the resting value of the pump activity (Figure 4).

The importance of the release process for the subsequent increase of the pump activity was further substantiated by the observation that when histamine release was inhibited by $CuCl_2$ there was no change in the pump activity. Copper was used to block the release of histamine since it has been shown to inhibit the release from rat peritoneal mast cells induced by compound 48/80 or by concanavalin A (Sharma & Jande, 1989), and the activation of concanavalin A resembles the activation by antigen (Healicon & Foreman, 1984). The mechanism by which the inhibition occurs is not known, but $CuCl_2$ does not damage the cells and as a consequence, does not cause a large spontaneous release of histamine. Apparently, there is no serious inhibition of cellular energy production (Sharma & Jande, 1989) and in presence of $CuCl_2$ we observed a large increase of the pump activity when the mast cells were loaded with sodium, indicating that $CuCl_2$ does not itself block the activity of Na^+/K^+-pump. The finding of an increased Na^+/K^+-pump activity after stimulation of rat peritoneal mast cells is in agreement with the findings of Bronner *et al.* (1991). These authors studied membrane potential changes following histamine release from mast cells stimulated with compound 48/80 or antigen. They observed that the late phase hyperpolarization of the plasma membrane occurring 2 min after stimulation of the cells was inhibited by ouabain. On the basis of this result, it was concluded that the late phase hyperpolarization was due to activation of the Na^+/K^+-pump following histamine release.

When mast cells are stimulated to exocytosis, they undergo major structural changes, which besides degranulation consist of cellular swelling and disappearance of the fingerlike projections of the plasma membrane (Holm-Nielsen *et al.*, 1981). However, studies carried out *in vivo* (Padawar, 1970; Kruger & Lagunoff, 1981) and *in vitro* (Holm-Nielsen *et al.*, 1981; Slutsky *et al.*, 1987; Levi-Schaeffer & Riesel, 1989) indicate that the mast cells survive the degranulation and reattain their normal structure and function. Holm-Nielsen *et al.* (1981) followed the recovery process of the mast cells by electron microscopy from 10 s to 48 h after application of compound 48/80. Immediately after the stimulation of the cells, their diameter was increased from 12 μm to 18 μm. During the first 2 h after the release, small exocytotic vesicles fused to form a large central vacuole in the cytoplasm. This was followed by a gradual reduction of the diameter of the cells, but even after 48 h, full morphological recovery was not obtained. Levi-Schaffer & Riesel (1989) were able to keep rat mast cells viable in a culture system. They demonstrated full structural, biochemical and functional reconstitution of the mast cell 14 days after stimulation of the cells with compound 48/80. The resting morphology of the cells were reattained within 3–4 h, while it took several days to replenish the histamine stores. In another culture system it has been shown that the recovery process includes a reduction of the cell size, a sealing of the exocytotic cavities and a transient displacement of the cell nucleus (Slutsky *et al.*, 1987). These authors also found that the recovery of the cells was dependent on an interaction between calcium and calmodulin, and on microfilaments and cellular energy.

Thus, although mast cells are known to recover from degranulation, very little is known about the cellular mechanisms involved in this process. The ouabain-sensitive $K^+(^{86}Rb^+)$-uptake is known to have a large reserve capacity, and it increases when intracellular sodium is increased (Knudsen & Johansen, 1990). We suggest that the activation of this uptake following stimulation of the cells reflects an increase in intracellular sodium following histamine release, and that activation of the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake is needed in order for the cells to remove the excess intracellular sodium. The ouabain-resistant $K^+(^{86}Rb^+)$-uptake was also increased following application of compound 48/80 or antigen. This may reflect the increased surface area of the cells that develops in relation to the histamine release (Holm-Nielsen *et al.*, 1981). There was a difference in the pattern of stimulation of the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake when the cells were exposed to compound 48/80 and antigen (egg albumin). With compound 48/80 the uptake declined to a level not significantly different from the control level in unstimulated cells after 2 h of incubation, while the uptake returned to the control level after 60 min when antigen was used. A different amount of histamine released by compound 48/80 and antigen was also observed in these studies. Mast cells from different species and tissues are heterogeneous (Pearce, 1986). This fact must be acknowledged in the interpretation of the results, since Wistar rats were used for immunological release and Sprague-Dawley rats for compound 48/80-induced release. However, our control experiments with compound 48/80-induced histamine release from Wistar mast cells showed that a similar degree of histamine release from the two different mast cell types led to a not significantly different stimulation of the Na^+/K^+-pump in both cells. Therefore, we suggest that the observed differences between immunological and compound 48/80-induced histamine release and the different pattern in the poststimulatory Na^+/K^+-pump activation reflects basic differences between the two secretagogues rather than differences between rat strains.

The incubation of the cells took place in the presence of the inducers and it could be argued that this would lead to a persistent influence on the cell membrane and thus explain the prolonged activation of the ouabain-sensitive $K^+(^{86}Rb^+)$-

uptake. However, this is not likely, since it has been shown that the recovery of the cell morphology takes place even in the presence of compound 48/80 (Slutsky *et al.*, 1987; Levi-Schaffer & Riesel-Yaron, 1990).

Activation of the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake requires energy in the form of ATP (Skou, 1986). Johansen & Chakravarty (1975) have shown that activation of the mast cells with compound 48/80 or antigen leads to an increased cellular ATP utilization during and after the histamine release. The increased activity of the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake may at least in part explain this finding. An activation of the Na^+/K^+-pump following stimulation of lymphocytes with concanavalin A has previously been observed (Prasad *et al.*, 1987), and activation of the Na^+/K^+-pump (the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake) may thus be a more general phenomenon following cell stimulation, not only confirmed to the mast cell.

In conclusion, stimulation of rat peritoneal mast cells with compound 48/80 or antigen increases the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake of the cells. We suggest that this reflects an increased concentration of intracellular sodium following the histamine release and that activation of the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake, which is equivalent to the activity of the Na^+/K^+-pump, plays a role in the early part of the recovery process of the cells.

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Effect of an intracellular calcium chelator on the regulation of electrically evoked [^{3}H]-noradrenaline release from rat hippocampal slices

[1]Bertil B. Fredholm & Ping-Sheng Hu

Department of Pharmacology, Karolinska Institutet, Box 60 400, S-104 01 Stockholm, Sweden

1 The electrically (3 Hz, 5 min) evoked [^{3}H]-noradrenaline ([^{3}H]-NA) release from rat hippocampal slices was reduced by prior treatment of the slices with 1,2-*bis*(2-aminophenoxy)ethane-N,N,N'N'-tetraacetomethylester (BAPTA/AM) in a concentration-(10 to 500 μM) dependent manner (40% at 30 μM). Reduction of medium calcium from 1.3 to 0.5 mM caused a larger (70%) decrease. BAPTA free acid (100 mM), a non-permeable Ca^{2+}-chelator had no significant effect.

2 Basal [^{3}H]-noradrenaline release was reduced by BAPTA/AM in a concentration-dependent manner (50% at 30 μM), but reduction of external Ca^{2+} from 1.3 to 0.5 mM did not alter basal release.

3 About 10% of total [^{3}H]-NA in the slices was released at 3 Hz stimulation in 1.3 mM Ca^{2+} buffer. Addition of the α_2-adrenoceptor antagonist, idazoxan (1 μM), increased electrically evoked [^{3}H]-NA release to 26% but stimulated release was not altered by the adenosine A_1-receptor antagonist, 8-cyclopentyl theophylline (8-CPT) (1 μM).

4 Evoked release was reduced by the α_2-receptor agonist, UK 14,304, in a concentration-dependent manner in the presence of 8-CPT (1 μM). The magnitude of this effect was not altered by the treatment of slices with 30 μM BAPTA/AM.

5 The adenosine A_1 receptor agonist, N^6-cyclohexyl adenosine (CHA) (1 μM) inhibited electrically evoked [^{3}H]-NA release by about 40% in the presence of idazoxan (1 μM). The effect of CHA was not significantly altered by treatment of slices with BAPTA/AM.

6 The N-type Ca^{2+} -channel antagonist, ω-conotoxin (30 nM), inhibited electrically evoked [^{3}H]-NA release by 30–50% and this was not altered by treatment of the slices with BAPTA/AM.

7 The present results show that spontaneous [^{3}H]-NA release is affected by reduction of intracellular Ca^{2+}, but not by reduction of extracellular Ca^{2+} or by the presynaptic agonists or ω-conotoxin. By contrast, electrically evoked release was affected more strongly by alterations of extracellular Ca^{2+} than by buffering intracellular Ca^{2+}. The reduction of electrically evoked [^{3}H]-NA release by agonists at the adenosine A_1-receptor and α_2-adrenoceptor is probably mediated through the control of Ca^{2+} entry via membrane ion channels or at a low affinity Ca^{2+}-site governing evoked release.

Keywords: Calcium chelator; presynaptic receptors; calcium channels; conotoxin; adenosine receptors; α_2-adrenoceptors

Introduction

The release of noradrenaline from central noradrenergic neurones is regulated by different presynaptic receptors including α_2-adrenoceptors (Starke, 1987) and adenosine A_1-receptors (Fredholm & Dunwiddie, 1988). Neurotransmitter release is in general a strongly Ca^{2+}-dependent process (Augustine *et al.*, 1987; Miller, 1988). There is direct evidence that both α_2-adrenoceptors and adenosine A_1-receptors can regulate neuronal calcium entry either by acting directly on Ca^{2+}-channels (Holz & Rane, 1986; Madison *et al.*, 1987) or indirectly by altering membrane potential via an action on K^+-channels (Williams *et al.*, 1985; Trussell & Jackson, 1985). Thus, it is generally believed that these presynaptic agonists limit neurotransmitter release by decreasing calcium entry (see Starke, 1987; Fredholm & Dunwiddie, 1988), but this may be achieved by actions on several different types of ion channels (Nicoll, 1988). However, there is also evidence that neurotransmitter release may be regulated by altering not calcium entry, but the sensitivity of the release machinery in the cytoplasm to calcium (Silinsky, 1984; 1986), and even by mobilization of intracellular calcium (Kramer *et al.*, 1991). In addition there is the so-called calcium-voltage hypothesis (Parnas *et al.*, 1986; Hochner *et al.*, 1989), according to which the voltage change induces a conformational adaptation of the membrane rendering release sites more sensitive to calcium, so that in the presence of high intracellular calcium levels, depolarization directly triggers transmitter release. This theory has, however, been strongly contested (Mulkey & Zucker, 1991).

Intracellular calcium buffers can be used to study the role of changes in intracellular calcium. In elegant studies the two chelators ethyleneglycol-*bis*(β-amino-ethylether)-N,N'-tetraacetic acid (EGTA) and 1,2-*bis*(2-aminophenoxyl)ethane-N,N,N',N'-tetraacetic acid (BAPTA) have been injected directly into the squid giant synapse (Adams *et al.*, 1985; Adler *et al.*, 1991). However, it is not possible to use this technique in the very small noradrenergic nerve terminals in the central nervous system. Instead it is possible to use a non-disruptive loading technique (Tsien, 1981), which depends on the use of permeant derivatives of the calcium chelators that are taken up by cells where they are subsequently hydrolyzed to form the active chelating compounds intracellularly. BAPTA/AM (1,2-*bis*(2-aminophenoxy)ethane-N,N,N',N'-tetraacetomethylester) is such a compound (Tsien, 1980). BAPTA/AM has been used to study the role of intracellular calcium changes in the regulation of neurotransmitter release from the crayfish neuromuscular junction (Charlton & Iwanchyshyn, 1986) and the frog endplate (Tanabe & Kijima, 1989).

In the present experiments we have therefore studied the effect of BAPTA loading of hippocampal slices on the release

[1] Author for correspondence.

of noradrenaline following electrical field stimulation. We have also examined how such treatment alters the regulation of neurotransmitter release by adenosine A_1-receptor agonists and α_2-adrenoceptor agonists. Finally, we have compared the effect of these presynaptic agonists with the effect of a compound, ω-conotoxin, that is known to interact specifically with calcium entry, mainly through N-type Ca^{2+}-channels (Olivera *et al.*, 1984; Reynolds *et al.*, 1986; Miller, 1988).

Methods

The electrically evoked release of [^{3}H]-NA was studied essentially as described by Jonzon & Fredholm (1984). 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 operated manually. The slices were incubated twice for 30 min at room temperature and once for 30 min at 37°C. (−)-[^{3}H]-NA was added in the presence of 0.025 mg ml^{-1} pargyline chloride and 0.02 mg ml^{-1} ascorbic acid and incubated for 45 min at 37°C. In experiments with BAPTA/AM all the procedures were the same as described above except that hippocampal slices were preincubated with BAPTA/AM for 30 min at 37°C before addition of [^{3}H]-NA. Then the slices were placed in glass chambers and superfused, starting 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 $CaCl_2$ 1.3. Pulses of 10 V, 2 ms duration were delivered by two platinum electrodes placed 2.5 cm apart. To measure the drug-induced changes in the electrically evoked [^{3}H]-NA release, we compared the amount of radioactivity released by a 5 min control simulation (S_1, delivered during collection of the 4th superfusate fraction) with the amount released by a second 5 min stimulation (S_2, delivered during collection of the 12th superfusate fraction) before and during which drugs were added to the superfusate. The basal release was subtracted and the S_2/S_1 ratio calculated. In all experiments the buffer contained 1 μM desipramine to inhibit noradrenaline reuptake. In the experiments examining the frequency-dependence of release, three stimulations, 0.3, 1, and 3 Hz for 5 min were given during the 4th, 11th and 17th fractions and the ^{3}H-outflow of five fractions were summed up for each stimulation. The released [^{3}H]-NA was expressed as percentage of total ^{3}H in the slices. 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. Statistical analysis was carried out by one way ANOVA with the Bonferroni test (GraphPad InStat) 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}) was obtained from the Radiochemical Centre, Amersham, U.K. *Bis*(*o*-aminophenoxyl)ethane-N,N,N′,N′-tetraacetomethylester (Quin2/AM) was obtained from Calbiochem, La Jolla, CA, U.S.A. and 1,2-*bis*(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) and its tetraacetomethyl ester (BAPTA/AM), ascorbic acid and pargyline hydrochloride were from Sigma, St. Louis, MO, U.S.A.; desipramine from Ciba-Geigy, Summit, NJ, U.S.A.; UK 14,304 (5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline) and yohimbine hydrochloride were gifts from Dr S.Z. Langer. N^6-cyclohexyl adenosine (CHA) was obtained from Boehringer, Mannheim, Germany. 8-Cyclopentyl theophylline (8-CPT) was from Research Biochemicals Inc., Wayland, MA, U.S.A. Idazoxan (RX 781094; 2-(2-(1,4-benzodioxanyl)2-imidazoline HCl)) was a gift from Reckitt & Colman, Kingston-upon-Hull, U.K. ω-Conotoxin was from Peninsula Laboratories, Inc. Belmont, CA, U.S.A.

Results

Electrical field stimulation evoked a frequency-dependent increase in the outflow of [^{3}H]-NA from the hippocampal slices (Figure 1). The electrically evoked neurotransmitter release was reduced either by decreasing the extracellular calcium concentration from 1.3 to 0.5 mM or by pretreating slices with BAPTA/AM, which after uptake and hydrolysis is converted to the intracellular calcium chelator BAPTA (Figure 1). The magnitude of the reduction depended on the concentration of BAPTA/AM present during preincubation (Figure 2). An apparently maximal reduction was obtained at 100

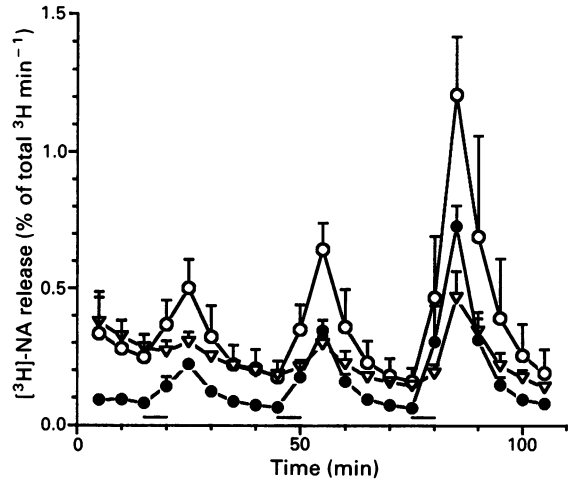


Figure 1 The time course of [^{3}H]-noradrenaline ([^{3}H]-NA) release from rat hippocampus at 0.3, 1 and 3 Hz stimulations. The methods are described in the text. The results are expressed as percentage of total tissue ^{3}H released per min. The circles are the experiments carried out in 1.3 mM Ca^{2+} buffer: (○) control; (●) experiments using slices pretreated with 100 μM BAPTA/AM; (▽) experiments in 0.5 mM Ca^{2+} buffer. The lines at the bottom represent the periods of stimulation: from left to right at 0.3, 1 and 3 Hz respectively. Mean ± s.e.mean (vertical bars) of at least four observations.

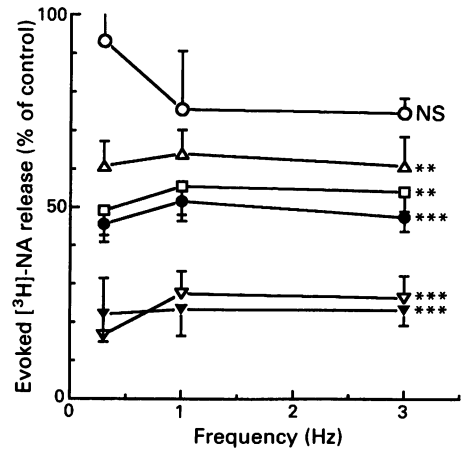


Figure 2 The effect of BAPTA/AM treatment or of reduction in extracellular Ca^{2+} on [^{3}H]-noradrenaline ([^{3}H]-NA) release from rat hippocampal slices induced by electrical stimulation at increasing frequencies. The results are expressed as a percentage of control release at the same frequency. The magnitude of [^{3}H]-NA release in these control experiments was: 2.77 ± 0.45, 4.81 ± 0.69 and 11.37 ± 1.40% of the total ^{3}H-radioactivity in the slices at 0.3, 1 and 3 Hz stimulation. (●, ○), (△), (□) and (●) are 10, 30, 100 and 500 μM BAPTA/AM respectively; (▽, ▼) experiments in 0.5 mM Ca^{2+} buffer; (▽) experiments in the absence and (▼) in the presence of 30 μM BAPTA/AM. Mean ± s.e.mean (vertical bars) of at least four experiments.
$^{**}P<0.01$ and $^{***}P<0.001$ compared with corresponding control.

Table I Comparison of BAPTA free acid (FA; 100 μM) and BAPTA/AM (30 μM), loaded during 30 or 90 min, with Quin2/AM (30 μM) on electrically evoked [^{3}H]-noradrenaline ([^{3}H]-NA) release from rat hippocampal slices

	Incubation (min)	*Frequency* (Hz) 0.3	1	3	*No. of exp.*
Control	30	2.77 ± 0.45	4.81 ± 0.69	11.37 ± 1.40	15
BAPTA-FA	30	2.20 ± 0.51	4.60 ± 0.39	9.30 ± 0.49	4
BAPTA/AM	30	1.83 ± 0.17	3.00 ± 0.37	6.40 ± 0.71**	6
	90	1.45 ± 0.32	2.43 ± 0.28	5.00 ± 0.67**	4
Quin2/AM	30	1.86 ± 0.32	4.10 ± 0.66	8.41 ± 1.25	4

The methods used are described in the Methods section. Five fractions of ^{3}H-release after each stimulation were summed and the background was subtracted. The release is expressed as percentage of total ^{3}H in the slices. Mean ± s.e.mean.
**$P < 0.01$ compared with the control in the same column.

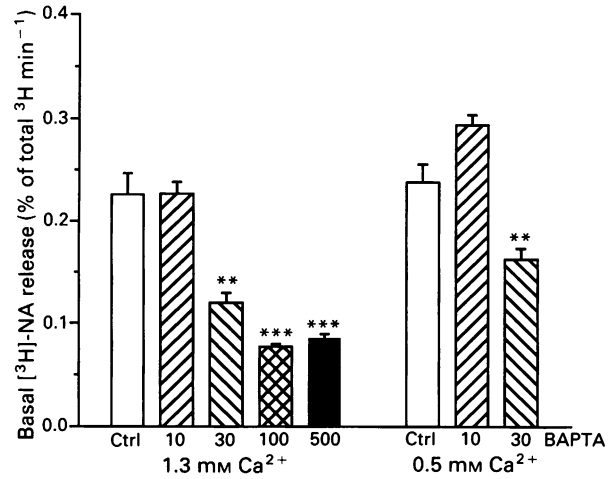


Figure 3 The effects BAPTA/AM treatment and reduction of medium Ca^{2+} on the basal [^{3}H]-noradrenaline ([^{3}H]-NA) release from rat hippocampal slices. The data are from the same experiments as those shown in Figure 2. Seven fractions under unstimulated condition (three before simulation 1, two before stimulation 2 and 3 respectively) were summed and the results are expressed as percentage of total [^{3}H]-NA per min. Mean ± s.e.mean (vertical bars) of at least four observations.

and 500 μM BAPTA when the evoked release was reduced by about 50% at all three frequencies employed. The magnitude of the evoked neurotransmitter release was more substantially reduced by reduction of the extracellular calcium concentration from 1.3 to 0.5 mM calcium (Figure 2). This caused an approximately 75% inhibition of the evoked neurotransmitter release. The effect of the BAPTA ester treatment was not mimicked by incubating the slices with BAPTA free acid (Table 1), which is not taken up by the cells.

In agreement with previous results, the basal outflow of noradrenaline was essentially unaltered by lowering the extracellular calcium concentration from 1.3 to 0.5 mM. By contrast, BAPTA treatment caused a concentration-dependent reduction in the basal release of noradrenaline (Figure 3).

The effect of BAPTA/AM was increased when the incubation time was prolonged from 30 to 90 min (Table 1). Despite this, the remaining experiments were performed during a 30 min incubation because of a desire to limit the time of preincubation of the slices before experiments. Another calcium chelator, Quin-2/AM, was less potent than BAPTA/AM in reducing electrically evoked neurotransmitter release (Table 1).

In the remaining experiments, two periods of stimulation were given. During the second stimulation a presynaptic inhibitor of transmitter release, either the α_2-receptor agonist, UK 14,304 or the adenosine A_1-receptor agonist, N^6-cyclohexyladenosine (CHA) was given. When the effect of the α_2-adrenoceptor agonist was studied the adenosine receptors were blocked by the adenosine A_1-receptor antagonist, 8-cyclopentyltheophylline (1 μM). When the effect of the A_1 agonist was studied, the α_2-adrenoceptor antagonist idazoxan (1 μM) was given. Idazoxan is known to increase the evoked release of NA. In order to eliminate the influence of changes in the biophase NA (by about 2.6 fold), the magnitude of the evoked release in the presence of idazoxan was matched to that induced by a 3 Hz stimulation under control conditions either by reducing the stimulation frequency to 0.5 Hz or by reducing the calcium concentration from 1.3 to 0.43 mM.

As seen in Table 2, the α_2-receptor agonist, UK 14,304, caused a concentration-dependent inhibition of the evoked release of ^{3}H radioactivity. The magnitude of this inhibition was not significantly different when the slices had been pretreated with BAPTA/AM, even though the BAPTA/AM treatment did reduce transmitter release. For comparison, the effect of the N-channel inhibitor, ω-conotoxin, was studied. The effect of 30 nM ω-conotoxin was essentially equivalent to the effect of 0.1 μM UK 14,304. This was true, irrespective of whether BAPTA/AM was present in the preincubation or not.

As seen in Tables 3 and 4, the adenosine A_1-receptor agonist, CHA (1 μM), caused an approximately 40% inhibition of evoked neurotransmitter release in the presence of an α_2-adrenoceptor antagonist both in untreated slices and in slices incubated with BAPTA/AM. The magnitude of the response was similar to that observed with 30 nM ω-conotoxin both when the experiments were carried out at a low stimulation frequency (Table 3) and when the extracellular calcium concentration was reduced from 1.3 to 0.43 mM (Table 4). The effect of the toxin was essentially the same in control slices and in slices treated with BAPTA/AM. The two presynatic agonists and the toxin were without effect on the basal release of [^{3}H]-NA (Table 5).

Discussion

We have confirmed previous observations (Charlton & Iwanchyshyn, 1986; Tanabe & Kijima, 1989) that treatment with the permeant calcium chelator BAPTA/AM can reduce electrically evoked neurotransmitter release from an intact preparation. Quin-2/AM, another permeant intracellular Ca^{2+} chelator (Tsien, 1981) also tended to reduce the [^{3}H]-NA release by buffering the intracellular Ca^{2+}. The magnitude of the BAPTA effect was dependent on the concentration of BAPTA/AM in the medium, reaching a maximum at about 100 μM and was also increased by prolonging the time of exposure to BAPTA/AM from 30 to 90 min. A similar inhibition was not achieved by incubation with BAPTA free

Table 2 Effects of UK 14,304 and ω-conotoxin (CTX) on [^{3}H]-noradrenaline ([^{3}H]-NA) release from rat hippocampal slices by electrical stimulation at 3 Hz in the presence of 8-cyclopentyl theophylline (1 μM) with and without prior treatment with BAPTA/AM (30 μM)

Drugs	*Conc.* (μM)	*Control* S_1	S_2	S_2/S_1	*% inhib.*	*No.*
Control		10.64 ± 0.98	10.65 ± 0.85	1.01 ± 0.10	0	10
UK 14,304	0.01	10.92 ± 1.66	10.11 ± 1.66	0.94 ± 0.07	7	5
	0.1	9.90 ± 0.70	7.42 ± 0.81	0.73 ± 0.04*	28	9
	1	9.58 ± 0.98	4.56 ± 0.48	0.48 ± 0.04**	52	5
CTX	0.03	11.29 ± 0.99	7.32 ± 0.83	0.67 ± 0.04**	34	8
		Treatment with BAPTA/AM				
Control		8.27 ± 1.07	8.64 ± 1.06	1.03 ± 0.05	0	9
UK 14,304	0.01	7.70 ± 1.29	6.90 ± 1.54	0.90 ± 0.05	13	5
	0.1	8.17 ± 1.60	5.25 ± 1.95	0.62 ± 0.14*	40	8
	1	7.67 ± 0.79	4.41 ± 0.67	0.57 ± 0.04**	45	4
CTX	0.03	8.67 ± 1.06	6.37 ± 0.73	0.74 ± 0.06**	28	9

The inhibitory effects of UK 14,304 and ω-conotoxin added before second stimulation are expressed as a percentage of S_2/S_1 in the presence of 8-CPT alone. Mean ± s.e.mean.
*$P<0.05$ and **$P<0.01$ compared to the control.

Table 3 Effect of N^6-cyclohexyl adenosine (CHA) and ω-conotoxin (CTX) on [^{3}H]-noradrenaline ([^{3}H]-NA) release from rat hippocampal slices with and without treatment with BAPTA/AM (30 μM) at 0.5 Hz stimulation in the presence of 1 μM idazoxan and 1.3 mM Ca^{2+} in the medium

Drugs	*Conc.* (μM)	*Control* S_1	S_2	S_2/S_1	*% inhib.*	*No.*
Control		10.21 ± 3.10	10.58 ± 3.39	1.07 ± 0.10	0	5
CHA	1	9.00 ± 2.10	6.02 ± 2.05	0.66 ± 0.08*	38	3
CTX	0.03	11.30 ± 1.74	5.72 ± 1.17	0.51 ± 0.06**	52	5
		Treatment with BAPTA/AM				
Control		7.70 ± 0.73	7.76 ± 0.38	1.01 ± 0.07	0	4
CHA	1	7.74 ± 0.78	4.61 ± 0.54	0.59 ± 0.05**	42	5
CTX	0.03	7.39 ± 1.91	3.34 ± 0.70	0.45 ± 0.04***	55	5

The effects of CHA and ω-conotoxin are expressed as percentage inhibition of S_2/S_1 in control. Mean ± s.e.mean. *$P<0.05$; **$P<0.01$ and ***$P<0.001$ compared with the control.

Table 4 Effect of N^6-cyclohexyl adenosine (CHA) and ω-conotoxin (CTX) on [^{3}H]-noradrenaline ([^{3}H]-NA) release from rat hippocampal slices with and without treatment with BAPTA/AM (30 μM) at 3 Hz stimulation in the presence of 1 μM idazoxan and 0.43 mM Ca^{2+} medium

Drugs	*Conc.* (μM)	*Control* S_1	S_2	S_2/S_1	*% inhib.*	*No.*
Control		9.40 ± 1.62	10.15 ± 1.97	1.08 ± 0.12	0	12
CHA	1	8.31 ± 0.44	5.09 ± 0.34	0.61 ± 0.16*	44	6
CTX	0.03	9.69 ± 0.57	5.17 ± 0.72	0.53 ± 0.07*	51	5
		Treatment with BAPTA/AM				
Control		4.85 ± 0.73	4.92 ± 0.79	0.98 ± 0.05	0	8
CHA	1	3.21 ± 0.64	1.99 ± 0.34	0.61 ± 0.02***	38	5
CTX	0.03	3.81 ± 0.56	2.25 ± 0.38	0.59 ± 0.06***	40	7

The effects of CHA and ω-conotoxin added before the second stimulation are expressed as percentage inhibition of S_2/S_1 in control. Mean ± s.e.mean. *$P<0.05$; and ***$P<0.001$ compared with the control.

acid, which does not penetrate cell membranes. Thus, the effect of BAPTA/AM cannot simply be ascribed to a reduction of the calcium in the medium due to extracellular chelation. This is also shown by the fact that a reduction in extracellular Ca^{2+}, although having a more potent effect than BAPTA/AM on electrically evoked transmitter release, could not decrease spontaneous release in the same way as BAPTA/AM. Our findings also agree in principle with those where BAPTA has been directly injected into the nerves (e.g. Adler *et al.*, 1991). It therefore seems reasonable to assume that the effects observed are in fact due to chelation of intracellular Ca^{2+} following the BAPTA/AM treatment.

When BAPTA is injected into the squid giant neurone to a concentration of 4 mM, the postsynaptic response, and by inference the transmitter release, was reduced by 80% (Adler *et al.*, 1991). Using the present non-disruptive loading technique we never observed more than a 50–55% inhibition of transmitter release and at the most commonly used concentration of BAPTA/AM (30 μM) it was about 40%. Assuming that the relationship between intracellular BAPTA concentra-

Table 5 The effects of N^6-cyclohexyl adenosine (CHA), UK 14,304 and ω-conotoxin (CTX) on the basal [^{3}H]-noradrenaline ([^{3}H]-NA) release from rat hippocampal slices

8-CPT (1 μM), 1.3 mM Ca^{2+}			
Control		0.095 ± 0.003 (10)	
UK 14,304	0.01 μM	0.090 ± 0.005 (5)	NS
	0.1	0.086 ± 0.007 (9)	NS
	1	0.090 ± 0.005 (5)	NS
CTX	0.03	0.104 ± 0.007 (8)	NS
Idazoxan (1 μM), 0.43 mM Ca^{2+}			
Control		0.85 ± 0.007 (12)	
CHA	1 μM	0.082 ± 0.004 (6)	NS
CTX	0.03	0.088 ± 0.058 (5)	NS

The data are expressed as percentage of total [^{3}H]-NA in the slices at the 11th fraction (10 min after addition of drugs). The data in the upper panel of the table are from the same experiments as those shown in Table 3 and those in the lower panel from the experiments shown in Table 4.

tion and inhibition of transmitter release is similar in the central noradrenergic neurones and in the squid giant synapse, we may use data of Adler and coworkers (1991) to estimate that we probably reached an intracellular concentration of 0.25–0.5 mM BAPTA in the present experiments. From this estimate and the equation and rate constants for BAPTA/Ca^{2+} binding given by Adler *et al.* (1991) it can be calculated that the time constant of BAPTA/Ca^{2+} equilibrium would be of the order of 20–50 μs under the present conditions. Thus, it seems reasonable to assume that equilibrium should be achieved well within the 200 μs that has been shown to be the delay between influx of calcium and onset of transmitter release (Llinas, 1991). However, it has been convincingly argued that a substantial part of this delay is due to the time taken for the actual release process (Zucker *et al.*, 1991; Augustine *et al.*, 1991) and that the Ca^{2+}-receptor involved in transmitter release must bind Ca^{2+} within tens of microseconds. The significant, but incomplete, inhibition of evoked [^{3}H]-NA release we observed is entirely consistent with this view.

One important corollary to this rapidity of calcium binding to the relevant receptors is that the latter must be located very close to the sites of Ca^{2+} entry, i.e. the Ca^{2+}-channels (Zucker *et al.*, 1991; Augustine *et al.*, 1991). These authors have calculated that at these sites the Ca^{2+} concentration is likely to be very high and close to 100 μM. Direct evidence for such very high concentrations at the release sites has been obtained in hair cells (Roberts *et al.*, 1990), by using the Ca^{2+} activated K^+-channel as the indicator. It can be shown that this model predicts the extremely high dependence of neurotransmitter release on the concentration of extracellular Ca^{2+} (Augustine *et al.*, 1991) that has been observed in numerous preparations including the rat hippocampal noradrenergic nerve terminals (Hu & Fredholm, 1991). Furthermore, it predicts that a prolongation of the action potential by a potassium channel inhibitor markedly increases the calcium flux and hence the transmitter release. It is therefore of interest that the dependence of transmitter release on extracellular calcium is markedly altered by the inclusion of the potassium channel inhibitor, 4-aminopyridine (Hu & Fredholm, 1991).

In contrast to the electrically evoked release the basal rate of release was not markedly influenced by altering the extracellular Ca^{2+} concentration, but strongly reduced by BAPTA treatment. This basal release may be similar to the spontaneous transmitter release in the squid giant synapse, which can be studied by following the miniature endplate potentials (Llinas, 1991). This process has been shown to be highly correlated with the degree of synapsin phosphorylation by calcium-calmodulin-dependent protein kinase II (McGuiness *et al.*, 1989; Llinas, 1991). It has been convincingly argued that the effect is related to the mobilization of vesicles to release sites close to the cell membrane. Since the enzyme is localized in the cytosol and the substrate is associated both with the cytoskeleton and with storage vesicles that are localized away from the immediate vicinity of the cell membrane it is reasonable to assume that the relevant calcium pool is the cytosolic one that is markedly influenced by BAPTA, but less strongly by modification of extracellular calcium (Zucker *et al.*, 1991; Augustine *et al.* 1991).

Even though the absolute magnitude of the electrically evoked [^{3}H]-NA release was reduced by BAPTA-treatment of the slices, the effect of the Ca-channel inhibitor, ω-conotoxin, was essentially unaltered. This suggests that a direct blockade of the calcium entry through the channel by a presynaptic agonist should be similarly unaltered by the BAPTA treatment. Therefore it was interesting to note that the extent of inhibition of NA release by both the α_2- and adenosine A_1-agonist was unaffected. Since there is no evidence for the involvement of a dihydropyridine-sensitive L-type Ca-channel in the regulation of NA release in the hippocampus (Fredholm *et al.*, 1990) the present results could be explained if both types of presynaptic agonist affect the Ca-influx through N-type channels. There is direct evidence that both adenosine A_1-receptors (Gross *et al.*, 1989) and α_2-receptors (Lipscombe *et al.*, 1989) regulate these channels. However, it is not possible to say how this is brought about. A direct inhibition of the channel is one possibility, an alteration of the voltage dependence of the channel as suggested e.g. by Bean (1989) is another. The possibility that the membrane potential is altered via an action on a potassium conductance (Trussell & Jackson, 1985; Williams *et al.*, 1985) is also not ruled out.

In addition the studied presynaptic agonists could have some action that is independent of a regulation of calcium entry. It is clear that adenosine may, under some circumstances have such actions. Silinsky (1984, 1986) showed that spontaneous transmitter release could be affected by adenosine in a manner independent of changes in steady state calcium changes in the nerve terminal. Similarly, Scholz & Miller (1992) have recently shown that spontaneous transmitter release in cultured hippocampal neurones activated by α-latrotoxin can be affected by a process 'downstream' of calcium influx. Presynaptic α_2-adrenoceptor agonists may also under some circumstances affect transmitter release independently of Ca^{2+} entry (Jackish *et al.*, 1992). However, we found no evidence that the presynaptic agonists affected spontaneous release of transmitter and in this regard their action mimicked the lowering of external calcium of the action of the N-channel blocker. As discussed above, there appear to be major differences in the regulation of spontaneous and electrically evoked transmitter release, at least in the short term. Even though presynaptic agonists may, under some circumstances, affect basal neurotransmitter release independently of changes in calcium entry, this action appears not to be of major importance under conditions when transmitter release is strongly regulated by calcium entry processes. Under such circumstances the presynaptic agonists studied appear to regulate NA release either by limiting Ca^{2+} entry or by controlling a step thereafter that is different from those involved in the basal release of transmitter.

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Mediation by the same muscarinic receptor subtype of phasic and tonic contractile activities in the rat isolated portal vein

[1]M. Pfaffendorf & P.A. Van Zwieten

Department of Pharmacotherapy, Academic Medical Centre, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

1 The effects of several agonists on the phasic and tonic contractile responses to muscarinic receptor stimulation have been investigated in the rat portal vein *in vitro*.

2 Neither chemical denervation with 6-hydroxydopamine nor the presence of the α_1-adrenoceptor antagonist, prazosin, influenced the spontaneous or the stimulated myogenic activity of the portal vein.

3 Indomethacin and N^G-nitro-L-arginine were used to investigate the influence of vasoactive factors in this preparation. They slightly increased the frequency and the amplitude of the spontaneous myogenic activity of the portal vein, respectively. N^G-nitro-L-arginine but not indomethacin enhanced the maximal phasic response to carbachol. Both indomethacin and N^G-nitro-L-arginine failed to influence the tonic response to carbachol.

4 Muscarinic agonists increased phasic activity according to the rank order of potency: acetylcholine > muscarine > methacholine > carbachol > aceclidine > bethanechol. These effects were superimposed on a sustained contracture at higher concentrations. Oxotremorine was more potent than arecoline in increasing the mechanical phasic activity, without inducing a sustained contracture. Pilocarpine and McN A 343 were weak agonists, producing submaximal effects only on phasic activity.

5 The muscarinic antagonists AF-DX 116, 4-diphenylacetoxy-N-methylpiperidine (4-DAMP), *P*-fluorohexahydrosiladiphenidol (pFHHSiD) and pirenzepine antagonized the phasic and tonic mechanical responses to carbachol. Although the tonic contracture was slightly more sensitive to all antagonists studied, the rank order of potency: 4-DAMP > pFHHSiD > pirenzepine > AF-DX 116 was the same for both types of response, which is indicative of a M_3-receptor subtype.

6 The tonic contractile response of the rat portal vein to carbachol was more susceptible to partial receptor inactivation with propylbenzilylcholine mustard than the phasic contractile response. The dissociation constants (K_A) obtained from an analysis according to Furchgott & Bursztyn (1967) were found to be 4.32 ± 0.31 μM for the phasic and 3.56 ± 0.21 μM for the tonic type of carbachol-induced response, respectively. Since the EC_{50}-values for both carbachol-induced effects were different (phasic 0.232 ± 0.02 μM; tonic 2.75 ± 0.1 μM) the phasic type of response appears to involve a large receptor reserve.

Keywords: Rat portal vein; muscarinic receptor subtypes; receptor inactivation; receptor reserve

Introduction

In intact vascular tissue acetylcholine (ACh) may trigger vasoconstriction (Demy & Vanhoutte, 1982) as well as vasodilatation, the latter by inducing the release of relaxing factors from the endothelium (Furchgott & Zawadzki, 1980). In most vessels the net effect is a pronounced vasodilatation, although there are exceptions, for instance, the mesenteric portal vein of several species (Hughes & Vane, 1967; Uematsu *et al.*, 1984). The contraction implies the existence of muscarinic receptors on the smooth muscle cells which subserve an excitatory function. Although cholinergic innervation in the mammalian vascular system usually has a low density, it has been demonstrated in portal and mesenteric veins (Yoshioka *et al.*, 1988).

The longitudinal smooth muscle of the rat isolated portal vein maintained *in vitro* exhibits spontaneous intermittent contractions of varying amplitude and duration. Muscarinic agonists such as ACh or carbachol administered in a cumulative manner (Van Rossum, 1963) induce two different types of response in this preparation. Low concentrations of the agonist amplify the spontaneous phasic contractions (phasic response), while at higher concentrations the basal tone of the smooth muscle is increased to a stable tonic contraction (tonic response). This contractile pattern of the portal vein is also elicited by other excitatory transmitters such as angiotensin II (Blair-West *et al.*, 1971) or noradrenaline (Funaki & Bohr, 1964).

The aim of the present study was to investigate whether the two types of response to muscarinic stimulation in the longitudinal smooth muscle of the rat portal vein are due to activation of one receptor subtype.

Preliminary accounts of the results have been presented elsewhere (Pfaffendorf, 1992; Pfaffendorf & Van Zwieten, 1992).

Methods

Portal veins were taken from male Wistar rats, weighing 300–400 g. The rats were killed by stunning and exsanguination. After an abdominal midline incision the portal vein was exposed, stripped of surrounding tissue and isolated over a length of about 10 mm. The preparations were fixed longitudinally in a 20 ml organ bath. Tissues were maintained in a modified Tyrode solution, of the following composition (mM): NaCl 136, KCl 2.5, $NaHCO_3$ 11.9, $MgCl_2$ 0.5, $CaCl_2$ 0.9, NaH_2PO_4 0.42 and glucose 5.5. The solution was maintained at 37°C and the pH was kept at 7.4 by bubbling with 95% O_2 and 5% CO_2. A passive tension of 10mN was applied and continuously adjusted during the experiment. The contractions were measured isometrically by a force-

[1] Author for correspondence.

displacement transducer (Kyowa, Tokyo, Japan) connected to a d.c. amplifier (Kyowa) and recorded on a thermal pen recorder (WKK, Kaltenbrunn, Germany).

Experimental protocol

The preparations were allowed to equilibrate for 2 h with or without a particular pretreatment. During this period the medium was exchanged every 30 min. Subsequently a cumulative concentration-response curve was constructed by increasing agonist concentration in logarithmic increments by injecting volumes less than 40 μl/concentration directly into the organ bath. An exposure period of 5 min was maintained in order to reach an equilibrium effect. The contractile response was measured during the last 2.5 min of the equilibration period and 4 min after each increase of the agonist concentration, respectively. Changes in phasic myogenic contractile force were measured as the maximum difference between the average peak force at the end of the equilibration period and at the equilibrium effect of each agonist. Changes in tonic contraction were established as the stable increase of the mechanical tension above the baseline. Only one cumulative concentration-response curve was constructed for each preparation.

All experiments with ACh were performed after 1 h pretreatment with physostigmine (10^{-5} M). This procedure influenced neither the basal contraction amplitude (1.85 ± 0.50 *vs.* 1.65 ± 0.15 mN, $n = 16$, NS) nor the basal contraction (contr) frequency (2.67 ± 0.32 *vs.* 3.56 ± 0.24 contr. min^{-1}, $n = 6$, NS). Indomethacin and N^G-nitro-L-arginine were used to evaluate the influence of prostaglandin synthesis and endothelium-derived nitric oxide, respectively, on rat portal vein responses to the muscarinic agonist carbachol. The preparations were incubated with indomethacin (10^{-5} M) or N^G-nitro-L-arginine (10^{-4} M) for 1 h before the administration of carbachol.

Prazosin (10^{-7} M) and hexamethonium (10^{-5} M) were used to evaluate a possible role of noradrenaline release and ganglionic stimulation, respectively.

Experiments were performed with chemically denervated preparations according to Aprigliano & Hermsmeyer (1976). To destroy the adrenergic nerve endings, the preparations were incubated for 10 min with 6-hydroxydopamine 300 μg ml^{-1}, at pH 4 in a bicarbonate-free Krebs solution (in mM: NaCl 118, KCl 2.5, $MgCl_2$ 0.5, $CaCl_2$ 0.9 and tris(hydroxymethyl)-aminomethane 30).

In order to characterize the muscarinic receptor subtype in the rat portal vein we used three concentrations of the antagonists, pirenzepine, AF-DX 116, *p*-fluorohexahydrosiladiphenidol (pFHHSiD), 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) and of the non-selective antagonist, atropine. The preparations were incubated with each antagonist for 2 h. Only one antagonist concentration was tested per individual preparation.

Receptor inactivation

A solution of propylbenzilylcholine mustard (PrBCM, 10^{-3} M in a buffer containing NaCl 140 mM, Na_2HPO_4 9.2 mM and NaH_2PO_4 1.3 mM with a pH of 7.6) was kept for 60 min at room temperature to allow the formation of the active aziridinium ion, as described by Burgen *et al.* (1974). The mustard was then added to the organ bath at the desired concentrations (10^{-9}, 10^{-8} or 10^{-7} M) and the preparations were incubated for 20 min. Subsequently, the preparations were washed three times and allowed to equilibrate another 30 min until the carbachol concentration-response curves were constructed.

Calculations

Using a computer programme (GraphPad, Institute for Scientific Information, USA), concentration-response curves based on the relationship

$$E = E_{max} \cdot A^P \cdot (A^P + EC_{50}{}^P)^{-1}$$

were fitted to averaged log concentration-response data of 6 individual experiments. Since there were only small interindividual variations the responses were expressed and calculated as unnormalized values (in mN).

In the equation, E is the response obtained with a given concentration A, E_{max} is the maximally attainable response, EC_{50} is the concentration required for the half maximal effect and the exponent P describes the slope of the relationship (Hill-coefficient). pA_2 values were calculated according to Arunlakshana & Schild (1959).

The dissociation constants (K_A) of carbacol for the phasic and the tonic responses to muscarinic stimulation as well as the percentage of inactivated receptors were determined according to Furchgott & Bursztyn (1967). Concentration-response curves with and without partial receptor inactivation were constructed as described above, and a computer was programmed to calculate 17 pairs of equieffective carbachol concentrations with 3% increments between 40 and 90% which formed the basis for a regression analysis of a double reciprocal plot of equieffective carbachol concentrations with or without the receptor-inactivating agent. The K_A was calculated as slope-1 divided by the ordinate-intercept, and the fraction of unalkylated receptors was calculated as the reciprocal slope of the regression line. The range between 40 and 90% response was chosen, because points near the top of the depressed curve give a more reliable estimation of the K_A value (Kenakin, 1987). From the dissociation constants and the concentration-response relationships, the percentage receptor occupation was calculated for a given effect.

Statistics

The results are expressed as means ± s.e.mean. The statistical significance of differences was evaluated by two-tailed, unpaired Student's *t* test. *P* values less than 0.05 were considered to be significant. n = number of preparations.

Drugs

The following compounds were purchased from Sigma (St. Louis, MO, USA): acetylcholine chloride, arecoline hydrobromide, acetyl-β-methacholine chloride, atropine sulphate, bethanechol chloride, carbachol chloride, hexamethonium chloride, 6-hydroxydopamine hydrochloride, indomethacin, McN A 343 (4-(N-[3-chlorophenyl]-carbamoyloxy)-2-butynyltrimethylammonium chloride, (±)-muscarine chloride, oxotremorine sesquifumarate, pilocarpine hydrochloride, physostigmine hemisulphate, pirenzepine dihydrochloride and prazosin hydrochloride. AF-DX 116 (11-2[[2-[(diethylamino) methyl]-1-piperidyl]acetyl]-5,11-dihydro- 6H[2,3-b] [1,4] benzodiazepine-6-one) and 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methobromide were a gift from Dr H. Doods, Dr Karl Thomae GmbH, Biberach, Germany). *p*FHHSiD (*p*-fluorohexahydrosiladiphenidol) was a gift from Dr H. Tacke, Dept. Inorganic chemistry, Technische Universität, Karlsruhe, Germany. Aceclidine hydrochloride was purchased from Merck Sharp & Dohme, Haarlem, The Netherlands and N^G-nitro-L-arginine from Aldrich Chemical Comp., Milw., WI, U.S.A.

Indomethacin was dissolved in 0.15 M $NaHCO_3$. N^G-nitro-L-arginine was first dissolved in 100 μl 1 N HCl/25 mg and then diluted in saline. All other drugs were dissolved in distilled water before being added to the medium.

Results

At an extracellular Ca^{2+} concentration of 0.9 mM and a K^+ concentration of 2.5 mM, spontaneous phasic myogenic activity was observed with a frequency of 3.56 ± 0.24 ($n = 42$)

contr. min^{-1} and an amplitude of 1.65 ± 0.15 mN ($n = 42$). ACh, when administered in a cumulative manner (10^{-8} to 10^{-6} M) at 5 min intervals caused concentration-dependent increases of the amplitude of the rhythmic contractions, while their frequency was reduced. At concentrations higher than 10^{-6} M a concentration-dependent, stable increase of the base line was observed (tonic contracture) while the rhythmic activity disappeared. An original recording of a cumulative concentration-response curve to carbachol which causes a similar pattern of contractile response is depicted in Figure 1. The ACh concentration-response relationship was steeper (see Figure 2) for the tonic contraction than for the increase in amplitude of the phasic contractions (slope 2.6 ± 0.08 ($n = 6$) *vs.* 1.25 ± 0.10 ($n = 6$), $P < 0.05$). This dual action on the contractile behaviour of the rat portal vein was also observed for the muscarinic agonists aceclidine, bethanechol, carbachol, methacholine and muscarine. Arecoline and oxotremorine increased the amplitude of the spontaneous contractions to the same extent as did acetylcholine and the other full agonists but they were unable to induce a tonic contracture (see Figure 1). McN A 343 and pilocarpine only showed weak activity, even on the phasic response (see Figure 1). The characteristics of the average agonist concentration-response curves are listed in Table 1.

Indomethacin (10^{-5} M) did not influence the magnitude of the spontaneous myogenic contractions but enhanced the contraction frequency (4.63 ± 0.35 ($n = 6$) *vs.* 3.56 ± 0.24 contr min^{-1} ($n = 42$), $P < 0.05$). The concentration-response curves for the phasic and the tonic responses to carbachol were not altered by indomethacin.

N^G-nitro-L-arginine (10^{-4} M) increased the spontaneous myogenic contractions (2.20 ± 0.08 ($n = 6$) *vs.* 1.65 ± 0.15 mN ($n = 42$), $P < 0.05$) but had no effect on the contraction frequency. The maximal response of the phasic contractions to carbachol was significantly increased (15.9 ± 1.16 ($n = 6$) *vs.* 12.5 ± 0.51 mN ($n = 6$), $P < 0.05$) whereas the maximal tonic response to carbachol was unaltered (14.3 ± 0.50 ($n = 6$) *vs.* 14.2 ± 0.51 mN ($n = 6$), NS) by N^G-nitro-L-arginine pretreatment.

Chemical denervation of the portal vein preparations with 6-hydroxydopamine influenced neither the frequency (3.01 ± 0.35 ($n = 6$) *vs.* 3.56 ± 0.24 contr min^{-1} ($n = 42$), NS) nor the amplitude of the spontaneous myogenic activity (1.47 ± 0.10 ($n = 6$) *vs.* 1.65 ± 0.15 mN ($n = 42$), NS). The concentration-response curve to carbachol was unaltered by this procedure.

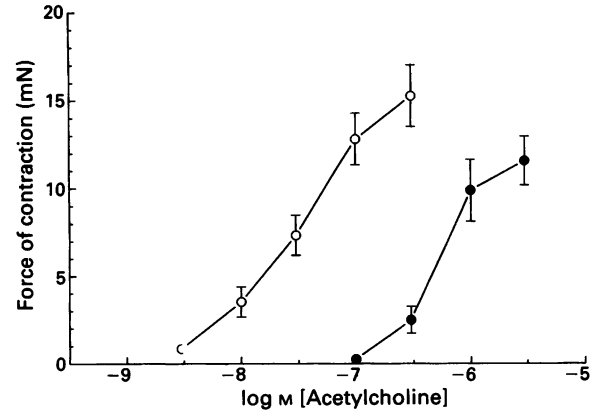


Figure 2 Concentration-response curves for the acetylcholine-induced increases of the phasic (○) and the tonic (●) contraction; means ± s.e.mean (vertical bars) $n = 6$.

Neither pretreatment with prazosin (10^{-7} M) nor with hexamethonium (10^{-5} M) influenced the basal or the carbachol-stimulated myogenic activity of the rat portal vein.

In order to investigate the interaction between the full agonist carbachol and the partial agonist oxotremorine, a maximally effective carbachol concentration (10^{-5} M) that induced a pronounced and stable contracture of the portal vein, was applied. After 5 min the same concentration of oxotremorine was added. The contracture waned and was replaced by phasic contractions with amplitudes equal to the maximal oxotremorine-induced effect (see Figure 3).

None of the muscarinic antagonists *per se* influenced the

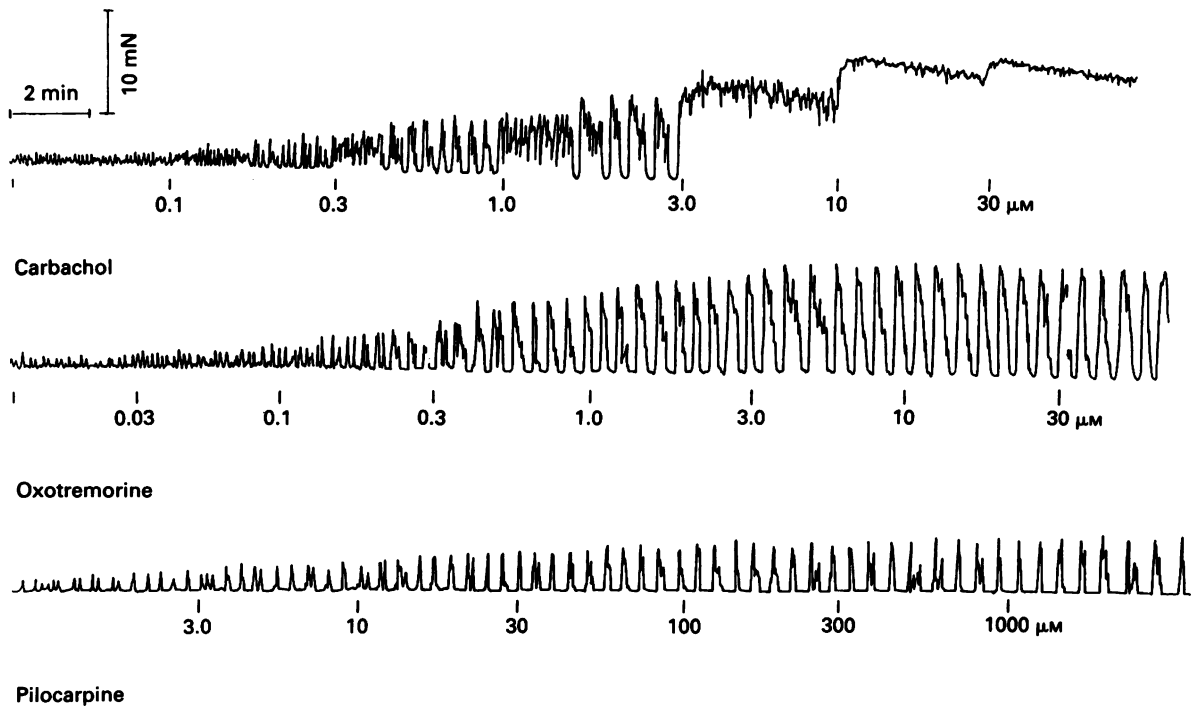


Figure 1 Original recordings of isometric contractions following the cumulative addition of muscarinic agonists to the rat portal vein preparation.

Table 1 Characteristics of the concentration-response relationships of some muscarinic receptor agonists on the phasic and tonic responses of the rat portal vein determined by fitting logistic function (see Methods) to experimental data

	Phasic response E_{max} (mN)	pEC_{50}[a]	*Tonic response* E_{max} (mN)	pEC_{50}[a]
Acetylcholine	15.3 ± 2.5	7.5 ± 0.02	11.6 ± 1.4	6.3 ± 0.01
Aceclidine	15.5 ± 1.8	5.7 ± 0.04	11.6 ± 1.3	4.6 ± 0.05
Arecoline	16.1 ± 0.75	5.7 ± 0.02	no effect	
Bethanechol	15.4 ± 1.75	5.2 ± 0.04	11.5 ± 1.5	4.3 ± 0.05
Carbachol	12.5 ± 0.51	6.6 ± 0.04	13.9 ± 0.5	5.6 ± 0.02
McN A 343	7.1 ± 0.93	4.8 ± 0.03	no effect	
Methacholine	12.3 ± 0.60	6.7 ± 0.05	11.0 ± 1.7	5.4 ± 0.02
Muscarine	13.6 ± 1.6	7.1 ± 0.09	16.4 ± 2.3	5.7 ± 0.03
Oxotremorine	15.2 ± 2.24	6.7 ± 0.03	no effect	
Pilocarpine	7.1 ± 1.00	5.6 ± 0.03	no effect	

Values are means ± s.e.mean, $n = 6$
[a]Negative logarithm of the agonist concentration needed to produce half maximal effect.

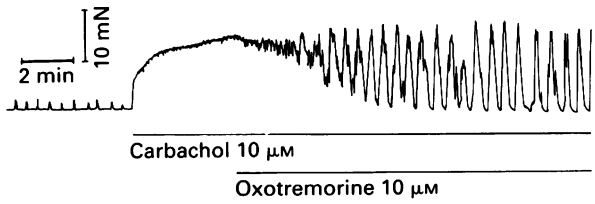


Figure 3 Original recording of isometric contractions after the addition of carbachol 10^{-5} M followed by oxotremorine 10^{-5} M to a rat portal vein preparation.

spontaneous myogenic contraction of the preparations. All antagonists shifted the concentration-response curves to carbachol, for both the phasic and the tonic contractions, to the right and in a parallel manner. No depression of the maximal responses to carbachol was observed. The slopes of the linear regression relationships obtained according to Arunlakshana & Schild (1959) were not different from unity for any of the antagonists, suggesting competitive interaction (see Figure 4) for both types of responses. Although the tonic response of rat portal vein to carbachol showed a somewhat higher sensitivity to the antagonists used, the rank order of potency was the same for both types of response: atropine > 4-DAMP > *p*FHHSiD > pirenzepine > AFDX 116 (see Table 2).

Pretreatment with PrBCM (up to 10^{-7} M) did not influence the spontaneous myogenic activity of the rat portal vein preparations.

The concentration-response curves for carbachol-induced phasic myogenic contractions and tonic contractures were shifted rightward following irreversible receptor inactivation by PrBCM treatment (see Figure 5).

This procedure was more effective in reducing the maximal tonic response compared to the phasic response (E_{max} values in % of control – 10^{-8} M: 38.9 ± 10.5 *vs.* 100 ± 10.7, $n = 6$; 10^{-7} M: no response *vs.* 71 ± 11.2, $n = 6$). Nevertheless, the dissociation constants (K_A), obtained from the double reciprocal plot of equieffective carbachol concentrations with and without PrBCM pretreatment were not different for both effects (phasic response 4.32 ± 0.30 μM, $n = 6$; tonic response 3.56 ± 0.21 μM, $n = 6$, NS).

From occupancy-response relationships for both types of carbachol-induced responses in the rat portal vein, using the dissociation constants and the concentration-response data, the percentage receptor occupation for a given effect was calculated. To induce 90% of the maximal phase response 15.2 ± 1.36% of the receptors have to be occupied whereas 60.5 ± 2.72% are needed for 90% of the maximal tonic response. The carbachol-induced tonic contraction had a lower receptor reserve compared to the increase of the phasic contractions.

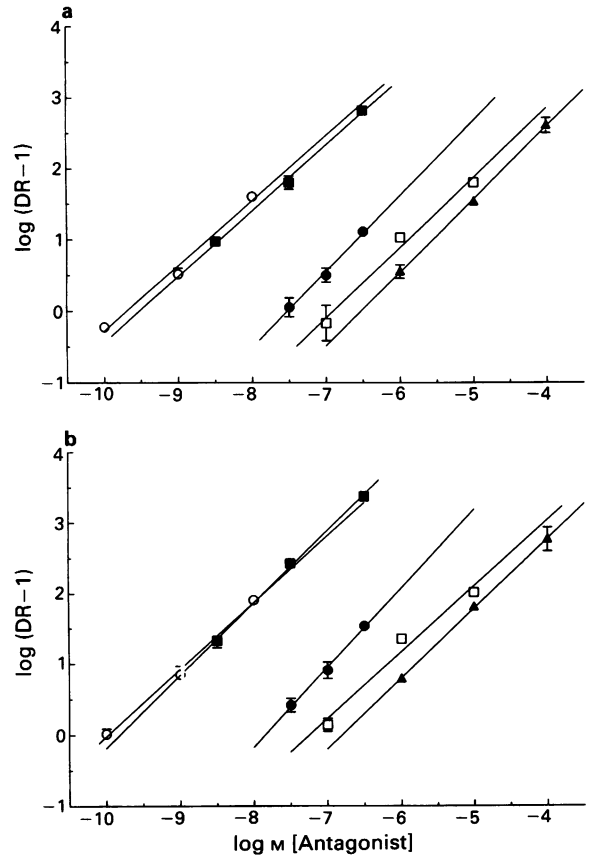


Figure 4 (a) Schild regressions for atropine (○), 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) (■) *p*-fluorohexahydrosiladiphenidol (p-FHHSiD) (●), pirenzepine (□) and AF-DX 116 (▲) against the carbachol-induced increase of the phasic contractions in the rat portal vein. (b) Schild regressions for atropine (○), 4-DAMP (■), pFHHSiD (●), pirenzepine (□) and AF-DX 116 (▲) against the carbachol-induced tonic contracture in the rat portal vein. Values are means ± s.e.mean (vertical bars) $n = 6$ for both (a) and (b).

Discussion

All muscarinic agonists investigated in the present study were able to enhance the mechanical smooth muscle activity of the

Table 2 Schild regression parameters for muscarinic receptor antagonists against the carbachol-induced phasic and tonic responses of the rat portal vein

Antagonist	*Phasic response* pA_2	Slope	*Tonic response* pA_2	Slope
Atropine	9.68 ± 0.07	0.93 ± 0.10	9.98 ± 0.11	0.95 ± 0.07
4-DAMP	9.55 ± 0.14	0.93 ± 0.06	9.84 ± 0.08	1.02 ± 0.04
pFHHSiD	7.53 ± 0.10	1.06 ± 0.11	7.87 ± 0.15	1.11 ± 0.11
Pirenzepine	6.90 ± 0.15	0.99 ± 0.12	7.25 ± 0.25	0.93 ± 0.16
AF-DX 116	6.54 ± 0.06	1.03 ± 0.03	6.78 ± 0.17	0.99 ± 0.16

For abbreviations, see text.
Means ± s.e.mean, $n = 6$.

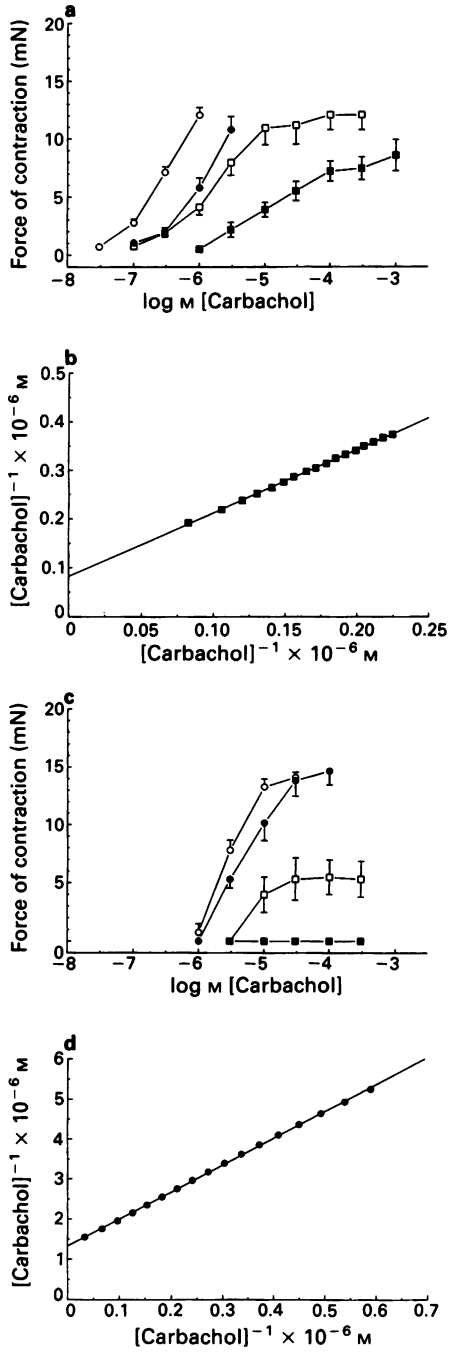


Figure 5 (a) Effect of propylbenzilylcholine mustard (PrBCM) treatment on the concentration-response curve of the carbachol-induced increase of the phasic myogenic contractions in the rat portal vein. The preparations were pretreated for 20 min with PrBCM at 0 nM (○), 1 nM (●), 10 nM (□), or 100 nM (■). Values are means ± s.e.mean (vertical bars), $n = 6$. (b) Equieffective concentrations of carbachol on the phasic response in control preparations and preparations pretreated with 10 nM PrBCM were calculated and their reciprocals were plotted. The straight line fitted to the data by linear regression analysis had a correlation coefficient of 0.99 and yielded values of $K_A = 4.32$ μM, q = 0.148. (c) Effect of PrBCM treatment on the concentration-response curve of the carbachol-induced tonic contracture in the rat portal vein. The preparations were pretreated for 20 min with PrBCM at 0 nM (○), 1 nM (●), 10 nM (□), or 100 nM (■). Values are means ± s.e.mean (vertical bars), $n = 6$. (d) Equieffective concentrations of carbachol on the tonic response in control preparations and preparations pretreated with 1 nM PrBCM were calculated and their reciprocals were plotted. The straight line fitted to the data by linear regression analysis had a correlation coefficient of 0.99 and yielded values of $K_A = 3.56$ μM, q = 0.77.

rat portal vein. By contrast, most other intact vascular tissues are known to relax as a response to the stimulated release of relaxing factors from the endothelium (Furchgott & Zawadzki, 1980).

In vitro pretreatment with 6-hydroxydopamine has been reported to produce a rapid adrenergic denervation in the rat isolated portal vein (Aprigliano & Hermsmeyer, 1976). In contrast, we found no transient increase of spontaneous myogenic activity after the addition of 6-hydroxydopamine. The α_1-adrenoceptor antagonist, prazosin and the ganglion blocker, hexamethonium were likewise ineffective. These findings suggest that both the spontaneous myogenic activity and the contractile response induced by muscarinic agonists are independent of the release of catecholamines. This observation and the insensitivity of the spontaneous myogenic contractions to physostigmine or muscarinic antagonists suggest that there is insignificant basal release of ACh and catecholamines in the rat isolated portal vein preparation. Muscarinic receptor-induced relaxation of most intact vascular tissues results from the release of relaxing factors (e.g. nitric oxide, Ignarro *et al.*, 1986; Palmer *et al.*, 1987) from the vascular endothelium (Furchgott & Vanhoutte, 1989). Pretreatment with N^G-nitro-L-arginine, an inhibitor of nitric oxide synthesis (Palmer *et al.*, 1988; Mulsch & Busse, 1990), increased the basal spontaneous myogenic contractions as well as the maximal phasic contractions produced by carbachol, while the basal contraction frequency and the maximal tonic response were not influenced. These findings suggest either a weak influence of endothelium-derived nitric oxide on the basal and the carbachol-stimulated muscular tone or a more pronounced excitatory influence of muscarinic stimulation in this preparation compared to other vascular tissues. Nevertheless, the small but significant influence of N^G-nitro-L-arginine indicates the presence of functional endothelium in the rat portal vein.

Acetylcholine can stimulate the production of various prostaglandins in vascular tissue. Kato *et al.* (1990) reported the formation of vasoconstrictor prostaglandin H_2 in the rat aorta whereas ACh-dependent synthesis of vasorelaxant prostacyclin was described by Jaiswal & Malik (1990) in rabbit aorta. Pretreatment with indomethacin (10^{-5} M) did not

significantly alter the basal myogenic contractions nor the concentration-response curve of carbachol in the rat isolated portal vein, although a significant increase of the spontaneous contraction frequency was observed. These results suggest that prostaglandins are not involved in the contractile responses elicited by muscarinic agonists, although they modulate the spontaneous mechanical activity of the rat portal vein.

There were agonist-dependent differences in the maximal effect on the phasic contractions. The weak efficacy of McN A 343 and pilocarpine, both agonists for which M_1-subtype selectivity has been claimed (Caulfield & Stubley, 1982; Hammer & Giachetti, 1984; Watson *et al.*, 1986) is remarkable. Arecoline and oxotremorine are both agonists which induce a full response on the phasic contractions but like McN A 343 and pilocarpine they were unable to induce a tonic contracture. Oxotremorine was suggested to possess moderate selectivity for M_2-receptors (Potter *et al.*, 1984). The question arose therefore, whether the phasic and the tonic types of responses are mediated by different muscarinic receptor subtypes. This explanation seems unlikely, since bethanechol, also an agonist with a certain M_2-selectivity (Rattan & Goyal, 1984), maximally stimulates both types of response. Furthermore, oxotremorine, a partial agonist with a higher affinity for muscarinic receptors compared to carbachol (Ringdahl, 1984) proved able to counteract the carbachol-induced contracture and to convert it into maximally increased phasic contractions. This finding clearly indicates that carbachol and oxotremorine have access to the same receptor population and marked differences in subtype selectivity between these two compounds can be ruled out as an explanation for the observed discrepancy of the functional responses. On the basis of the experiments with agonists it seems more likely that the different patterns of response observed for McN A 343, pilocarpine, arecoline and oxotremorine are caused by partial agonist properties and concomitantly by the availability of receptors and the nature of the coupling between receptor activation and response (Kenakin, 1984).

A serious drawback with respect to muscarinic receptor subtype classification by agonists is the lack of highly selective compounds. Potencies of pirenzepine, AF-DX 116, 4-DAMP and pFHHSiD were slightly greater against the tonic compared to the phasic response to carbachol although the rank order of potency was the same.

The non-selective antagonist, atropine (Mitchelson, 1984) was the most potent of those investigated. The pA_2-value for the phasic (9.7) and the tonic (10.0) response of the portal vein was higher compared to other studies on muscarinic receptors mediating vascular contraction *in vitro* ($pA_2 = 9.5$, Van Charldorp *et al.*, 1988; 9.3, Garcia-Villalon *et al.*, 1991).

Pirenzepine showed a relatively low affinity for the muscarinic receptors mediating the phasic ($pA_2 = 6.9$) and the tonic ($pA_2 = 7.25$) contractile responses. This finding is in agreement with studies in other vascular tissues ($pA_2 = 7.3$, Van Charldorp, 1988), and it indicates that M_1-receptors do not play an important role in the rat portal vein. This finding appears to be in accordance with the results obtained with the agonists, McN A 343 and pilocarpine. AF-DX 116 shows a moderate selectivity for cardiac muscarinic receptors (M_2). The pA_2-values in our study are relatively high (6.4 and 6.78) when compared to other vascular preparations (6.2, Van Charldorp, 1988) but nevertheless lower than those in cardiac preparations (7.3, Giachetti *et al.*, 1986).

The presence of muscarinic M_3-receptors in the longitudinal smooth muscle of the rat portal vein which mediate both the phasic and the tonic contractile responses was emphasized by the potent inhibitory activities of the M_3-selective antagonists 4-DAMP and pFHHSiD. Although the ability of 4-DAMP to discriminate between M_1- and M_3-receptor subtypes is limited (Doods *et al.*, 1987) its high potency argues against functional M_2-receptors in the portal vein.

pFHHSiD, a compound with a higher M_3-subtype selectivity (Lambrecht *et al.*, 1989) had pA_2-values (7.5, 7.87) close to those reported from preparations with an M_3-subtype population such as the endothelium of the rabbit isolated jugular vein (7.8, Eglen *et al.*, 1990).

The antagonist profile appears to be consistent with the presence of muscarinic M_3-receptors. In contrast to our results, O'Rourke & Vanhoutte (1987) concluded that the M_1-receptor subtype mediated contractile responses of canine saphenous vein to muscarinic agonists. Similar findings have been reported by Eglen *et al.* (1990) for the canine femoral vein. In this respect the rat portal vein appears to resemble arterial vessels for which the M_3-subtype mediates the contractile responses to muscarinic stimulation *in vitro* (Van Charldorp *et al.*, 1988; Duckles, 1988).

In contrast to the competitive antagonists, alkylation of the receptor with PrBCM was able to discriminate between the phasic and the tonic types of response to carbachol. At 10^{-9} M and 10^{-8} M PrBCM, the concentration-response curve for the phasic type of contraction was shifted to the right. A 30% depression of the maximal response was observed at 10^{-7} M PrBCM. The tonic type of response was clearly more sensitive to receptor alkylation. A rightward shift of the carbachol concentration-response curve with a concomitant depression of the maximum was observed. At 10^{-7} M PrBCM no tonic response was obtained. Similar dissociation constants (K_A) for carbachol for the two types of response (phasic 4.32 ± 0.3 μM; tonic 3.56 ± 0.21 μM, $n = 6$, NS) were calculated by the method of Furchgott & Bursztyn (1961). On the other hand, a 10 fold difference was found between the pEC_{50} values for the two types of contraction (see Table 1). This resulted in two distinct occupancy-response relationships. If the pEC_{50} values for carbachol for the phasic and tonic type of contraction represent the affinity at the same muscarinic receptor subtype, this would mean that there are two different receptor reserves for carbachol for the two types of response. Differences in the receptor reserve could account for the disparity in the potency of carbachol and the partial agonist properties of oxotremorine in inducing these two types of functional response.

Although the method of receptor-inactivation is a convenient technique for estimating agonist dissociation constants, its results must be treated with caution. One prerequisite for getting accurate K_A-values is that the receptor transduction characteristics are not altered by the treatment (Leff *et al.*, 1990). This might not be the case if receptor coupling proteins (G-proteins) are involved in the signalling pathway (Kenakin, 1990). Since no data are available on the transduction of muscarinic stimulation in the rat portal vein the presented K_A-values must be interpreted as apparent agonist dissociation constants.

It is tempting to speculate that the same muscarinic receptor could be coupled to two distinct signalling-pathways which mediate different types of response as shown in cardiac tissue of various species (Imai & Ohta, 1982; Brown & Goldstein, 1986; Eglen *et al.*, 1988). In conclusion, evidence has been presented that both the muscarinic agonist-induced increase of the phasic myogenic contractions and the tonic contracture in the longitudinal smooth muscle of the rat portal vein are mediated by M_3-receptors.

Neither the release of catecholamines nor the formation of prostanoids and nitric oxide can account for the contractile effect of muscarinic agonists in this preparation.

Among the muscarinic agonists investigated, full and partial agonists were found for the phasic and tonic contractile response.

Although different EC_{50}-values were observed for the two types of response the dissociation constants, as determined by means of irreversible receptor blockade, were nearly identical, which suggests differences in the functional receptor reserve.

Our data suggest that in the rat portal vein a homogeneous muscarinic receptor population mediates two types of mechanical responses with different efficiency.

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Selective I_K blockade as an antiarrhythmic mechanism: effects of UK66,914 on ischaemia and reperfusion arrhythmias in rat and rabbit hearts

Siân A. Rees & [1]Michael J. Curtis

Cardiovascular Research Laboratories, Department of Pharmacology, Division of Biomedical Sciences, King's College, University of London, Manresa Road, London SW3 6LX

1 UK66,914 is a specific and selective blocker of the delayed rectifying potassium current (I_K). The effectiveness of I_K block as a mechanism for prevention of ischaemia- and reperfusion-induced arrhythmias was tested by use of UK66,914: its actions in rat, a species deficient in cardiac I_K were compared with its actions in rabbit, a species possessing functional cardiac I_K. Antiarrhythmic actions in rabbit but none in rat is the only outcome possible if selective I_K blockade is responsible for the antiarrhythmic actions of the drug during ischaemia and/or reperfusion.

2 During 30 min regional ischaemia, 0.3 and 1 μM UK66,914 had no influence on the incidence of ventricular fibrillation (VF) in rat ($n = 9$/group), values being 78% in controls, 100% in 0.3 μM-treated hearts and 78% in 1.0 μM-treated hearts (NS). UK66,914 also had no effect on reperfusion-induced VF incidence (100% in each group), nor on the latency to onset of ischaemia- or reperfusion-induced arrhythmias. In contrast, in rabbit ($n = 13$/group), similar concentrations of drug reduced the incidence of reperfusion-induced VF from 77% in controls, to 38% and 31% ($P<0.05$) respectively. The incidence of ischaemia-induced arrhythmias was too low in controls to permit detection of an antiarrhythmic effect in rabbit; however no drug-induced proarrhythmia was seen.

3 QT interval was not affected by UK66,914 in rat (NS), values (mean ± s.e.mean) being 114 ± 8, 121 ± 8, and 113 ± 6 ms in control, 0.3 and 1 μM groups respectively, 10 min after the onset of ischaemia. However, in the rabbit the same concentrations of UK66,914 produced concentration-dependent widening of QT interval, values being 172 ± 14, 194 ± 9 (NS) and 233 ± 18 ms ($P<0.05$), with increasing concentrations of drug, 10 min after the onset of ischaemia.

4 UK66,914 had no substantial effects on haemodynamics (heart rate, coronary flow or recovery of flow during reperfusion), at either concentration, in either species. Occluded zones sizes were similar with increasing concentrations of drug in rat (42 ± 4, 40 ± 2 and 40 ± 2% of total ventricular weight) and rabbit (46 ± 2, 43 ± 2 and 46 ± 3%). Thus, the species-dependent antiarrhythmic effects of UK66,914 were not attributable to differences in these variables.

5 In conclusion, the rat versus rabbit data indicate that for UK66,914 to inhibit ventricular arrhythmias associated with reperfusion, I_K blockade is sufficient and necessary. The rat data indicate that no antiarrhythmic effects occur with the drug during ischaemia when there is no scope for I_K blockade. Whilst other mechanisms may be unlikely to be involved, they cannot be excluded completely. Together the rat and rabbit studies indicate that the antiarrhythmic effects of UK66,914 during ischaemia and reperfusion are adequately explained by I_K blockade.

Keywords: UK66,914; ventricular fibrillation; myocardial ischaemia; reperfusion; I_K

Introduction

Ischaemic heart disease remains a serious problem despite intensive research in recent years. In the U.S.A. alone, 400,000 people die each year from this pathological condition (Lucchesi, 1984). Death is generally attributed to cardiac arrhythmias, especially ventricular fibrillation (Adgey *et al.*, 1971), which can be elicited by ischaemia (Campbell *et al.*, 1981) and by reperfusion (Tzivoni *et al.*, 1983). The findings of the CAST study (1989) illustrate that better antiarrhythmic agents devoid of serious side effects are required. Targetting drugs for arrhythmias may be facilited by combining specificity with selectivity; once a beneficial pharmacological action has been described, drugs with a selective ability to produce this effect can be sought. The present study is concerned with the potential of specific and selective blockade of the delayed rectifier potassium current (I_K) as an antiarrhythmic mechanism.

Antiarrhythmic drugs which block potassium (K^+) currents are generally classified as Class III agents (Colatsky *et al.*, 1990), although this activity is primarily defined on the basis of prolongation of action potential duration (APD) and/or QT interval, in the absence of significant actions on conduction (Vaughan Williams, 1970). In ischaemic heart disease, when re-entrant excitation is an important mechanisms of abnormal conduction (Janse *et al.*, 1986; Curtis & Hearse, 1989a), antiarrhythmic effects of Class III agents can be expected, via prolongation of APD and refractoriness (Singh & Nademanee, 1985; Gibson & Kersten, 1990). However, until recently, most Class III agents have been non-selective, possessing other actions. For example, amiodarone inhibits fast and slow inward currents (Mason *et al.*, 1983), as well as I_K and the inwardly rectifying (I_{K1}) K^+ current (Colatsky *et al.*, 1990). In the setting of acute ischaemia and reperfusion, the utility of Class III agents is equivocal (see Tsuchihashi & Curtis, 1991). Recently, new agents have been developed which not only affect APD selectively, but affect particular cardiac repolarizing K^+ currents selectively. This raises the possiblity of revising the classification of agents erstwhile part of the broad Class III group, in terms of the specific channel with which they interact, as recently proposed by Colatsky (1992) in accordance with the principles outlined in the Sicilian Gambit (Task Force of the Working

[1] Author for correspondence.

Group on Arrhythmias of the European Society of Cardiology, 1991). Such new agents include tedisamil, which blocks the transient outward current (I_{to}; Dukes *et al.*, 1990), RP58866, an I_{K1} blocker (Escande *et al.*, 1989), UK66,914, an I_K blocker (Gwilt *et al.*, 1988) and glibencamide which blocks the ATP-dependent K^+ channel ($I_{K(ATP)}$; Kantor *et al.*, 1990), the latter acting as a Class III agent in ischaemic tissue only. These agents therefore make it possible to test not only whether selective Class III activity is a useful antiarrhythmic mechanism in particular settings such as ischaemia and reperfusion, but also whether selective block of a particular K^+ current is more effective than blockade of another for suppression of such arrhythmias.

In order to examine the potential utility of I_K blockade as an antiarrhythmic mechanism in the setting of acute regional ischaemia and reperfusion, we have used two species (rat and rabbit) for study. Rabbit ventricle possesses I_K (Carmeliet, 1991), whereas rat ventricle lacks functional I_K (Gwilt *et al.*, 1986; Tande *et al.*, 1990). The drug chosen, UK66,914, is a specific and selective I_K blocker, at concentrations with no actions on I_{K1}, I_{Na} or I_{Ca} (Gwilt *et al.*, 1991). Recent work on the anaesthetized dog indicates that UK66,914 prolongs ventricular effective refractory period (ERP), widens QT interval and increases electrically-induced ventricular fibrillation threshold (Gwilt *et al.*, 1988). The hypothesis that I_K block represents a specific antiarrhythmic mechanism in the settings of acute ischaemia and reperfusion predicts that UK66,914 should prevent arrhythmias in rabbit, but not in rat.

Our findings have been presented in a preliminary form to the British Pharmacological Society (Rees & Curtis, 1991a,b) and to the International Society for Heart Research (Rees & Curtis, 1992).

Methods

The perfusion technique, methods for induction of ischaemia and reperfusion, methods for verification and quantification of occluded zone size and the techniques for recording, quantifying and analysing data, have all been described previously (Curtis & Hearse, 1989a,b).

Animals and general experimental methods

Rats (male Wistar; Bantin and Kingman, UK; 250–300 g; $n = 9$/group) were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.). Hearts were excised 30 s after an i.v. injection of 250 iu sodium heparin and placed into ice-cold perfusion solution containing (in mM): NaCl 118.5, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.4, KCl 3.0 and glucose 11.1. Hearts were perfused according to Langendorff (1895), with perfusion solution delivered at 37°C and pH 7.4. All solutions were filtered (5 μm pore size) before use. Perfusion pressure was constant and equivalent to 100 cmH_2O. A unipolar electrogram (ECG) was recorded by implanting one stainless-steel wire electrode into the centre of the region to become ischaemic with a second connected to the aorta. A traction-type coronary occluder consisting of a silk suture (Mersilk, 4/0) threaded through a polythene guide was used for coronary occulsion. The suture was positioned loosely around the left main coronary artery beneath the left atrial appendage. Regional ischaemia was induced by tightening the occluder, and reperfusion by releasing it.

Rabbits (male New Zealand White; Froxfield, UK; 0.9–1.2 kg; $n = 13$/group) were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.v.) with sodium heparin (250 iu, i.v.). Perfusion was as described for the rat except that the Ca^{2+} concentration of the perfusion solution was raised to 2.8 mM. Although blood Ca^{2+} concentration in rabbit is slightly higher than that in rat (Galinanes & Hearse, 1990), our primary reason for using a higher Ca^{2+} concentration in rabbit studies was to raise the susceptibility to arrhythmias in this species, since baseline incidence of ischaemia and reperfusion arrhythmias is lower in rabbit than in rat (Coker, 1989; Tanaka & Hearse, 1988; Tsuchihashi & Curtis, 1991). In preliminary studies in which rabbit hearts were perfused with solution containing 1.4 mM Ca^{2+}, reperfusion-induced VF occurred in 13% of hearts ($n = 8$) compared with 77% of hearts ($n = 13$) perfused with solution containing 2.8 mM Ca^{2+} ($P < 0.05$). Reperfusion arrhythmia score, as defined by Curtis & Walker (1988), was also significantly higher in hearts perfused with 2.8 mM Ca^{2+} (3.5 ± 0.3 versus 1.1 ± 0.7 for hearts perfused with 1.4 mM Ca^{2+}; $P < 0.05$). Thus to provide maximum scope for detection of antiarrhythmic effects of UK66,914, we perfused rabbit hearts with solution containing 2.8 mM Ca^{2+}. The ECG was recorded in the same way as for rat studies. The electrode arrangement in rabbit and rat studies gives an ECG dominated by the local signal, as expected, and as demonstrated by regional recording from adjacent perfusion beds (Curtis, 1991b).

In further experiments the antiarrhythmic effects of the highest concentration of UK66,914 (1.0 μM) were investigated in rat hearts ($n = 9$/group) perfused with solution containing an elevated Ca^{2+} concentration (2.8 mM) to match that used in rabbit studies.

Experimental protocol

Hearts were perfused for an initial 5 min with control solution, then the solution was changed in a randomised, 'blind' fashion to one of 3 solutions: control, 0.3 μM UK66,914 or 1.0 μM UK66,914. Control solution contained an appropriate volume of vehicle (50% ethanol/50% water). After a further 5 min perfusion, the left coronary artery was occluded. After 30 min ischaemia the occluder was released to achieve reperfusion.

The choice of drug concentration was based on published data which shows that UK66,914 has little effect on cardiac electrophysiology at 0.5 μM, but has a concentration-dependent ability to prolong APD and increase ERP (without affecting fast or slow inward currents), over the range of 0.3 to 1.0 μM (Gwilt *et al.*, 1991).

Measurement of occluded zone size

Two independent methods were used to verify occlusion and to delineate the occluded zone from uninvolved tissue. First, coronary flow was measured by collecting the coronary effluent in a graduated cylinder; occlusion was verified by comparing flow at 1 min before occlusion with flow at 1 min after occlusion and was quantified in terms of the percentage reduction in flow. Second, at the end of 5 min of reperfusion, readmission of flow was verified and the size of the formerly occluded zone was quantified by the disulphine blue dye exclusion method (Curtis & Hearse, 1989a). Occluded zone size was quantified as a % of total ventricular weight. Values of coronary flow in the uninvolved tissue and the reperfused zone were calculated from the total coronary flow and the weights of the occluded zone and the uninvolved zone, as described previously (Curtis & Hearse, 1989b).

Exclusion criteria

Any rat heart with a sinus rate less than 250 beats min^{-1} or a coronary flow more than 18 ml min^{-1} or less than 8 ml min^{-1} 5 min before the onset of ischaemia (and before randomisation) was excluded. Likewise, any rabbit heart with a sinus rate less than 200 beats min^{-1} or a coronary flow greater than 80 ml min^{-1} or less than 35 ml min^{-1} was excluded. Furthermore, any heart not in sinus rhythm during the 2 s before reperfusion was excluded from the reperfusion sample.

Arrhythmia diagnosis and ECG analysis

A digital storage type oscilloscope (Gould, model DSO400) and a Gould chart recorder (model RS3200) were used in the

identification and analysis of waveforms and diagnosis of arrhythmias. Arrhythmias were defined according to the Lambeth Conventions (Walker *et al.*, 1988) with slight modification (Tsuchihashi & Curtis, 1991). Ventricular premature beats (VPB) were defined as discrete and identifiable premature QRS complexes; a run of 4 or more VPBs was defined as ventricular tachycardia (VT). Ventricular fibrillation (VF) was defined as a signal from which individual QRS deflections vary in amplitude and coupling interval on a cycle to cycle basis. From the ECG, the incidence and the time to onset of ventricular tachyarrhythmias, the RR interval and the QT interval (measured at the point of 100% repolarization with on-screen cursors) were obtained. QT interval is corrected for heart rate in man, using Bazett's correction ($QTc = QT/\sqrt{RR}$), in order to accomodate for rate-dependent APD alterations (Bazett, 1920). However, it is not known whether QT is rate-dependent in rat or rabbit, thus the appropriate corrections for these species are unknown. For this reason we have presented QT in three ways: uncorrected (QT), corrected according to Bazett's formula (QTc) and corrected according to Zbinden *et al.* (1980) ($QTz = QT/RR$).

Measurement of all variables was performed in a 'blind' manner permitting use of sampling-based statistics (see below).

Drugs and materials

UK66,914 (N-(4-{1-hydroxy-2-[4-(4-pyridinyl)-1-piperazinyl] ethyl}phenyl)methanesulphonamide; batch number 66914/03R/1) was a gift from Pfizer Central Research (Sandwich, Kent, UK). It was stored as a 10^{-3} M stock solution in acidified 50% ethanol/50% H_2O. All salts were reagent grade chemicals from Sigma Chemical Co. Water for preparing perfusion solution was obtained by use of a reverse osmosis system (Milli-RO 10 and Milli-Q 50, Millipore Ltd).

Statistics

Gaussian distributed variables were expressed as mean ± s.e.mean and were subjected to analysis of variance. If treatment constituted a significant source of variance, each group was compared with the control by Dunnett's test. The time to onset of first arrhythmias was $\log_{10}$ transformed to generate a Gaussain distributed variable as described previously (Curtis & Hearse, 1989a). Binomially distributed variables were compared by the Chi^2 test with Yates' correction where appropriate (Gad & Weil, 1989). $P<0.05$ was taken as indicative of a statistically significant difference between values. NS indicates no statistically significant difference.

Results

Effects of UK66,914 in rat

According to the hypothesis that UK66,914 exerts its antiarrhythmic effects via I_K blockade, the drug should produce no effects on arrhythmias or on QT interval in the rat, since this species does not possess cardiac I_K (Gwilt *et al.*, 1986; Tande *et al.*, 1990).

UK66,914 had no significant effect on the incidence of ischaemia- or reperfusion-induced arrhythmias (Figures 1 and 2). Furthermore, it neither delayed nor hastened the onset of ischaemia- or reperfusion-induced arrhythmias (Table 1). It was also without effect on QT interval (Figure 3). Thus UK66,914 had no significant effects on any of the variables studied.

UK66,914 also had no significant effects on haemodynamic variables. RR interval was 246 ± 13, 243 ± 17 and 249 ± 13 ms in controls, 0.3 μM and 1.0 μM groups respectively (NS). Coronary flow in these groups 1 min before the onset of ischaemia was 10.9 ± 0.5, 11.1 ± 0.6 and 11.0 ± 0.5 ml min^{-1} g^{-1} respectively (NS) and recovery of coronary flow was

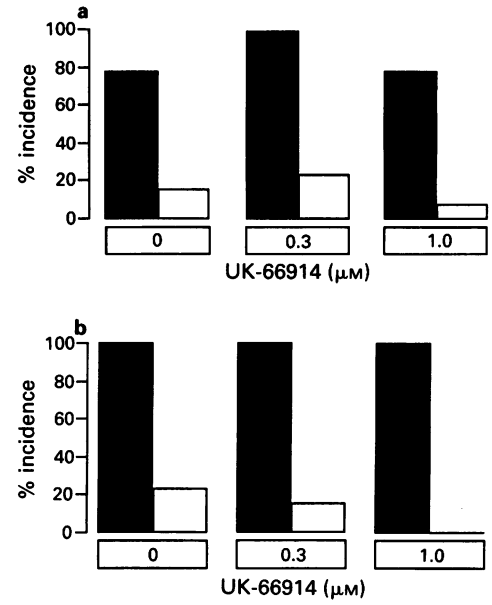


Figure 1 Effect of UK66,914 on % incidence of ischaemia-induced VF (a) and VT (b) in rat (solid columns) and rabbit (open columns). There were no statistically significant differences between groups within each species ($n = 9$ per group of rats; $n = 13$ per group of rabbits).

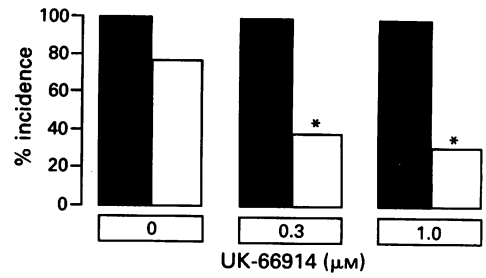


Figure 2 Effect of UK66,914 on % incidences of reperfusion-induced VF in rat (solid columns) and rabbit (open columns). *$P<0.05$ vs controls (0 μM) within each species ($n = 9$ per group of rats; $n = 13$ per group of rabbits).

9.7 ± 1.0, 9.1 ± 0.8 and 10.3 ± 1.5 ml min^{-1} g^{-1} of reperfused tissue respectively (NS). The occluded zone size was also similar in each group, values being 42 ± 4, 40 ± 2, 40 ± 2% of total ventricular weight, with increasing concentrations of drug (NS).

In rat hearts perfused with solution containing 2.8 mM Ca^{2+}, UK66,914 (1.0 μM) had no significant effects on ischaemia- or reperfusion-induced VF or VT, QT interval or RR interval (Table 2). The drug was also without significant effect on coronary flow measured 1 min before ischaemia (14.5 ± 0.9 ml min^{-1} g^{-1} total ventricular weight in control group versus 15.1 ± 0.9 in drug-treated hearts) or on recovery of flow on reperfusion (12.0 ± 1.9 ml min^{-1} g^{-1} total ventricular weight in control group versus 11.6 ± 1.9 in drug-treated hearts). Occluded zone sizes were similar in both groups, values being 42 ± 3% in control hearts versus 42 ± 2% in UK66,914-treated hearts.

Table 1 Effect of UK66,914 on latency to ischaemia- or reperfusion-induced arrhythmias (Arr), relative to the onset of ischaemia or reperfusion, respectively, in rat (in $\log_{10}$ s)

	Control	0.3 μM *UK66,914*	1.0 μM *UK66,914*
1st Ischaemic Arr	2.78 ± 0.04	2.80 ± 0.03	2.72 ± 0.06
1st Ischaemic VF	3.10 ± 0.03	3.13 ± 0.04	3.16 ± 0.02
1st Reperfusion VF	1.05 ± 0.09	0.87 ± 0.21	0.81 ± 0.15

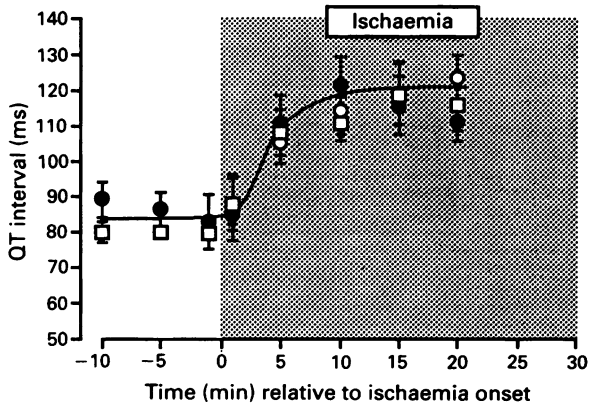


Figure 3 Effect of UK66,914 on QT interval (ms) in rat ($n = 9$/group) before and at various times after the onset of ischaemia. Values are mean ± s.e.mean (vertical bars) for control (○), 0.3 μM UK66,914 (●) and 0.1 μM UK66,914 (□) groups. Background shading indicates ischaemia. There were no statistically significant differences between groups at any timepoint during the experiment. The line indicates change in values before and during the timecourse of ischaemia.

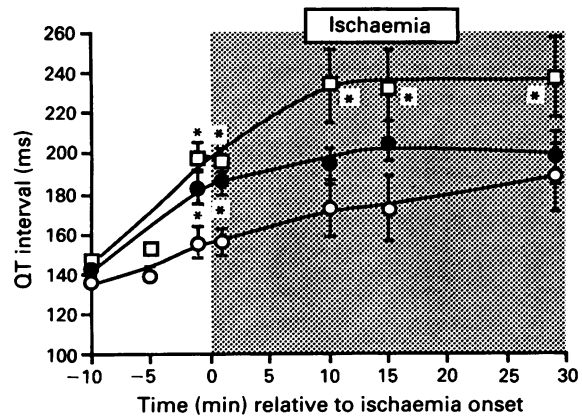


Figure 4 Effect of UK66,914 on QT interval (ms) in rabbit ($n = 13$/group) before and at various times after the onset of ischaemia. Values are mean ± s.e.mean (vertical bars) for control (○), 0.3 μM UK66,914 (●) and 0.1 μM UK66,194 (□) groups. Background shading indicates ischaemia. Statistically significant differences versus control: $^*P < 0.05$.

Effects of UK66,914 in rabbit

The hypothesis under test predicts that UK66,914 will inhibit arrhythmias and prolong QT in rabbit, since rabbit ventricle possesses I_K (Carmeliet, 1991).

The control incidence of ischaemia-induced VT and VF was very low (VT 23% incidence and VF 15% incidence; Figure 1). Thus it was not possible to examine the effects of UK66,914 against ischaemia-induced arrhythmias in the rabbit heart. Proarrhythmic effects could, however, be examined. Figure 1 shows that UK66,914 had no proarrhythmic activity.

Reperfusion-induced VF occurred with a high incidence (10/13) in control rabbit hearts. This permitted analysis of the antiarrhythmic effects of UK66,914. Figure 2 shows that 0.3 μM and 1.0 μM UK66,914 significantly reduced the incidence of reperfusion-induced VF by more than 50% ($P < 0.05$). There was no significant effect on the time to onset of reperfusion-induced VF, values (in log s) being 1.99 ± 0.16, 1.95 ± 0.11 and 1.80 ± 0.21 in control, 0.3 μM and 1.0 μM groups respectively. VT was not affected by the drug (incidence was 46%, 62% and 69%, in control, 0.3 μM and 1.0 μM UK66,914 groups respectively; NS)

UK66,914 caused slight bradycardia which reached statistical significance at the high (1.0 μM) concentration (RR interval values 10 min after the start of ischaemia were 282 ± 14 ms in controls versus 331 ± 16 ms with 1.0 μM UK66,914; $P < 0.05$). UK66,914 prolonged QT at each concentration (Figure 4) and changes were significant at the highest concentration ($P < 0.05$), irrespective of whether QT was corrected for bradycardia or which correction was used (Table 3).

UK66,914 did not affect any other variable. Coronary flow 1 min before the onset of ischaemia was 13.8 ± 0.8, 12.3 ± 0.5, and 13.4 ± 0.6 ml $min^{-1}g^{-1}$ in groups with increasing concentrations of drug (NS). Recovery of coronary flow was 11.4 ± 1.6, 10.4 ± 1.3 and 12.0 ± 1.3 ml $min^{-1}g^{-1}$ of reperfused tissue with increasing concentration of drug (NS). Occluded zone sizes were similar to values in rat, and were 46 ± 2, 43 ± 2 and 46 ± 3% ventricular weight respectively (NS).

Thus, in the rabbit isolated perfused heart UK66,914 prolongs QT (corrected for bradycardia) and suppresses reperfusion-induced VF, without substantial haemodynamic changes.

Discussion

Antiarrhythmic mechanisms in ischaemia and reperfusion

Development of new antiarrhythmic drugs for prevention of ventricular arrhythmias associated with acute ischaemia and reperfusion may be done rationally by the design of drugs with highly selective cellular actions. However, this requires knowledge that the chosen mechanism (for example, block of I_K) is, in fact, a mechanism that will lead to arrhythmia suppression. Surprisingly, there is scanty proof that current antiarrhythmic drugs suppress arrhythmias in acute ischaemia or reperfusion by the cellular mechanisms commonly attributed. For example, although it may be commonly accepted that quinidine suppresses VF via block of sodium channels, its lack of selectivity for these channels legitimizes the suggestion, should it be made, that other actions play a greater or lesser role in mediating its antiarrhythmic effects.

Table 2 Effect of UK66,914 on arrhythmias (Arr), QT interval and RR interval in rat, in the presence of 2.8 mM Ca^{2+}

	Incidence of arrhythmias (%)			*Onset time of arrhythmias* (log s)		
	Isch VF	Isch VT	Rep VF	Isch Arr	Isch VF	Rep VF
Control	56	100	83	2.16 ± 0.24	2.86 ± 0.12	1.42 ± 0.26
UK66,914	44	100	83	2.33 ± 0.10	2.95 ± 0.03	1.00 ± 0.26
	QT interval (ms)			*RR interval* (ms)		
	QT-10	QT-1	QT + 10	RR-10	RR-1	RR + 10
Control	88 ± 5	95 ± 6	114 ± 9	184 ± 5	208 ± 5	252 ± 7
UK66,914	88 ± 3	89 ± 3	131 ± 9	177 ± 5	199 ± 7	257 ± 10

Isch = ischaemia-induced, Rep = reperfusion-induced. QT-10 and RR-10 are readings taken before randomization and 10 min before ischaemia; QT-1 and RR-1 are readings taken after randomization but 1 min before ischaemia; QT + 10 and RR + 10 are readings taken after 10 min of ischaemia. There were no significant differences between the two groups ($P > 0.05$).

In the case of quinidine I_K, I_{K1} or I_{to} block are possible mechanisms (Colatsky, 1990) which may contribute to its antiarrhythmic activity. Drug development is therefore hampered by the difficulty in establishing proof of an antiarrhythmic mechanism in terms of specific cellular targets. Part of the problem in establishing proof of a mechanism is that, for ion channel blocking drugs (especially Na^+ and K^+ channel blockers) one cannot readily use a conventional approach to pharmacological analysis. For example it is not possible to shift the dose-response curve for any antiarrhythmic effect to the right by raising agonist (Na^+ or K^+) concentration since the simple agonist/antagonist paradigm does not apply. Nor can one measure ion current by patch clamp in the whole heart and relate this to arrhythmias induced contiguously in the same preparation owing to technical restrictions. Therefore, if one wishes to establish a cellular action as a specific antiarrhythmic mechanism in the settings of ischaemia and reperfusion, alternative approaches are required.

The present study is concerned with the hypothesis that I_K blockade is a specific antiarrhythmic mechanism during ischaemia and reperfusion. We have tested the hypothesis using a novel approach. We used a highly selective I_K blocker (UK66,914) and compared its actions in rat and rabbit, making use of the fact that the rat ventricle is devoid of functional cardiac I_K (Tande *et al.*, 1990) whereas the rabbit possesses this current (Carmeliet, 1991).

I_K *blockade as a specific antiarrhythmic mechanism*

We found that UK66,914 did not suppress ischaemia- or reperfusion-induced arrhythmias in rat at any of the concentrations studied; neither were there effects on QT interval, haemodynamic variables (heart rate and coronary flow), nor on independent determinants of arrhythmogenesis (occluded zone size). The drug was also ineffective when the Ca^{2+} concentration of the perfusion solution was raised to match that used in rabbit studies (2.8 mM). Therefore, we deduce that when there is no scope for cardiac I_K blockade, UK66,914 is devoid of antiarrhythmic activity during ischaemia and reperfusion.

In contrast, in rabbit, UK66,914 reduced the incidence of reperfusion-induced arrhythmias and prolonged QT interval (irrespective of the heart rate correction used). The higher concentration of drug (1.0 μM) also caused slight bradycardia, in accordance with other studies *in vitro* (Tande *et al.*, 1990; Yang *et al.*, 1991) which have reported negative chronotrophy with I_K blocking agents. UK66,914 did not alter coronary flow or occluded zone size, so arrhythmia suppression was not secondary to changes in these variables. Therefore we deduce that when there is scope for cardiac I_K blockade, UK66,914 causes QT prolongation and arrhythmia suppression.

Taken together, the rat and rabbit data indicate that for UK66,914 to inhibit reperfusion-induced VF, I_K blockade and associated QT prolongation are necessary and sufficient. Lack of protective action in rat is not merely negative evidence since, should protection have been observed then this would have falsified the I_K hypothesis by indicating that a non-specific mechanism is sufficient to explain the protective action of the drug, and I_K block (which does not occur in the rat ventricle) unnecessary. Had reperfusion-induced VF not been suppressed in rabbit then we would have deduced that I_K block by UK66,914 is not sufficient to constitute an antiarrhythmic mechanism during reperfusion.

In the setting of ischaemia, direct comparison of VF suppression in rat versus rabbit was not possible because the control rabbit hearts exhibited very few ischaemia-induced arrhythmias compared with the rat. However, in the rat, the observed lack of action of UK66,914 on ischaemia-induced VF was exactly as expected if I_K blockade is necessary for the drug to suppress this arrhythmia.

Indirect support of the I_K *hypothesis from QT changes*

During ischaemia UK66,914 prolonged QT in rabbit but not rat. QT prolongation by UK66,914 can be explained by (and is evidence of) I_K block in rabbit, and its absence in rat can be explained by (and is evidence of) absence of I_K block. QT prolongation during reperfusion could not be examined meaningfully owing to the rapid occurrence of arrhythmias upon reperfusion, particularly in control hearts. It could be argued that QT differences at the end of ischaemia (29 min after occlusion) may reflect better any tendency of the drug to delay repolarization during reperfusion than QT differences recorded earlier (e.g. at 10 min after the onset of ischaemia). However, since reperfusion causes rapid washout of K^+ from the extracellular space (Watanabe *et al.*, 1988) and rapid hyperpolarization (Ferrier *et al.*, 1985) then QT values during early reperfusion (which we cannot measure) may be better reflected by equivalent values during early ischaemia (e.g. after 10 min at which time extracellular K^+ is less elevated; Hirche *et al.*, 1980) than values at 29 min (when K^+ values reach as much as 30 mM; Hirche *et al.*, 1980).

It has been reported that under conditions of global hypoxia (MacKenzie *et al.*, 1990) and global ischaemia (Adams *et al.*, 1990) UK66,914 and other Class III agents lose their ability to prolong APD. Our QT data measured under conditions of regional ischaemia, were not completely in accordance with these earlier studies. This may perhaps reflect differences between the global and regional models used. We observed, however, a tendency for the effects of the drug to diminish after sustained (29 min) ischaemia.

Recently Sanguinetti & Jurkiewicz (1990) suggested that in guinea-pig ventricular myocytes, I_K may be subclassified (on the basis of channel kinetics and sensitivity to blockade by certain K^+ channel blockers) as I_{Kr} ('rapid') and I_{Ks} ('slow'). I_{Kr} activates very rapidly and is sensitive to blockade by the benzenesulphonamide, E-4031. I_{Ks}, in contrast, is characterized by a delayed onset of activation that occurs over a voltage range typical of the classically described cardiac I_K and is not blocked by E-4031. UK66,914 blocks both I_{Kr} and I_{Ks}, but blocks the former at lower concentrations (Gwilt *et al.*, 1991). Dissection of the relative effectiveness of I_{Kr} and I_{Ks} block as antiarrhythmic mechanisms has previously not been attempted. In the present study, all the deduction we have made regarding the role of I_K in mediating the antiarrhythmic effects of UK66,914 can be extended, in the case of the rabbit, to I_{Kr}. This is because rabbit ventricle possess functional I_{Kr} but not I_{Ks} (Carmeliet, 1991). Whether I_{Ks} block by UK66,914 contributes to its antiarrhythmic activity in other species in which functional I_{Ks} is present cannot be assessed from the present studies and this will be resolved only when novel agents with pure I_{Ks} blocking action have been developed.

Table 3 Effect of UK66,914 on QT interval (corrected and uncorrected) in rabbit

	QT			*QTc*			*QTz*		
	Control	0.3 μM	1.0 μM	Control	0.3 μM	1.0 μM	Control	0.3 μM	1.0 μM
−10	139.2 ± 3.9	140.7 ± 3.9	145.5 ± 2.7	8.8 ± 0.3	9.1 ± 0.2	9.4 ± 0.2	0.57 ± 0.02	0.59 ± 0.02	0.60 ± 0.02
−1	155.6 ± 8.0	183.3 ± 7.5*	199.3 ± 6.8*	9.5 ± 0.5	10.8 ± 0.4*	11.7 ± 0.4*	0.59 ± 0.03	0.64 ± 0.03	0.68 ± 0.03*
+10	172.8 ± 14.0	193.6 ± 8.8	232.8 ± 18.2*	10.2 ± 0.6	11.0 ± 0.3	12.7 ± 0.7*	0.61 ± 0.02	0.63 ± 0.01	0.70 ± 0.02*

Time refers to min before (−) and after (+) the onset of ischaemia

I_K block and proarrhythmia in rabbit

The low baseline incidence of ischaemia-induced VF in rabbits means that if the drug were to have had proarrhythmic effects then there would have been scope for such effects to be revealed. Class III antiarrhythmics have long been associated with proarrhythmia, especially torsades de pointes (McKibben *et al.*, 1984; Curtis, 1991a). Torsades de pointes was originally described by Dessertenne (1966) as a type of ventricular tachyarrhythmia intermediate between VT and VF, although it may better be described as a syndrome (Curtis, 1991a). Rabbit hearts are unusually susceptible to torsades de pointes when exposed to Class III agents in the presence of α_1-adenoceptor agonists (Carlsson *et al.*, 1991). However, we found no proarrhythmic effects of UK66,914 in terms of either the incidence of arrhythmias or the time to onset of arrthymias. This suggests that in the absence of raised α_1-tone, I_K block is not a specific mechanism for proarrhythmia in hearts subjected to acute ischaemia.

Conclusion

I_K blockade appears to be a specific antiarrhythmic mechanism in reperfusion. It remains to be seen whether specific and selective blockade of I_K leads to suppression of ischaemia- or reperfusion-induced ventricular arrhythmias in man.

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Neurokinin-induced changes in pial artery diameter in the anaesthetized guinea-pig

[1]D.T. Beattie, C.M. Stubbs, H.E. Connor & W. Feniuk

Department of Neuropharmacology, Glaxo Group Research Ltd, Park Road, Ware Herts SG12 0DP

1 The effects of selective neurokinin agents on pial artery diameter, measured with an on-line image analyser, have been studied in anaesthetized guinea-pigs in order to characterize the neurokinin receptors present on pial arteries.

2 Perivascular injection of either substance P (0.01–1 μM) or the selective NK_1 receptor agonists, substance P methyl ester (SPOMe, 0.01–1 μM) and GR73632 (0.1 μM), increased pial artery diameter.

3 In contrast, the selective NK_2 receptor agonist, GR64349 (1 μM), produced a small vasoconstriction while the NK_3 receptor-selective agonist, senktide (1 μM) was inactive.

4 Co-administration of GR82334 (1 μM), a selective NK_1 receptor antagonist, inhibited the vasodilatation produced by SPOMe (0.1 μM) but not that caused by calcitonin gene-related peptide (CGRP, 0.01 μM).

5 The results are consistent with an involvement of NK_1 receptors in the neurokinin-induced increase in guinea-pig pial artery diameter.

Keywords: Pial artery; vasodilatation; substance P; neurokinin receptors; calcitonin gene-related peptide (CGRP); GR73632; GR64349; GR82334

Introduction

The neuropeptides, substance P and calcitonin gene-related peptide (CGRP), have been implicated in the pathogenesis of vascular headache (Moskowitz, 1984; Moskowitz *et al.*, 1989). Cranial blood vessels possess a dense distribution of substance P- and CGRP-like immunoreactive nerve fibres originating in the trigeminal ganglion (Edvinsson *et al.*, 1983; Liu-Chen *et al.*, 1983; Skofitsch & Jacobowitz, 1985) and stimulation of these fibres results in a release of substance P and CGRP (Goadsby *et al.*, 1988; Moskowitz *et al.*, 1983). Substance P causes both local oedema and plasma protein extravasation by increasing vascular permeability (Markowitz *et al.*, 1987) and, in common with CGRP, produces vasodilatation of the intracranial vasculature (Edvinsson *et al.*, 1981; McCulloch *et al.*, 1986). This vasodilatation may result in the initiation of local axon reflexes and thus further activation of trigeminal perivascular sensory nerves contributing to the perception of pain (Humphrey & Feniuk, 1991).

It is now clear that neurokinin receptor-mediated responses, such as those implicated in vascular headache, may involve at least three different receptors, classified NK_1, NK_2 and NK_3 according to relative potencies of neurokinin agonists (Lee *et al.*, 1986; Watson *et al.*, 1983). Although substance P has greater affinity for NK_1 receptors (Regoli *et al.*, 1989; Guard & Watson, 1991), compounds with a much greater selectivity for each neurokinin receptor type have recently been introduced, allowing a more definitive classification of neurokinin-induced responses. Thus, there are selective neurokinin receptor agonists, such as SPOMe (Watson *et al.*, 1983) and GR73632 (both NK_1), GR64349 (NK_2) (Hagan *et al.*, 1991b) and senktide (NK_3) (Wormser *et al.*, 1986), and antagonists, such as the NK_1-sensitive, GR82334 (Hagan *et al.*, 1991a), each exhibiting 100–1000 fold selectivity for the particular receptor type implicated in, for example, neurokinin-induced constriction of the rat portal vein (NK_3) or plasma protein extravasation (NK_1). GR73632, GR64349 and GR82334 are resistant to metabolism by peptidases (Hagan *et al.*, 1991a,b) and are therefore useful for *in vivo* investigation of neurokinin-mediated effects. The use of these compounds in an earlier study (Stubbs *et al.*, 1992) indicated that substance P-induced relaxations of the dog middle cerebral artery, *in vitro*, were mediated via NK_1 receptor activation. The aim of the present study was to characterize the neurokinin receptor(s) mediating dilatation of guinea-pig pial arteries *in vivo* by use of these selective drug tools.

[1] Author for correspondence.

Methods

Adult male Dunkin-Hartley guinea-pigs (300–450 g) were anaesthetized (i.p.) with ketamine (40 mg kg^{-1}), pentobarbitone (18 mg kg^{-1}) and xylazine (6 mg kg^{-1}). The trachea was cannulated and animals artificially respired (60 strokes min^{-1}, 12 ml kg^{-1}) with room air. The right carotid artery and jugular vein were cannulated to permit, respectively, the continuous measurement of arterial blood pressure and infusion of anaesthetic (15 mg kg^{-1} h^{-1} pentobarbitone). The acid/base status in the arterial blood was checked during the experiment by blood gas analysis (Radiometer ABL 30) and pH, $PaCO_2$ and PaO_2 maintained at physiological levels (7.36 ± 0.01, 38.2 ± 2.0 mmHg and 98.3 ± 5.2 mmHg respectively (mean ± s.e.mean, $n = 53$)) by adjusting the stroke volume and/or supplementing the inspired air with O_2. Rectal temperature was monitored and maintained at 37–38°C with a heated blanket and thermistor (CFP 8185).

The head of the guinea-pig was placed in a stereotaxic frame (David Kopf) and a longitudinal incision made in the scalp. A craniotomy measuring, approximately, 1 cm × 0.5 cm was made over the left parietal cortex with a saline-cooled dental drill and the area covered with mineral oil (37°C). The dura was then carefully removed to expose the underlying pial blood vessels. Changes in pial artery calibre were recorded with an automatic image analyser (HVS SP143) attached via a camera (Ikegami) to a binocular microscope (Meiji) and displayed, together with blood pressure on a chart recorder (Lectromed M19). The image was continuously displayed on a videomonitor (Hitachi VM-910E/K).

Drugs were dissolved in artificial cerebrospinal fluid (ACSF) immediately before use and were applied (approx. 0.5 μl in 10 s) by pressure ejection (WPI PV 830) through glass micropipettes (6–8 μm tip, outer diameter) placed, by use of a micromanipulator, in the perivascular space close to the artery under investigation. ACSF was applied to each artery prior to any drug application, and in most experiments 10 mM K^+ was ejected to ascertain the normal vessel reactivity. Responses were expressed as a percentage change from the pre-injection diameter. Antagonist/agonist interactions were studied by co-administration from the same micropipette after having first determined, on different arteries, the responses to the agonist and antagonist alone.

Drugs and solutions

The ACSF had the following composition (mmol l^{-1}): Na^+ (159.5), K^+ (2.5), Ca^{2+} (1.5), Cl^- (150.5), HCO_3^- (14.5) (Wahl *et al.*, 1989) and was bubbled with 5% CO_2 in O_2 giving a pH of 7.2. The following drugs were used in the study: substance P (Peninsula Laboratories), human α-calcitonin gene-related peptide (CGRP, Peninsula Laboratories), substance P methyl ester (SPOMe, Cambridge Research Biochemicals Ltd), senktide (Cambridge Research Biochemicals Ltd). GR73632 (δAva [L-Pro^9, N-MeLeu^{10}] substance P (7–11)), GR64349 ([Lys^3,Gly^8-**R**-γ-Lactam-Leu^9] neurokinin A (3–10)) and GR82334 ([D-Pro^9[Spiro-γ-Lactam]Leu^{10},Trp^{11}] physalaemin (1–11)) were synthesized by the Medicinal Chemistry Department, Glaxo Group Research Ltd, Greenford. Each compound was dissolved in 10% (v:v) acetic acid (10 mM) in distilled water and stock solutions (1 or 5 mM) stored in aliquots at −20°C until needed. Solutions were diluted to the final concentrations with ACSF.

Statistical analysis

Values shown are expressed as means ± s.e.mean and n values quoted refer to the number of arteries studied, from at least 3 animals (exact number shown in square brackets in the figure legends). Comparisons between treatments were made by Student's t test for unpaired samples.

Results

The guinea-pigs had a mean resting blood pressure of 44 ± 1 mmHg (n = 40). Following perivascular administration, none of the compounds used in the study had any effect on systemic blood pressure. The diameter of pial arteries studied ranged from 22 to 110 μm (mean = 58 ± 2 μm, n = 96) and responses elicited by either the neurokinin agonists or CGRP did not appear to be dependent on initial artery calibre. Under resting conditions, the diameter of the majority of arteries remained constant, although in approximately 10% of the vessels small spontaneous myogenic changes in tone were apparent. Arteries which were spontaneously active responded to CGRP and neurokinins in a similar manner to those vessels which demonstrated no myogenic activity.

The perivascular injection of ACSF (0.5 μl in 10 s) produced little change (2.0 ± 0.8%, n = 39) in diameter of most (>90%) pial arteries (Figure 1). Vessels which exhibited dilatation in response to ACSF, probably reflecting effects of the pressure ejection *per se*, were excluded from any further study. Ejection of 10 mM K^+ in ACSF produced a transient, reproducible vasodilatation (Figure 1).

The neuropeptides, substance P (0.01–1 μM) and CGRP (0.01 and 0.1 μM) dose-dependently increased pial artery diameter (Figure 1). Substance P and CGRP had similar potencies, the concentrations (with 95% confidence limits) producing a 30% increase in pial artery diameter were 0.04 (0.02–0.09) and 0.01 (0.01–0.03) μM, respectively. While the vasodilatations produced by both peptides were immediate in onset, the duration of their responses differed markedly (Figure 2); those evoked by substance P were short lasting with the time required to reach a 50% reduction in response amplitude ($t_{1/2}$) of approximately 0.5 min at 0.1 μM, while those to CGRP were of a much longer duration ($t_{1/2}$ > 15 min at 0.01 μM). Dose-response curves to either compound were not constructed from the same artery as repeated application of substance P and to a lesser extent CGRP resulted in desensitization. In addition, the long duration of the CGRP-induced response made further injections of this agent at the same site impractical. The effects of only a single concentration of each drug were therefore examined in any one vessel.

Selective neurokinin receptor agonists were used to characterize the neurokinin-induced responses. The NK_1 receptor agonists, SPOMe (0.01–1 μM) and GR73632 (0.1 μM), but not GR64349 or senktide (NK_2 and NK_3 agonists, respec-

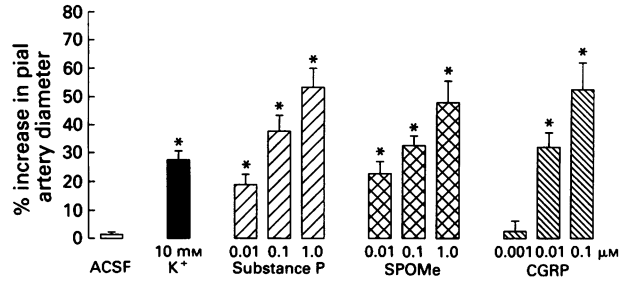


Figure 1 The effect of ACSF (n = 39 [19]), K^+ (10 mM, n = 25[13]), substance P (0.01 (n = 12 [5]), 0.1 (n = 6 [4]) and 1 μM (n = 3 [3])), substance P methyl ester (SPOMe, 0.01 (n = 5[3]), 0.1 (n = 4 [3]) and 1 μM (n = 4 [3])) and calcitonin gene-related peptide (CGRP, 0.001 (n = 6 [3]), 0.01 (n = 10 [6]) and 0.1 μM (n = 7 [3])), injected perivascularly, on guinea-pig pial artery diameter, expressed as a percentage increase (mean ± s.e.mean, vertical bars) (n = number of arteries [number of guinea-pigs])) over pre-injection tone. K^+ (10 nM), substance P (0.01–1 μM), SPOMe (0.01–1 μM) and CGRP (0.01–0.1 μM) induced a significant increase in pial artery diameter (*P<0.05 compared to ACSF).

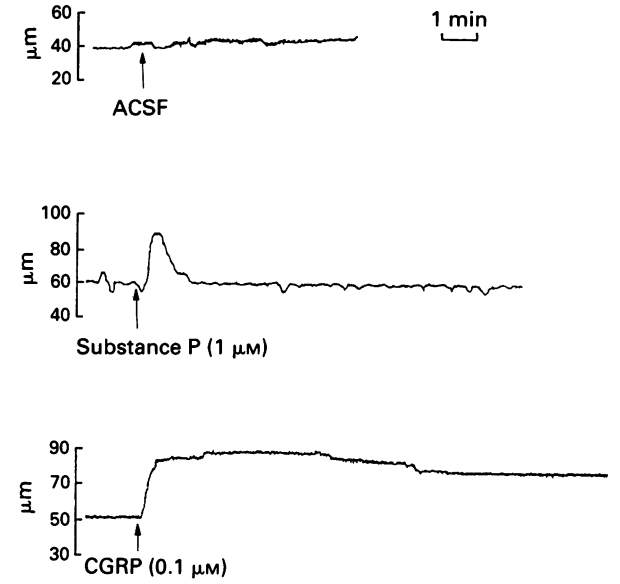


Figure 2 Responses evoked by ACSF, substance P (1 μM) and calcitonin gene-related peptide (CGRP, 0.1 μM) in guinea-pig pial arteries (resting diameters 40, 60 and 52 μm, respectively), following perivascular bolus injection of ACSF or drug in ACSF. ACSF had no effect on artery diameter, while substance P produced a short lasting, and CGRP a maintained vasodilatation.

tively, both 1 μM), significantly increased pial artery diameter (Figures 1 and 3). Indeed, GR64349 (1 μM) significantly reduced artery calibre while senktide (1 μM) was relatively inactive, producing an increase in diameter of 8.0 ± 3.7% (not significantly different from ACSF injection). The vasoconstriction produced by GR64349 had a slow onset (15–30 s), relative to the neurokinin-induced vasodilatations and a $t_{1/2}$ of approximately 3 min at 1 μM. The vasodilatations evoked by SPOMe and GR73632 had a $t_{1/2}$ of approximately 0.5 min at 0.1 μM. The NK_1 receptor antagonist, GR82334 (1 μM), had no apparent effect on artery calibre in its own right, but significantly attenuated vasodilator responses to SPOMe (0.1 μM). In contrast, GR82334 (1 μM) had no effect on the vasodilatation produced by CGRP (0.01 μM) (Figure 4).

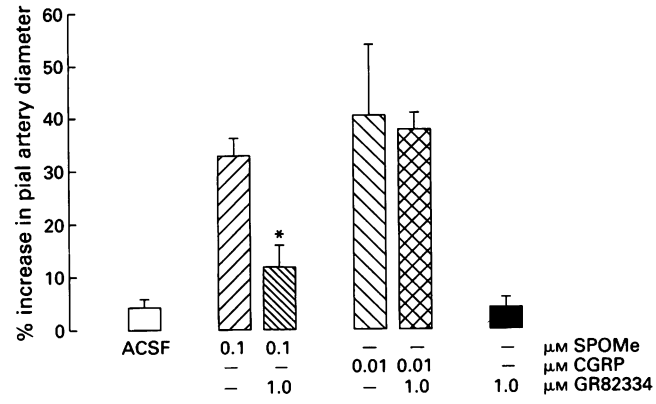


Figure 4 The effect of co-administration of GR82334 (1 μM, $n = 3,4$ [3]) on the percentage increase in pial artery diameter produced by substance P methyl ester (SPOMe, 0.1 μM, $n = 4$ [3]) and calcitonin gene-related peptide (CGRP, 0.01 μM, $n = 3$ [3]). GR82334 reduced significantly ($^*P < 0.05$) SPOMe-, but not CGRP-, induced vasodilatation. GR82334 (1 μM, $n = 7$ [4]) alone had no effect on artery calibre.

Discussion

A number of nociceptive agents, such as substance P, CGRP, bradykinin, prostaglandins and 5-hydroxytryptamine may be involved in the pathogenesis of vascular headache (Moskowitz, 1984; Moskowitz *et al.*, 1989). Evidence for the involvement of substance P is particularly strong; it is stored in, and released from, trigeminal nerve terminals in the intracranial artery wall where it produces plasma protein extravasation and vasodilatation (Edvinsson *et al.*, 1983; Moskowtiz *et al.*, 1989). In the present study, substance P markedly dilated guinea-pig pial arteries when injected perivascularly, an effect consistent with its vasodilator activity in a variety of species, both *in vitro* and *in situ* (Regoli *et al.*, 1989; Jansen *et al.*, 1991; Stubbs *et al.*, 1992). Moreover, the potency with which substance P increased pial artery diameter in the guinea-pig was similar to that observed in cat cerebral vessels (Edvinsson *et al.*, 1981; Jansen *et al.*, 1991). Such a vasodilatation could activate trigeminal nerve endings and via the initiation of local axon reflexes lead to the sensation of pain (Humphrey & Feniuk, 1991).

CGRP, which is co-localized with substance P in trigeminal sensory nerves (Skofitsch & Jacobowitz, 1985; Goadsby *et al.*, 1988), also increased pial artery diameter, in keeping with its recognized potent vasodilator activity in the cerebrovasculature of the cat (McCulloch *et al.*, 1986) and in other tissues (Brain *et al.*, 1985). As in the cat (McCulloch *et al.*, 1986), CGRP-induced responses in the guinea-pig had a longer duration than those produced by substance P.

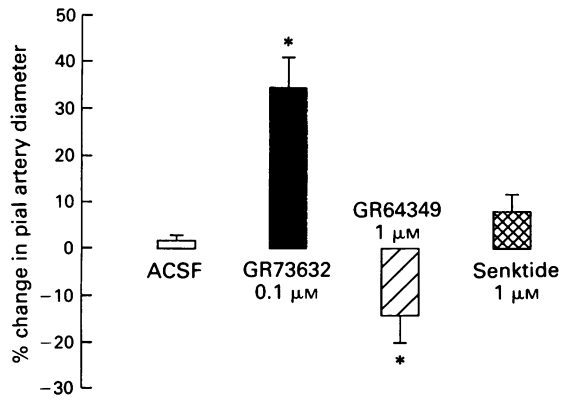


Figure 3 The effect of perivascular injections of receptor-selective neurokinin agonists on guinea-pig pial artery diameter, expressed as the percentage change from resting levels. GR73632 (0.1 μM, $n = 6$ [3]) increased while GR64349 (1 μM, $n = 13$ [4]) decreased and senktide (1 μM, $n = 8$ [3]) had no effect on artery calibre. $^*P < 0.05$ compared to ACSF.

The ability of the selective NK_1 receptor agonists, SPOMe and GR73632 (Guard & Watson, 1991; Hagan *et al.*, 1991b), to dilate pial arteries suggested the involvement of NK_1 receptors. Consistent with this proposal, the selective NK_1 receptor antagonist, GR82334 (Hagan *et al.*, 1991a), inhibited the SPOMe-induced vasodilatation. GR82334 is a highly selective NK_1 receptor antagonist, having little affinity for other peptide receptors, such as those activated by neurokinins A and B, cholecystokinin, bradykinin or bombesin (Hagan, personal communication). The specificity of the compound was also evident in this study, as CGRP-induced vasodilatation was unaffected by GR82334 at a concentration that caused a marked reduction in the response to SPOMe.

The results obtained with selective NK_2 and NK_3 receptor agonists suggested that neurokinin-induced dilatation of pial arteries may not involve NK_2 or NK_3 receptors. Indeed, while senktide (1 μM), a NK_3 receptor agonist (Laufer *et al.*, 1986) was inactive, the selective NK_2 receptor agonist, GR64349 (Hagan *et al.*, 1991b) reduced guinea-pig pial artery diameter. This, however, contrasts with the finding (Jansen *et al.*, 1991) that in cat pial arteries, neurokinin A (0.01–1 μM), a high affinity NK_2 receptor agonist produced only dilatation. This apparent anomaly may suggest that species differences exist but seems more likely to reflect the poor selectivity of neurokinin A for NK_2 receptors (Guard & Watson, 1991). In support of the present findings, NK_2 receptor activation resulted in contraction of the rabbit pulmonary artery (D'Orléans-Juste *et al.*, 1986) and the endothelium-denuded rabbit thoracic aorta (Ireland *et al.*, 1991). The relevance of the vasoconstriction noted in guinea-pig pial arteries, which occurred at only a high concentration (1 μM), remains to be determined.

The results of this study suggest therefore, that NK_1, and possibly not NK_2 or NK_3, receptors mediate the neurokinin-induced vasodilatation of guinea-pig pial arteries and in so doing raises the possibility that NK_1 receptors may have a role in the pathogenesis of vascular headache.

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Influences of the endothelium and hypoxia on neurogenic transmission in the isolated pulmonary artery of the rabbit

[1]Margaret R. MacLean, K.M. McCulloch, J.B. MacMillan & J.C. McGrath

Autonomic Physiology Unit, Institute of Physiology, Glasgow University, Glasgow G12 8QQ

1 The effects of nitric oxide (10^{-6} M), N^{ω}-nitro-L-arginine methylester (L-NAME, 10^{-4} M, an inhibitor of nitric oxide synthase), endothelium removal, hypoxia and selective α-adrenoceptor antagonists on responses to nerve electrical field-stimulation (EFS) were studied in the rabbit isolated pulmonary artery.

2 EFS induced frequency-dependent contractions which were abolished by prazosin (α_1-adrenoceptor antagonist) and unaffected by rauwolscine (α_2-adrenoceptor antagonist). EFS-induced responses were potentiated by L-NAME and inhibited by nitric oxide. The effect of L-NAME was reversed by the presence of L-arginine (2×10^{-4} M), which had no effect on its own. In the presence of L-NAME, the EFS-induced responses were reduced by rauwolscine and the residual responses were abolished by prazosin.

3 Removal of the vascular endothelium increased the maximum contractile response to EFS but did not inhibit the ability of L-NAME to potentiate contractile responses to EFS.

4 Hypoxia inhibited the contractile response to EFS. This effect of hypoxia was also seen in the presence of L-NAME and in endothelium rubbed preparations.

5 In conclusion, the endothelium modulates EFS-induced contractions in the rabbit pulmonary artery. The contraction induced by EFS was inhibited by nitric oxide, but potentiated by the nitric oxide-synthase inhibitor, L-NAME. The effect of L-NAME was not mediated solely through the endothelium and revealed involvement of α_2-adrenoceptors in EFS-induced contraction. Hypoxia inhibited neurogenic responses in rabbit isolated pulmonary arteries.

Keywords: Hypoxia-induced vasoconstriction; pulmonary arteries; nitric oxide; vascular smooth muscle; endothelial cells

Introduction

A decrease in oxygen tension induces a vasodilatation in most systemic arteries, but induces a vasoconstriction in the pulmonary arterial bed (Staub, 1985). Hypoxia-induced pulmonary vasoconstriction (HPV) aids in the regulation of ventilation-perfusion matching in normal subjects in that it selectively re-distributes blood towards better ventilated alveoli (Dawson, 1984). However, this reflex contributes to the pulmonary hypertension occurring in patients with hypoxia-related pulmonary abnormalities, such as chronic obstructive pulmonary disease, cystic fibrosis, chronic bronchitis and emphysema (Rubin, 1984).

HPV is an endothelium-dependent phenomenon (Holden & McCall, 1984). The vascular endothelium has an inhibitory effect on adrenergic nerve stimulation in the rabbit carotid artery (Tesfamariam *et al.*, 1987) and rat caudal artery (Hynes *et al.*, 1988) and it is believed that endothelium-released vasorelaxant factors are responsible (Tesfamariam *et al.*, 1987). Recently, Liu *et al.* (1991) implicated nitric oxide as a modulator of neurogenic responses in guinea-pig pulmonary arteries. We have shown that transient inhibition of nitric oxide is responsible for the transient vasoconstrictor response to acute hypoxia in rabbit pulmonary arteries (MacLean & McGrath, 1991). Inhibition of nitric oxide synthase with N^{ω}-nitro-L-arginine methylester (L-NAME) potentiates post-junctional response to noradrenaline in rabbit pulmonary arteries and the rat perfused lung and in the former, this effect appears to involve α_2-adrenoceptor stimulation (Shaw *et al.*, 1992; MacLean *et al.*, 1993).

The action of endothelium-derived relaxing factor is inhibited by hypoxia in bovine pulmonary artery endothelial cells and in rabbit pulmonary arteries (Warren *et al.*, 1989; Johns *et al.*, 1989). It has recently been shown that moderate hypoxia can inhibit the activity of an isoform of nitric oxide synthase present in vascular endothelium, primarily through depletion of oxygen, one of the substrates for the enzyme (Rengasamy & Johns, 1991). Here we study the effect of nitric oxide on responses of rabbit pulmonary arteries to electrical field stimulation (EFS) and the α-adrenoceptors involved. The ability of endothelium removal to mimic the effects of nitric oxide synthase inhibition, through mechanical removal of a site of nitric oxide synthesis, is also studied. In addition we have investigated whether hypoxia can mimic the effects of L-NAME through an inhibitory effect on nitric oxide synthesis.

Methods

NZW rabbits (2–3 kg) were killed by stunning and cervical dislocation. The lungs and heart were removed and the left pulmonary artery dissected out and cleaned. The arteries were cut into 4–5 mm rings. Rings were suspended in 40 ml organ baths in Krebs saline (37°C) between parallel platinum electrodes for transmural stimulation. The rings were connected via cotton thread, under 2 g wt tension, to Grass FTO3c transducers for isometric recording on a Linseis L2065 chart recorder. The Krebs solution was gassed with 5% O_2, 6% CO_2 balance nitrogen to mimic the *in vivo* situation and the tissues allowed to equilibrate for at least 1 h before starting the experiment. When hypoxia was induced the Krebs solution was gassed with 0% O_2, 6% CO_2 balance nitrogen (bath O_2 tension equivalent to 2.5%).

Frequency-response curves (FRCs) were established using a Square One Instruments stimulator. Stimulation parameters used were 4, 8, 16, 32 and 64 Hz, 1 s train duration, 0.1 ms pulse width, 35 volts. In some experiments, following control FRC stimulations, rauwolscine (10^{-6} M) was added for 45 min and the FRC repeated. Prazosin (10^{-7} M) was then added for 45 min before repeating the FRC for a third time. Time controls were also carried out, repeating FRCs in the absence of any added drugs. In other experiments, the vascular endothelium was disrupted by rubbing with a pair of

[1] Author for correspondence.

forceps on a Krebs-soaked tissue. FRCs were then established in these rings as well as intact preparations. At the end of these experiments, tone was raised with 10^{-7} M noradrenaline and substance P (10^{-8} M) used as a stimulant of nitric oxide production to assess the viability of the endothelium. Fluorescent dyes were also used to examine histologically the structure of rubbed endothelial cells in at least ten random preparations (Daly *et al.*, 1992). In further experiments (in vessels with and without a functional endothelium), after establishing FRCs, 10^{-4} M L-NAME was added for 20 min and the FRCs repeated. In some of these experiments, 2×10^{-4} M L-arginine was introduced in the presence of L-NAME and a third CRC constructed. Nitric oxide (10^{-5} M) was added at the same time as a 64 Hz stimulation in nine preparations. To study the effect of hypoxia on FRCs, the vessels were equilibrated in the normoxic conditions, FRCs performed and the tissues were then re-equilibrated under hypoxic conditions, before establishing another FRC. A third FRC was then constructed after returning the vessels to normoxic conditions. These experiments were also carried out on vessels devoid of a functional endothelium and also in the presence of L-NAME.

The gas tensions and pH were determined regularly by use of both an oxygen electrode inserted into one of the organ baths and a blood gas analyser (Instrumentation Laboratory system 1302).

Drugs and solutions

The composition of the Krebs-bicarbonate saline was as follows (in mM): NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2. $CaCl_2$ 2.5, glucose 11, Na_2EDTA 0.023, propranolol 0.001, cocaine 0.01. The following drugs were used: prazosin (Pfizer), rauwolscine HCl (Roth), cocaine HCl (Macarthys), propranolol HCl (Sigma), N^{ω}-nitro-L-arginine methylester (Sigma). All drugs were dissolved in distilled water. Nitric oxide was prepared immediately before use in the following way: ensuring total exclusion of air, 38 µl of nitric oxide (99.9%, Air Products Ltd) gas was drawn into a syringe and injected at atmospheric pressure into 17 ml of nitrogen-deoxygenated distilled water which completely filled a rubber-sealed bottle. This produced a stock solution of 10^{-4} M nitric oxide. At all stages of this procedure oxygen contamination was prevented, i.e. all air in the tubing involved was replaced with nitric oxide gas.

Analysis of results

As the tension transducer was calibrated with a 2 g weight, contractions were expressed in equivalent weight units, i.e. mg wt. tension (see Miller, 1988). Control EFS-induced contractions are expressed as a percentage of their maximum response to 64 Hz. Subsequent contractions achieved, in the same preparations, after drug treatment or exposure to hypoxia are expressed as a percentage of these control maximum responses to 64 Hz. Statistical analysis of these results was performed using a paired Student's *t* test. In those experiments studying the effect of endothelium disruption, it was necessary to use two separate groups of preparations, rubbed and unrubbed. For both rubbed and unrubbed groups, EFS-induced contractions are expressed in absolute terms (mg wt. tension). An unpaired Student's *t* test was used for statistical analysis of the difference between unrubbed and rubbed preparations.

Results

Under normoxic conditions, the bath gas tensions and pH were as follows: $P_{O_2} = 57 \pm 0.5$ mmHg, $P_{CO_2} = 38.1 \pm 0.4$ mmHg and pH = 7.39 ± 0.03, $n = 6$. In hypoxic conditions, the bath gas tensions and pH were as follows: $P_{O_2} = 8.4 \pm 0.6$ mmHg, $P_{CO_2} = 37.7 \pm 0.5$ mmHg, pH = 7.37 ± 0.01, $n = 6$.

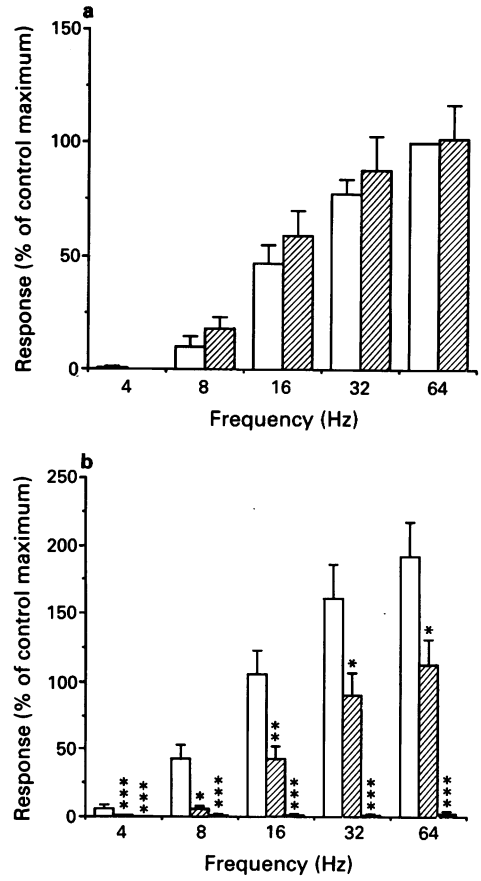


Figure 1 (a) Frequency-dependent contractions to electrical field stimulation in rabbit pulmonary arterial rings: the effect of 10^{-6} M rauwolscine. The open columns represent data from control preparations and the hatched columns represent contractions in the presence of rauwolscine. Each column represents data from 11 rings from $n = 6$ animals. The vertical bars show s.e.mean. (b) The effect of 10^{-6} M rauwolscine followed by 10^{-7} M prazosin on N^{ω}-nitro-L-arginine methylester (10^{-4} M)-treated rabbit pulmonary arterial rings (responses to electrical field stimulation). The open columns represent data from control preparations, the hatched columns demonstrate the effect of rauwolscine alone and the solid columns the effect of rauwolscine plus prazosin. Each column represents data from 10 rings from $n = 6$ animals. The vertical bars show s.e.mean. Significant difference from control was assessed with a paired Student's *t* test: $^{*}P<0.05$, $^{**}P<0.01$; $^{***}P<0.001$.

Nerve-responses and the effect of L-NAME, nitric oxide and α-adrenoceptor antagonists

EFS produced frequency-dependent contractions which were unaffected by rauwolscine (Figure 1a). Prazosin, alone or in the presence of rauwolscine, completely abolished all contractile responses. These contractions were also inhibited by tetrodotoxin (10^{-7} M) and are, therefore, described as being neurogenic. Time controls showed no effect of time on these frequency-dependent EFS-induced responses. In the presence of L-NAME, EFS-induced responses were potentiated. Rauwolscine now inhibited these responses and the residual contractile response was abolished by prazosin (Figure 1b) The potentiation of EFS-induced contractile responses is clarified in Figure 2a which shows that L-NAME potentiated contracile responses at every frequency, revealing a response previously not seen at 4 Hz. Figure 2a also shows that this effect of L-NAME was totally reversed by 2×10^{-4} M L-arginine. L-Arginine had no effect on its own. Responses to 64 Hz EFS, in the presence of L-arginine were $107 \pm 9.7\%$ of those in the same preparations prior to L-arginine administration (10 preparations from $n = 5$ animals, statistically

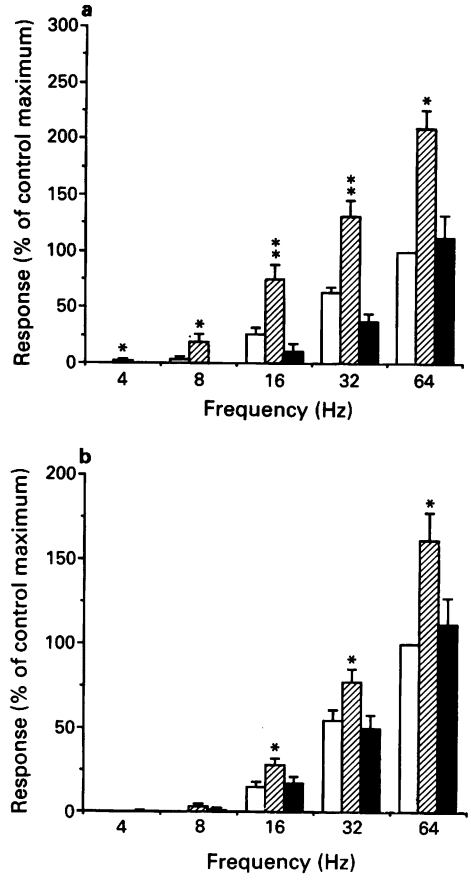


Figure 2 (a) The effect of 10^{-4} M N^{ω}-nitro-L-arginine methylester (L-NAME) and 2×10^{-4} M L-arginine on frequency-dependent responses to electrical field stimulation in rabbit pulmonary arterial rings. The open columns represent data from control preparations (17 rings from 12 animals), the hatched columns represent responses in the presence of L-NAME (17 rings from 12 animals) and the solid columns represent responses in the presence of both L-NAME and L-arginine (10 rings from 6 animals). The vertical bars show s.e.mean. Significant differences from control was assessed with a paired Student's t test: $^{*}P<0.05$, $^{**}P<0.01$. (b) The effect of endothelium removal on the influence of 10^{-4} M L-NAME and 2×10^{-4} M L-arginine on frequency-dependent responses to electrical field stimulation in rabbit pulmonary arterial rings. Open columns show control data from endothelium-rubbed preparations, hatched columns represent responses in the presence of L-NAME and solid columns represent responses in the presence of both L-NAME and L-arginine. Each column represents data from 15 rings from 8 animals. The vertical bars show s.e.mean. Significant difference from control was assessed with a paired Student's t test: $^{*}P<0.05$.

insignificant). Nitric oxide, 10^{-6} M, relaxed noradrenaline-precontracted vessels by 811 ± 314 mg wt. tension ($n = 9$ preparations, 3 animals) and reduced 64 Hz EFS-induced responses from 500 ± 196 mg wt. tension to -67 ± 198 mg wt. tension.

The effect of endothelium-removal

Acetylcholine, which is the standard agent used to show endothelium-dependent relaxations, induced contractions in precontracted rabbit pulmonary artery rings. Substance P was, therefore, used as an alternative. In control preparations, precontracted with noradrenaline, substance P induced a vasodilatation. For example, if tone was raised to 700 ± 30 mg wt. tension, 10^{-8} M substance P induced a vasodilatation of 92 ± 11 mg wt. tension. Removal of the vascular endothelium totally inhibited the ability of substance P to relax preconstricted vessels. In addition, in those preparations examined histologically, all endothelial cells appeared damaged by this rubbing procedure. We are confident, therefore, that this rubbing procedure produced maximal disruption of the vascular endothelium. The absence of a functional endothelium did not inhibit the ability of L-NAME to potentiate contraction to EFS or the ability of L-arginine to reverse the effects of L-NAME (Figure 2b). The sensitivity of the vessels to EFS was unaffected by endothelium removal while the maximum response achieved to 64 Hz was significantly increased (Figure 3).

The effect of hypoxia

Hypoxia reduced nerve-induced responses at every frequency tested. This effect of hypoxia was completely reversed after replacement of normoxic conditions (Figure 4). This effect of hypoxia was not altered by removal of the vascular endothelium. For example, in rubbed arteries, the maximum

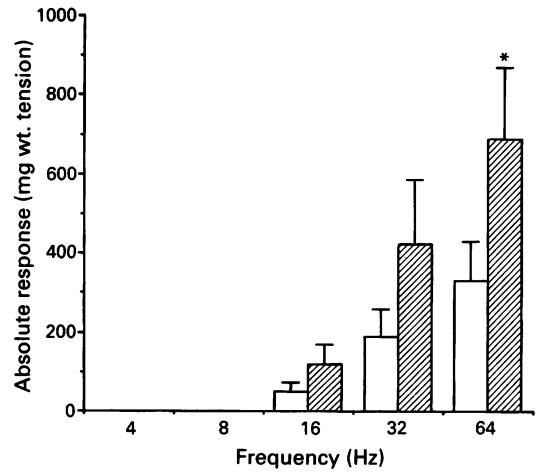


Figure 3 The effect of endothelial cell disruption on responses to electrical field stimulation in the rabbit pulmonary artery. Open columns show absolute data from unrubbed preparations and hatched columns show absolute data from endothelium-rubbed preparations. Each column represents data from 11 rings from 6 animals. The vertical bars show s.e.mean. Significant differences between data from rubbed and unrubbed preparations was assessed with an unpaired Student's t test: $^{*}P<0.01$.

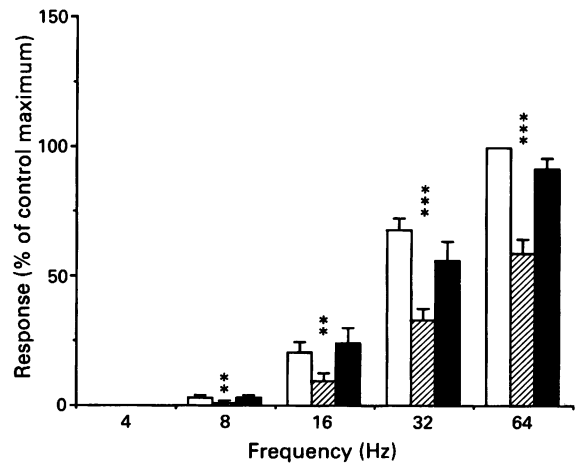


Figure 4 The effect of hypoxia on contractile responses to electrical field stimulation in the rabbit pulmonary artery. Open columns show control data from preparations in 5% O_2/6% CO_2 balance N_2; hatched columns represent responses after bubbling with 0% O_2/6% CO_2 balance N_2 and solid columns show contractile response recovery after re-bubbling with 5% O_2/6% CO_2 balance N_2. Each column represents data from 17 rings from 9 animals. The vertical bars show s.e.mean. Significant difference from control was assessed with a paired Student's t test: $^{**}P<0.01$; $^{**}P<0.001$.

contraction to 64 Hz EFS was 458 ± 139 mg wt. tension in normoxic conditions and 233 ± 80 mg wt. in hypoxic conditions (12 rings from $n = 6$ animals, statistical significance of difference in data: $P < 0.05$, paired t test). The effect of hypoxia was not altered by the presence of L-NAME. For example, in 10 rings from 5 animals, in normoxic conditions, L-NAME potentiated contractile responses to 64 Hz EFS to 150 ± 14% of control values. In hypoxic conditions, these responses were reduced to 35 ± 11% of control values.

Discussion

The results show that inhibition of nitric oxide synthase with L-NAME potentiates neurogenic responses in rabbit pulmonary arteries. L-Arginine, which is a substrate for this enzyme (Palmer *et al.*, 1988), totally reversed the effects of L-NAME. Nitric oxide itself caused a decrease in EFS-stimulated contractions. These observations have far reaching implications for the mechanisms underlying the development of pulmonary hypertension. Endothelial cell disruption has been observed in experimental pulmonary hypertension and in patients with hypoxia-related pulmonary hypertension (Rosenberg & Rabinovitch, 1986; Rabinovitch *et al.*, 1986). It has also been demonstrated that endothelium-dependent relaxation is impaired in isolated perfused lungs from patients with chronic lung disease (Cremona *et al.*, 1992). This is believed to be due to a lack of oxygen leading to reduced nitric oxide release (Dinh-Xuan, 1992). In support of this, it has recently been shown that moderate hypoxia, primarily through depletion of oxygen, one of the substrates for the enzyme, can inhibit the activity of an isoform of nitric oxide synthase present in vascular endothelium (Rengasamy & Johns, 1991). If reduced nitric oxide production leads to the increased sensitivity of pulmonary arterial smooth muscle to nerve-stimulation, this would contribute to the persistent pulmonary vasoconstriction typical in hypoxia-induced pulmonary hypertension. It has also been shown that nitric oxide production is increased during endotoxic shock which would aggravate the hypotension typical of endotoxaemia (Knowles *et al.*, 1990).

Surprisingly, mechanical disruption of the vascular endothelium (to an extent which prevented vasodilatation by an agonist acting via the endothelium-derived nitric oxide system) reduced, but did not abolish the ability of L-NAME to potentiate neurogenic responses. This suggests an extra-endothelial site for nitric oxide synthesis in rabbit pulmonary arteries. One site could be the vascular smooth muscle cell, as already suggested for rat ileac arteries by Gonzalez *et al.* (1992). Other sites of synthesis could be cells in the vascular adventitia. In pulmonary artery rings rubbed of their vascular endothelium and devoid of endothelium-dependent relaxations, the sensitivity of the tissues to nerve stimulation was not affected, though the maximum response attained by 64 Hz EFS was increased Hence, endothelium removal has similar effects to L-NAME.

Before administration of L-NAME, rauwolscine had no effect on nerve-induced responses whilst prazosin abolished them. This suggests that the neurogenic response was mediated entirely by α_1-adrenoceptors. In the presence of L-NAME, however, rauwolscine did reduce the neurogenic response, suggesting that nitric oxide may suppress responses mediated by a population of α_2-adrenoceptors which is uncovered upon inhibition of its synthesis. Nerve-mediated α_2-adrenoceptor responses in isolated arterial preparations have been hard to demonstrate, though their presence has been reported in venous preparations such as the rabbit isolated plantaris vein (Daly *et al.*, 1990) and the dog (Flavahan *et al.*, 1984) and human saphenous vein (Docherty & Hyland, 1985). In the rabbit isolated plantaris vein, the expression of α_2-adrenoceptor-mediated contraction is greatly enhanced by the presence of angiotensin II (Daly *et al.*, 1990). We have previously shown that, in order to observe responses mediated by post-junctional α_2-adrenoceptors, vascular tone must be increased. For example, in the rat tail artery, when vascular tone is raised with vasopressin, endothelin-1 or 5-hydroxytryptamine, a previously quiescent α_2-adrenoceptor population is uncovered (Templeton *et al.*, 1989; MacLean & McGrath, 1990). In addition, inhibition of neuronal uptake will reveal α_2-adrenoceptor-mediated nerve responses in the rat tail artery (Papanicolaou & Medgett, 1986). Hence, the results described here are compatible with the view that α_2-adrenoceptors require specific prevailing conditions before they are expressed. In the case of the pulmonary artery, one of these conditions is the inhibition of nitric oxide synthesis. The underlying mechanism for this phenomenon has still to be determined.

We have previously shown that inhibition of nitric oxide synthase also potentiates responses to noradrenaline and the α_2-adrenoceptor agonist UK 14304, in both rabbit isolated pulmonary arteries and the rat isolated perfused lung (Shaw *et al.*, 1992; MacLean *et al.*, 1993). The combined effect of the increased sensitivity to nerve stimulation, shown in the present study, and to circulating catecholamines would be likely to aggravate further pulmonary hypertension. Indeed, recent studies have shown that inhalation of nitric oxide in patients with chronic lung disease and pulmonary hypertension relieves the hypertension by a selective effect on the pulmonary circulation (Adnot *et al.*, 1992). This is somewhat of a breakthrough in the clinical approach to the treatment of this disorder, as the biggest problem facing clinicians is the lack of selectivity of most vasodilators used in the treatment of pulmonary hypertension. Due to pulmonary vascular remodelling, diseased lungs are relatively resistant to vasodilator therapy and the standard vasodilators exert greater effects on the systemic circulation leading to unwanted side-effects.

Acute experimental hypoxia did not mimic the effect of either L-NAME or endothelium removal in this study. In fact it inhibited the responses to nerve-stimulation. We have previously examined the integrity of the endothelium using fluroescent nuclear dyes (Daly *et al.*, 1992) after such an experiment and found it to be intact (unpublished observations). Disruption of the vascular endothelium did not influence the effect of hypoxia which is likely to be a metabolic one, occurring within the vascular smooth muscle cells. L-NAME did not alter the influence of hypoxia, indicating that the metabolic effects of hypoxia were sufficient to prevent the effect of L-NAME. Any inhibition of nitric oxide synthase caused by the depletion in oxygen were presumably obscured by these metabolic effects. *In vivo*, chronic hypoxia has been shown to induce endothelial disruption (Rosenberg & Rabinovitch, 1986; Rabinovitch *et al.*, 1986). The results presented here show that you cannot mimic these effects *in vitro* using the more acute hypoxic conditions prevalent in acute organ bath experiments. Caution must be taken, therefore, when interpreting results from *in vitro* studies.

In conclusion, the pulmonary vascular endothelium modulates EFS-induced contraction in rabbit pulmonary arteries. Responses to EFS are decreased by exogenous nitric oxide while inhibition of its synthesis with L-NAME potentiates contractile responses. In the rabbit isolated arteries, the effect of L-NAME is not mediated solely through endothelial nitric oxide synthase inhibition and involves recruitment of previously quiescent α_2-adrenoceptors. The results suggest that in conditions in which the pulmonary vascular endothelium is disrupted, or where the nitric oxide system is inhibited, conditions prevalent in pulmonary hypertension, neurogenic responses may be potentiated. Conversely, it may be inferred that, in conditions in which there is excess production of nitric oxide, such as endotoxic shock, neurogenic responses in the pulmonary circulation may be reduced.

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Influences of the endothelium and hypoxia on α_1- and α_2-adrenoceptor-mediated responses in the rabbit isolated pulmonary artery

[1]Margaret R. MacLean, K.M. McCulloch & J.C. McGrath

Autonomic Physiology Unit, Institute of Physiology, Glasgow University, Glasgow G12 8QQ

1 The effects of the inhibitor of nitric oxide synthase, N^{ω}-nitro-L-arginine methylester (L-NAME, 10^{-4} M), mechanical disruption of the endothelium and hypoxia on contraction to noradrenaline (α_1- and α_2-adrenoceptor agonist), phenylephrine (α_1-adrenoceptor agonist) and UK 14304 (α_2-adrenoceptor agonist) were compared in the rabbit isolated pulmonary artery. The effects of the selective antagonists rauwolscine (10^{-6} M, α_2-adrenoceptors) and prazosin (10^{-7} M, α_1-adrenoceptors) on the contractions to noradrenaline before and after exposure to L-NAME were also assessed.

2 Noradrenaline, phenylephrine and UK 14304 all produced concentration-dependent increases in vascular tone. The responses to noradrenaline were sensitive to both rauwolscine and prazosin (effect of prazosin >> rauwolscine). L-NAME increased the potency of both noradrenaline and UK 14304, and also the maximum tension achieved. It had no effect on the responses to phenylephrine. After L-NAME, contractions to noradrenaline, although still sensitivie to both rauwolscine and prazosin, were now more sensitive to inhibition by rauwolscine.

3 Endothelium removal augmented the potency and maximum contractions to noradrenaline, phenylephrine and UK 14304.

4 Hypoxia decreased both the potency of phenylephrine and its maximum contractile response, but increased the maximum response to noradrenaline without effecting responses to UK 14304.

5 In conclusion, in the rabbit pulmonary artery, augmentation of contractile responses to noradrenaline by L-NAME involves a potentiation of α_2-adrenoceptor-mediated contraction probably through an effect on the synthesis of endothelium-derived nitric oxide. Experimental hypoxia had differential effects on all three agonists and did not mimic the effect of nitric oxide synthase inhibition.

Keywords: Pulmonary artery; nitric oxide; adrenoceptors; N^{ω}-nitro-L-arginine methylester; hypoxia

Introduction

A decrease in oxygen tension induces a vasodilatation in most systemic arteries but induces a vasoconstriction in the pulmonary arterial bed (Staub, 1985). Hypoxia-induced pulmonary vasoconstriction (HPV) aids in the regulation of ventilation-perfusion matching in normal subjects, in that it selectively re-distributes blood towards better ventilated alveoli (Dawson, 1984). However, this reflex also contributes to the pulmonary hypertension occurring in patients with hypoxia-related pulmonary abnormalities such as chronic obstructive pulmonary disease, cystic fibrosis, chronic bronchitus and emphysema (Rubin, 1984).

In 1984, Holden & McCall demonstrated, using porcine pulmonary arteries, that disruption of the vascular endothelium inhibited hypoxia-induced contractions. HPV is, therefore thought to be an endothelium-dependent phenomenon. It has been known for some time that removal of the vascular endothelium can potentiate responses to exogenously applied noradrenaline (Cocks & Angus, 1983) and other α_1- and α_2-adrenoceptor agonists (Egleme *et al.*, 1984; Lues & Schumann, 1984). Nitric oxide (or a closely related compound) has been identified as the major endothelium-derived relaxing factor (Ignarro *et al.*, 1987; Palmer *et al.*, 1988; Moncada *et al.*, 1988; 1989). We have recently demonstrated that inhibition of the synthesis of nitric oxide with the nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine methylester (L-NAME) potentiated neurogenic responses in the rabbit isolated pulmonary artery, and that this involved α_2-adrenoceptors (MacLean *et al.*, 1993). We have also demonstrated, in a preliminary study, that L-NAME can potentiate contraction to exogenously applied noradrenaline in the rat perfused lung and in rabbit isolated pulmonary arteries although the adrenoceptor subtype(s) involved in this effect have not been clearly identified (Shaw *et al.*, 1992).

Transient inhibition of nitric oxide synthesis is responsible for the transient vasoconstrictor response to acute hypoxia in rabbit pulmonary arteries (MacLean & McGrath, 1991). Endothelium derived relaxing factor is inhibited by hypoxia in bovine pulmonary artery endothelial cells and in rabbit pulmonary arteries (Warren *et al.*, 1989; Johns *et al.*, 1989). Recently it has been shown that moderate hypoxia can inhibit the activity of the isoform of nitric oxide synthase present in vascular endothelium, primarily through depletion of oxygen, one of the substrates for the enzyme (Rengasamy & Johns, 1991).

The aim of this study was to determine the α-adrenoceptor subtype involved in the potentiating effect of L-NAME on rabbit pulmonary arteries. The ability of endothelial cell removal to mimic the effect of L-NAME on response to α-adrenoceptor agonists was also examined. We also determined if the degree of hypoxia enforced in day-long *in vitro* experiments mimicked the effects of nitric oxide synthase inhibition, by studying the effects of experimental hypoxia on responses to α-adrenoceptor agonists.

Methods

NZW rabbits (2–3 kg) were killed by stunning followed by cervical dislocation. The lungs and heart were removed and

[1] Author for correspondence.

the first left branch of the pulmonary artery (3–5 mm i.d.) dissected out and cleaned. The arteries were cut into rings which were suspended in 40 ml organ baths in Krebs saline under 2 g wt. tension. The Krebs solution was gassed with 5% O_2, 6% CO_2 balance nitrogen (to mimic the *in vivo* tensions in mixed arterio-venous pulmonary blood) and the tissues allowed to equilibrate for at least 1 h before starting the experiment. When hypoxia was induced the Krebs was gassed with 0% O_2, 6% CO_2 balance nitrogen (bath O_2 tension equivalent to 2.5%).

Concentration-response curves to noradrenaline, phenylephrine and UK 14304 were constructed before and after administration of L-NAME (10^{-4} M). In further experiments, concentration-response curves to noradrenaline were constructed before, then 45 min after, administration of rauwolscine (10^{-6} M). Prazosin (10^{-7} M) was then added, still in the presence of rauwolscine, and further concentration-response curves constructed 45 min later. This procedure with noradrenaline, rauwolscine and prazosin was also performed in the presence of 10^{-4} M L-NAME. In other experiments, the vascular endothelium was removed by gently rubbing the intimal surface with a pair of forceps on a Krebs-soaked tissue. Concentration-response curves to all three agonists were then established in these rings as well as intact preparations from the same animals. At the end of these experiments, tone was raised with 10^{-7} mM noradrenaline and substance P (10^{-8} M) used as a stimulant for nitric oxide production to show that only preparations with an intact endothelium relaxed. This ensured that endothelium removal had been successful. Fluorescent dyes were also used to examine histologically the structure of rubbed endothelial cells in at least ten random preparations (Daly *et al.*, 1992). To study the effect of hypoxia on agonist responses, the vessels were equilibrated in the normoxic conditions, concentration-response curves performed and the vessels were then re-equilibrated in the hypoxic conditions before constructing further concentration-response curves.

Drugs and solutions

The composition of the Krebs-bicarbonate saline was as follows (in mM): NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11, Na_2EDTA 0.023. The following drugs were used: prazosin (Pfizer), rauwolscine HCl (Roth), UK 14304 (5-bromo-6[2-imazolin-2-yl-amino] quinoxaline) bitartrate (Pfizer), N^{ω}-nitro-L-arginine methylester (Sigma). All drugs were dissolved in distilled water.

The gas tensions and pH were determined regularly by use of both an oxygen electrode inserted into one of the organ baths and a blood gas analyser (Instrumentation Laboratory system 1302).

Analysis of results

As the tension transducer was calibrated with a 2 g weight, contractions were expressed in equivalent weight units, i.e. mg wt. tension (see Miller, 1988). To standardize maximum responses for statistical comparison they are expressed, in the tables, as a percentage of the response obtained to 100 mM KCl in each preparation. For concentration-response curves, however, control contractions are expressed as a percentage of their maximum response to the relevant agonist. Subsequent contractions achieved after drug treatment or exposure to hypoxia are expressed as a percentage of their control maximum response. EC_{50}s for agonists were calculated for each preparation and meaned within groups. The EC_{50} is expressed as the $-\log$ concentration (M) and was calculated using a computer extrapolation. Statistical analysis of these results was performed by a paired Student's *t* test and confirmed by one-way analysis of variance where appropriate.

In the experiments comparing agonist responses in endothelium rubbed and unrubbed preparations, a slightly different analysis was required. In Table 3, to standardize maximum responses, for statistical comparison, contractions are expressed as a percentage of the maximum response obtained to 100 mM KCl in each preparation. For the concentration-response curves, however, in order to illustrate alterations in agonist potency, contractions achieved in endothelium-rubbed preparations are expressed as a percentage of their own maximum response to the relevant agonist. Statistical analysis of the difference between unrubbed preparations and rubbed ones was performed by use of unpaired Student's *t* test.

Results

In normoxic conditions, the bath gas tensions and pH were as follows: $P_{O_2} = 45.4 \pm 0.5$ mmHg, $P_{CO_2} = 35.6 \pm 0.3$ mmHg and pH $= 7.39 \pm 0.03$, $n = 6$.

The effect of 10^{-4} M L-NAME on concentration-response curves to noradrenaline, phenylephrine and UK 14304

L-NAME itself had variable effects on vascular tone. In some preparations (approximately 60%) it caused small increases in vascular tone (approximately 5% of the maximum contractile response to 100 mM KCl) whilst in others it had no effect on vascular tone. For example, in 8 preparations in which it did increase tone, it increased vascular tone by 206 ± 47 mg wt. tension. The maximum response to 100 mM KCl in these preparations was 4338 ± 535 mg wt. tension.

Figure 1 shows the effect of L-NAME on the concentration-response curves to noradrenaline, phenylephrine and UK 14304 in the rabbit isolated pulmonary artery by expressing contractile responses in the presence of L-NAME as a percentage of the maximum response achieved prior to its administration. Table 1 gives the corresponding EC_{50} values and maximum responses to each agonist expressed as a percentage of the maximum response obtained with 100 mM KCl.

Figure 1a and Table 1 show that L-NAME increased the potency of noradrenaline as well as increasing the maximum tension it was able to achieve in these vessels (by approximately 60% of control absolute maximum tensions). L-NAME augmented noradrenaline-evoked contractions regardless of whether or not it had any effect on vascular tone itself.

Figure 1b shows that L-NAME had no effect on the concentration-response curves to phenylephrine and, correspondingly, Table 1 shows no effect of L-NAME on the EC_{50} or on maximum responses to phenylephrine.

L-NAME increased the potency of UK 14304 as well as increasing the maximum tension it was able to achieve in these vessels by approximately 125% of control absolute maximum tensions (Figure 1c, Table 1).

Figure 2a shows the effect of 10^{-6} M rauwolscine and 10^{-7} M prazosin on the concentration-response curves to noradrenaline in the rabbit isolated pulmonary artery in control preparations. Figure 2b shows the effect of these antagonists on the concentration-response curves to noradrenaline in preparations pretreated with L-NAME. The EC_{50}s for noradrenaline in all conditions are summarized in Table 2. The results show that rauwolscine shifts the concentration-response curve to noradrenaline significantly to the right in both treated and untreated groups but it has a significantly greater effect on those preparations treated with L-NAME (see Table 2). To verify the statistical analysis of this effect of L-NAME, a one-way analysis of variance (ANOVA) was also performed on these data. This showed that the EC_{50} for noradrenaline in the presence of rauwolscine was significantly different from control values and also significantly different from the EC_{50} for noradrenaline obtained in the present of L-NAME as well as rauwolscine. Rauwolscine also decreased the maximum response to nora-

drenaline in the treated group (by approximately 30% of control absolute maximum tensions). Prazosin together with rauwolscine had a more profound effect on the noradrenaline concentration-response curves than rauwolscine alone, shifting them further to the right. Table 2 shows that prazosin plus rauwolscine had a greater effect than rauwolscine alone on the EC_{50}s to noradrenaline but this combination was less effective in the present of L-NAME. To verify the statistical analysis of this effect of L-NAME, a one-way analysis of variance (ANOVA) was also performed on these data. This showed that the EC_{50} for noradrenaline in the presence of both rauwolscine and prazosin was significantly different from control values and also significantly diferent from the EC_{50} for noradrenaline obtained in the presence of L-NAME, rauwolscine and prazosin.

The effect of endothelium-removal on concentration-response curves to noradrenaline, phenylephrine and UK 14304

In control preparations precontracted with noradrenaline, substance P induced a relaxation. For example, if tone was raised to 710 ± 30 mg wt. tension, 3×10^{-8} M substance P

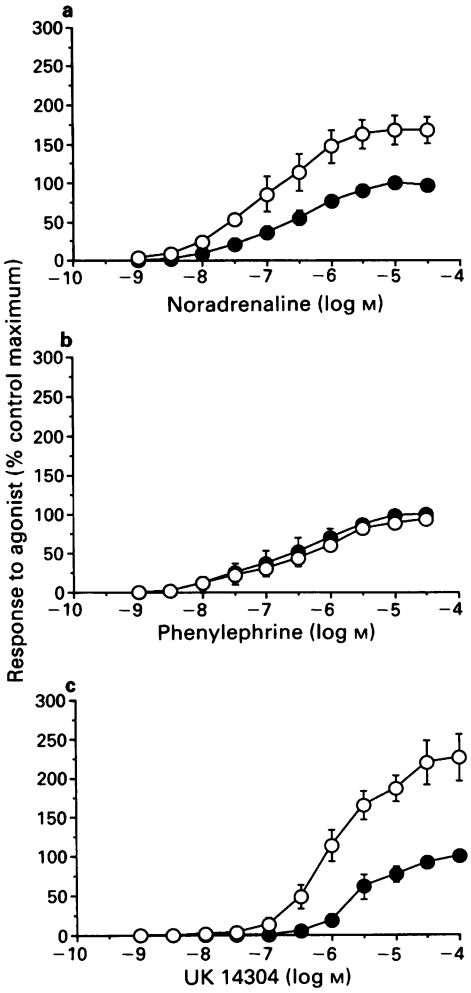


Figure 1 Vasoconstriction of rabbit isolated pulmonary arteries by noradrenaline (a), phenylephrine (b) and UK 14304 (c) in the absence (controls, ●) and presence of 10^{-4} M N^{ω}-nitro-L-arginine methylester (L-NAME, ○). Data are expressed as a percentage of the maximum response, to each agonist, achieved in the controls. Each point represents the mean response of 12 preparations from 6 animals with vertical bars representing ± s.e.mean.

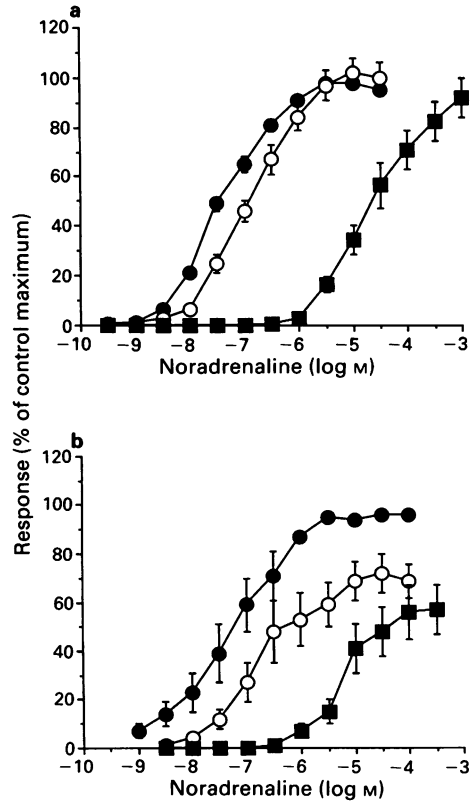


Figure 2 Vasoconstriction of rabbit isolated pulmonary arteries by noradrenaline (a) and after administration of 10^{-4} M N^{ω}-nitro-L-arginine methylester (L-NAME, b) and in the absence (●) and presence of 10^{-6} M rauwolscine (□) and 10^{-6} M rauwolscine plus 10^{-7} M prazosin (■). Data are expressed as a percentage of the maximum response achieved in the absence of either antagonist. Each point represents the mean responses of 10 preparations from 5 animals with vertical bars representing ± s.e.mean.

Table 1 The effect of N^{ω}-nitro-L-arginine methylester (L-NAME) on responses to α-adrenoceptor agonists in the rabbit isolated pulmonary artery

	Controls		*+ L-NAME* (10^{-4} M)		
Agonist	EC_{50}	*Maximum response to agonist*	EC_{50}	*Maximum response to agonist*	n
Noradrenaline	6.5 ± 0.2	44 ± 7	7.1 ± 0.4*	72 ± 12*	12
Phenylephrine	6.2 ± 0.4	51 ± 4	6.6 ± 0.3	45 ± 5	12
UK 14304	5.6 ± 0.1	17 ± 4	6.0 ± 0.2*	43 ± 4***	12

EC_{50} is expressed as the − log concentration (M) and the maximum response of each agonist as the percentage of the maximum response obtained with 100 mM KCl in each ring preparation. Data are expressed as mean ± s.e.mean. n = the number of preparations from six animals. Statistical comparisons with controls were carried out using Student's paired t test. *$P<0.05$; ***$P<0.001$

Table 2 The effect of rauwolscine and prazosin on the EC_{50} of noradrenaline in the rabbit isolated pulmonary artery in untreated preparations and preparations treated with 10^{-4} M N^{ω}-nitro-L-arginine methylester (L-NAME)

Antagonists	EC_{50} [-log concentration (M)] Untreated	+ L-NAME
Control	7.5 ± 0.1	7.3 ± 0.3
Rauwolscine (10^{-6} M)	6.9 ± 0.1*	6.4 ± 0.1*††
Rauwolscine (10^{-6} M) plus prazosin (10^{-7} M)	4.6 ± 0.4***	5.6 ± 0.1***††

Statistical comparisons with controls were carried out using a Student's paired t test. *$P<0.05$; ***$P<0.001$. Data are expressed as the mean (10 preparations from 5 animals) ± s.e.mean. Statistical comparisons between rauwolscine-treated preparations (untreated controls vs + L-NAME group) were carried out using a Student's unpaired t test. ††$P<0.01$.

induced a vasodilatation of 97 ± 11 mg wt. tension. Higher doses of substance P relaxed preconstricted rings by up to 50–60%, dependent on the level of preconstriction induced. Removal of the vascular endothelium totally inhibited the ability of substance P (10^{-7} M–10^{-6} M) to relax preconstricted vessels. In addition, in those preparations examined histologically, all endothelial cells appeared damaged by this rubbing procedure. We are confident, therefore, that rubbing produced maximal disruption of the vascular endothelium.

Figure 3 compares the potencies of noradrenaline, phenylephrine and UK 14304 in endothelium-intact and endothelium-rubbed preparations. Table 3 gives the calculated values for the EC_{50} and also gives the calculated maximum contraction to each agonist expressed as a percentage of the maximum response to 100 mM KCl.

Figure 3a shows that endothelial removal shifted the concentration-response curve for noradrenaline to the left, i.e. increased its potency. Table 3 shows the resultant effect of endothelium removal on EC_{50} values and also shows that there was an increase in the maximum tension induced by noradrenaline in endothelium rubbed vessels.

In a similar fashion, Figures 3b and 3c and Table 3 show that endothelial cell removal also increased both the potency of, and maximum contractile responses to, phenylephrine and UK 14304.

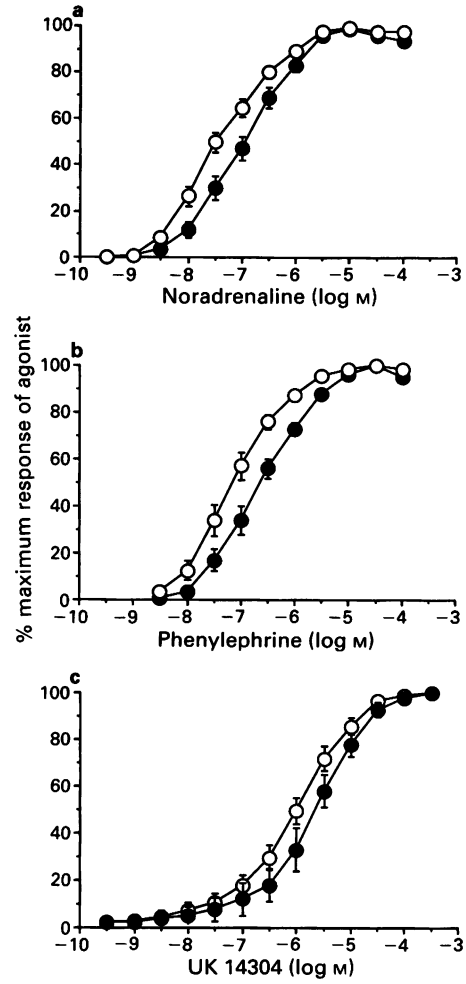


Figure 3 Vasoconstriction of rabbit isolated pulmonary arteries by noradrenaline (a), phenylephrine (b) and UK 14304 (c) in intact preparations (●) and preparations with a disrupted endothelium (○). Data are expressed as a percentage of the maximum response achieved by each agonist in each preparation. Each point represents the mean responses of 12 preparations (a,b) or 14 preparations (c) from 6 animals with vertical bars representing ± s.e.mean.

The effect of hypoxia on concentration-response curves to noradrenaline, phenylephrine and UK 14304

In hypoxic conditions, the bath gas tensions and pH were as follows: $P_{O_2} = 5.7 \pm 0.6$ mmHg, $P_{CO_2} = 35.8 \pm 0.5$ mmHg, pH = 7.37 ± 0.01, $n = 6$.

Figure 4 shows the effect of hypoxia on the concentration-response curves to noradrenaline, phenylephrine and UK 14304 in the rabbit isolated pulmonary artery by expressing contractile responses in hypoxic conditions as a percentage of the maximum response achieved in normoxic conditions. Table 4 gives the corresponding values for the EC_{50} and maximum responses to each agonist expressed as a percentage of the maximum response obtained with 100 mM KCl. Figure 4a and Table 4 show that hypoxia had no effect on the potency of noradrenaline but increased the maximum tension it was able to achieve in these vessels (by approximately 20% of control absolute maximum tensions).

Figure 4b and Table 4 show that hypoxia shifted the concentration-response curve to phenylephrine to the right

Table 3 The effect of mechanical disruption of the vascular endothelium on responses to α-adrenoceptor agonists in the rabbit isolated pulmonary artery

	Endothelium intact		*Endothelium disrupted*		
Agonist	EC_{50}	*Maximum response to agonist*	EC_{50}	*Maximum response to agonist*	n
Noradrenaline	7.0 ± 0.1	113 ± 6	7.4 ± 0.1*	142 ± 6**	12
Phenylephrine	6.7 ± 0.1	101 ± 5	7.2 ± 0.1**	124 ± 9*	12
UK 14304	5.6 ± 0.1	16 ± 5	6.0 ± 0.1*	49 ± 14**	14

EC_{50} is expressed as the − log concentration (M) and the maximum response of each agonist as the percentage of the maximum response obtained with 100 mM KCl in each ring preparation. Data are expressed as mean ± s.e.mean. n = the number of preparations from six animals. Statistical comparisons with controls were carried out using a Student's unpaired t test. *$P<0.05$; **$P<0.01$.

and hence decreased its potency. These results also show that the maximum response achieved by phenylephrine was likewise reduced by hypoxia (by approximately 20% of control absolute maximum tensions).

Figure 4c and Table 4 show the effect of hypoxia on the concentration-response curves to UK 14304. The results show that hypoxia had no significant effect on responses to UK 14304 in these vessels.

At the end of some experiments, arterial rings (six in total from three animals) that had been exposed to hypoxia were examined histologically by use of fluorescent dyes. There was no structural damage to the endothelial cells. In addition, pre-constricted arterial rings exposed to hypoxia for the duration of these experiments were still able to relax to substance P to the same extent as control preparations.

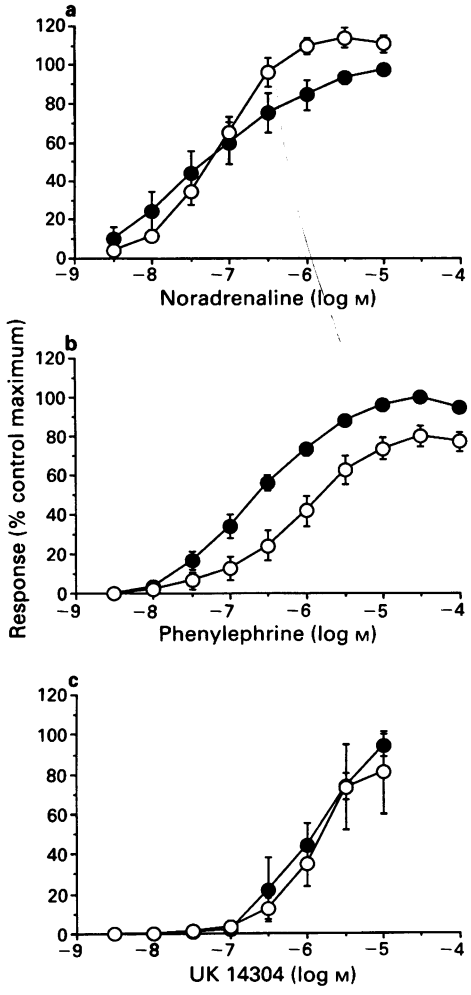


Figure 4 Vasoconstriction of rabbit isolated pulmonary arteries by noradrenaline (a), phenylephrine (b) and UK 14304 (c) in normoxia (Po_2 45 mmHg, ●) or hypoxia (Po_2 6 mmHg, ○). Data are expressed as a percentage of the maximum response achieved by each agonist in each normoxia. Each point represents the mean responses of 12 preparations (a,b) or 9 preparations (c) from 6 animals with vertical bars representing ± s.e.mean.

Discussion

The results show that inhibition of nitric oxide synthase with L-NAME potentiates responses to noradrenaline in rabbit pulmonary arteries. In a limited number of preliminary experiments we have also been able to demonstrate this phenomenon in human pulmonary artery rings (unpublished observations). This may have far reaching implications for our understanding of the mechanisms underlying the development of pulmonary hypertension. Endothelial cell disruption has been observed in experimental pulmonary hypertension, and in patients with hypoxia-related pulmonary hypertension (Rosenberg & Rabinovitch, 1986; Rabinovitch *et al.*, 1986). It has also been demonstrated that endothelium-dependent relaxation induced by acetylcholine is impaired in isolated perfused lungs from patients with chronic lung disease (Cremona *et al.*, 1992) and this is believed to be due to lack of oxygen leading to reduced nitric oxide release (Dinh-Xuan, 1992). If reduced nitric oxide production leads to the increased sensitivity of pulmonary arterial smooth muscle to circulating catecholamines, this would contribute to the persistent pulmonary vasoconstriction typical in hypoxia-induced pulmonary hypertension.

We have also shown that L-NAME can also potentiate the responses of rabbit pulmonary artery rings to nerve stimulation (MacLean *et al.*, 1993). The combined effect of the increased sensitivity to nerve stimulation and circulating catecholamines would further aggravate the pulmonary hypertension. Indeed, recent studies have shown that inhalation of nitric oxide in patients with chronic lung disease and pulmonary hypertension relieves the hypertension and exerts a selective effect on the pulmonary circulation (Adnot *et al.*, 1992). This is somewhat of a breakthrough in the clinical approach to treatment, as the biggest problem facing clinicians is the lack of selectivity of most vasodilators used in the treatment of pulmonary hypertension. Due to pulmonary vascular remodelling, diseased lungs are relatively resistant to vascular therapy and standard vasodilators exert greater effects on the systemic circulation leading to unwanted side-effects.

As with many other noradrenergic responses, this study shows that the response to noradrenaline in the rabbit pulmonary artery is mediated primarily by α_1-adrenoceptors. There was, however a significant effect of rauwolscine on these responses and some agonist activity of the selective α_2-adrenoceptor agonist, UK 14304. Together this suggests that there is a functional population of α_2-adrenoceptors in the rabbit pulmonary artery.

A parallel, as yet unpublished, study has demonstrated that, in the rabbit isolated pulmonary artery, 10^{-6} M rau-

Table 4 The effect of hypoxia on responses to α-adrenoceptor agonists in the rabbit isolated pulmonary artery

	Normoxia (Po_2 45 mmHg)		*Hypoxia* (Po_2 6 mmHg)		
Agonist	EC_{50}	*Maximum response to agonist*	EC_{50}	*Maximum response to agonist*	n
Noradrenaline	7.3 ± 0.3	78 ± 11	7.0 ± 0.2	97 ± 14**	12
Phenylephrine	6.6 ± 0.1	101 ± 5	6.1 ± 0.2**	83 ± 6 **	12
UK 14304	6.0 ± 0.2	24 ± 6	5.0 ± 1.0	22 ± 7	9

EC_{50} is expressed as the − log concentration (M) and the maximum response of each agonist as the percentage of the maximum response obtained with 100 mM KCl in each ring preparation. Data are expressed as mean ± s.e.mean. n = the number of preparations from six animals. Statistical comparisons with controls were carried out using a Student's unpaired t test. $^{**}P<0.01$.

wolscine caused a ten fold rightward shift in UK 14304 concentration-response curves demonstrating a rauwolscine-sensitive, α_2-adrenoceptor-selective effect of UK 14304. There was, however, a component of the response of UK 14304 which was sensitive to 10^{-7} M prazosin. This study demonstrated that the rabbit pulmonary artery behaves in a similar fashion to the rabbit isolated distal saphenous artery in that the α_2-adrenoceptor response is dependent, to some extent, upon a positive influence from the α_1-adrenoceptor (Dunn *et al.*, 1991b).

In the present study, the effect of UK 14304 and rauwolscine were significantly enhanced in the presence of L-NAME. Indeed, rauwolscine also halved the effect of L-NAME on the maximum contractile response to noradrenaline. As L-NAME virtually doubled the maximum response to noradrenaline, this suggests recruitment of α_2-adrenoceptors contributed to this increase in maximum response.

This is consistent with previous studies in which we have shown that in order to observe responses mediated by postjunctional α_2-adrenoceptors in arterial preparations, the vascular conditions must be modified. For example, in the rat tail artery, when vascular tone is raised with vasopressin, endothelin-1 or 5-hydroxytryptamine, a previously quiescent α_2-adrenoceptor population is 'uncovered' (Templeton *et al.*, 1989; MacLean & McGrath, 1990). The presence of angiotensin II selectively facilitates responses mediated via postjunctional α_2-adrenoceptors in the saphenous artery and ear vein of the rabbit (Dunn *et al.*, 1991a). In addition, inhibition of neuronal uptake will reveal α_2-adrenoceptor-mediated nerve responses in the rat tail artery (Papanicolaou & Medgett, 1986). Hence, the results described here are compatible with the view that α_2-adrenoceptors require specific vascular conditions before their effect is revealed. In the case of pulmonary arteries, one way to reveal a contribution by α_2-adrenoceptors is to inhibit nitric oxide synthesis. This is the case regardless of whether or not L-NAME itself increases vascular tone. We have previously described the variable effect of L-NAME on vascular tone which seems to be related to vessel diameter, the smaller pulmonary arteries being influenced more (MacLean & McGrath, 1991). In this study, any tone increase was very small and unrelated to the effect of L-NAME on contractile responses. For example, L-NAME always augmented responses to α-adrenoceptor agonists regardless of whether or not it caused an increase in vascular tone. In addition, any increase in tone was negligible compared to the absolute increase in α-adrenoceptor-induced contractions.

As L-NAME had no effects on responses to phenylephrine and actually decreased the effectiveness of prazosin it may be that the effect of L-NAME is selective to α_2-adrenoceptors. This may, however, be species-dependent as we have shown potentiation of phenylephrine-induced responses by L-NAME in the rat perfused lung (Shaw *et al.*, 1992) and Liu *et al.* (1991) demonstrated that nitric oxide synthase inhibition augmented contractile responses to phenylephrine in guinea-pig isolated pulmonary arteries. Accordingly, we are currently investigating the receptors involved in noradrenergic responses in other mammalin species (human, bovine, ovine). Contractile responses to α_2-adrenoceptor activation are typically well-maintained and could contribute to persistent pulmonary hypertension. If selective α_2-adrenoceptor facilitation does exist in human pulmonary arteries, this would be a pharmacological advantage for selective therapeutic intervention. α_2-Adrenoceptor antagonists could be directed at the pulmonary circulation in pulmonary hypertensives in which there is endothelial cell damage and an associated reduction in nitric oxide synthase activity.

Mechanical disruption of the vascular endothelium mimicked the effect of L-NAME on contractile responses to noradrenaline and UK 14304 consistent with L-NAME inhibiting endothelium-derived nitric oxide synthase. In addition, it also augmented responses to phenylephrine. The vascular endothelium produces and releases many other factors such as the relaxant prostacyclin and the constrictor endothelin-1. As the response to phenylephrine was augmented by endothelium disruption, this implies that such endothelial cell derived vasoactive factors also exert effects on α_1-adrenoceptor activation. Hence, where disruption of endothelial cell structure is associated with pulmonary hypertension (Rabinovitch *et al.*, 1986), the pulmonary vascular responses to circulating catecholamines are likely to be enhanced through augmentation of both α_1- and α_2-adrenoceptor-mediated contractile responses. This would further aggravate the pulmonary hypertension.

Acute experimental hypoxia increased the maximum contractile response to noradrenaline. This was evidently not through an effect on α_1-adrenoceptor activation alone as phenylephrine-induced responses were significantly depressed by hypoxia. Neither does it appear to act through α_2-adrenoceptor activation alone, as it had no significant effect on the responses to UK 14304. Marriott & Marshall (1990) also reported that equivalent experimental hypoxia potentiated noradrenaline-evoked contractions in rabbit pulmonary arteries. They concluded, from their studies, that either hypoxia was facilitating noradrenaline-induced influx of calcium through receptor-operated mechanisms or that combined depolarization by hypoxia and noradrenaline may facilitate calcium influx through voltage-operated calcium channels. Our study indicates that whichever mechanism is responsible, to observe the effect of hypoxia seems to require the simultaneous stimulation of both α_1- and α_2-adrenoceptors. It has previously been suggested that, in certain vascular beds, simultaneous activation of α_1- and α_2-adrenoceptors is required before the responses to α_2-adrenoceptor stimulation are observed, e.g. in the rabbit saphenous artery (Dunn *et al.*, 1991b) and rat tail artery (Xiao & Rand, 1989). It is likely, therefore, that such interactions may involve complex facilitatory interactions between α_1- and α_2-adrenoceptor-induced calcium ion channel activation and membrane potential changes.

As discussed, chronic pulmonary hypoxia associated with hypoxia-related lung disease results in inhibition of endothelium-dependent relaxation and this is likely to be due to a reduction in nitric oxide synthesis. These results certainly do not prove that experimental hypoxia can inhibit nitric oxide synthesis, but they do not disprove this theory either. What they do show is an inability to mimic the effect of chronic hypoxia exposure using the experimental hypoxia prevalent in *in vitro* experiments. Hence, caution must be taken when comparing *in vivo* hypoxic conditions with *in vitro* ones. Experiments are planned to overcome these experimental problems by use of an appropriate chronic hypoxic animal model.

We have previously reported that, in rabbit pulmonary arteries, a sudden hypoxic challenge may transiently inhibit nitric oxide synthase (MacLean & McGrath, 1991). It is evident, however, that the degree of hypoxia produced in the organ bath, for the length of time described here, is not sufficient to inhibit nitric oxide synthase persistently.

The results suggest that in conditions in which the pulmonary vascular endothelium is disrupted, or where the nitric oxide system is inhibited (conditions prevalent in pulmonary hypertension), pulmonary vascular responses to circulating catecholamines may be potentiated and this may involve α_2-adrenoceptor-activation.

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Conversion of glyceryl trinitrate to nitric oxide in tolerant and non-tolerant smooth muscle and endothelial cells

[1]Daniela Salvemini, [2]Alessandra Pistelli & [3]John Vane

The William Harvey Research Institute, St Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ

1 Exposure of smooth muscle cells (SMC) to glyceryl trinitrate (GTN, 75–600 μM) for 30 min led to a concentration-dependent increase in nitrite (NO_2^-), one of the breakdown products of nitric oxide (NO). This was not affected by 30 min pretreatment of the cells with 0.5 mM of sulphobromophthalein (SBP) an inhibitor of glutathione-S-transferase (GST), by metyrapone or SKF-525A inhibitors of cytochrome P_{450}. These experiments were confirmed by organ bath studies using rabbit aortic strips denuded of endothelium and contracted with phenylephrine. Thus, a 30 min incubation of the strips with 0.5 mM SPB, metyrapone or SKF-525A did not affect the relaxations in response to GTN (10^{-10}–10^{-6} M).

2 Potentiation of the anti-platelet effect of GTN (44 μM) by endothelial cells (EC, 40×10^3 cells) was not affected by prior incubation of EC with SBP, metyrapone or SKF-525A (all at 0.5 mM).

3 Potentiation of the antiplatelet activity of GTN (11–352 μM) by small numbers of SMC (24×10^3 cells) or EC (40×10^3 cells) treated with indomethacin (10 μM) was attenuated when the SMC or EC were treated in culture with a high concentration of GTN (600 μM) for 18 h beforehand (referred to as 'tolerant' cells). In addition, tolerant SMC produced far less NO_2^- than non-tolerant SMC.

4 Exposure of non-tolerant SMC or EC (10^5 cells) to GTN (200 μM) for 3 min increased (3–4 fold) the levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP). This increase was much less ($\leqslant$ 1 fold) in the tolerant SMC or EC (10^5 cells). The basal levels of cyclic GMP were similar in normal or tolerant SMC or EC. Sodium nitroprusside (80 μM) or atrial natriuretic factor (ANF, 10^{-7} M) increased the levels of cyclic GMP in normal or tolerant SMC or EC to the same extent.

5 The anti-platelet effects of GTN (44 μM) were potentiated by the sulphydryl donor N-acetylcysteine (NAC, 0.5 mM). Incubation of GTN (150–1200 μM) for 30 min with NAC (0.1–1 mM) led to a concentration-dependent increase in NO_2^- formation. The reduced ability of tolerant SMC or EC to potentiate the anti-platelet activity of GTN was restored by NAC (0.5 mM). These anti-aggregatory effects were abolished by concurrent co-incubation with oxyhaemoglobin (10 μM) indicating that they were due to NO release.

6 Thus, in SMC or EC, metabolism of GTN to NO does not depend on glutathione-S-transferase or the cytochrome P_{450} system. Furthermore, when compared to normal cells, tolerant SMC or EC metabolize GTN to NO less effectively as assessed by the reduced capacity to potentiate the antiplatelet effects of GTN, to release NO_2^- and to increase the level of cyclic GMP. This decrease in NO formation shows that tolerance to GTN is mainly due to impaired biotransformation of GTN to NO. NAC, by directly forming NO from GTN, compensates for this failing mechanism.

Keywords: Glyceryl trinitrate; nitric oxide; N-acetylcysteine; smooth muscle cells; endothelial cells; tolerance; metyrapone; SKF-525A; sulphobromophthalein

Introduction

Glyceryl trinitrate (GTN) and other organic nitrates such as isosorbide dinitrate (ISDN) require bioconversion to nitric oxide in order to elicit vasodilatation (Ignarro *et al.*, 1981; Murad, 1986); they can therefore be considered as prodrugs. The hypothesis of GTN action proposes that during its denitration, it interacts in vascular smooth muscle cells (SMC) with reduced sulphydryl groups (SH, Needleman *et al.*, 1973) to form NO or a S-nitrosothiol (Ignarro *et al.*, 1981). The mechanisms by which SH-containing molecules form NO from GTN is at present not known. It may be due to a nucleophilic attack by a thiolate anion on the nitrogen atom of the ester group of GTN (Feelisch, 1991). NO stimulates soluble guanylate cyclase (GC) by interacting with the ferrohaeme centre of the enzyme resulting in generation of guanosine 3':5'-cyclic monophosphate (cyclic GMP, Ignarro *et al.*, 1981) which subsequently leads to vasodilatation (Ignarro *et al.*, 1981) and inhibition of platelet aggregation (Mellion *et al.*, 1980; Nishikawa *et al.*, 1982). Factors that affect the conversion of GTN to NO will determine the amounts of NO generated.

The reduction in the vasodilator response to GTN with prolonged exposure (nitrate tolerance) has been interpreted as being the result of impaired enzymic biotransformation of GTN to NO (Brien *et al.*, 1986) or as a consequence of depletion of the intracellular sulphydryl pool (Needleman & Johnson, 1973) but the latter has been questioned (Elkayam, 1991). Apart from the metabolism of GTN to NO by SMC (Benjamin *et al.*, 1991; Feelish & Kelm, 1991; Salvemini *et al.*, 1992) we and others (Feelish & Kelm, 1991; Salvemini *et al.*, 1992) have found that endothelial cells (EC) also convert GTN to NO. We have now investigated whether EC also develop tolerance after long term exposure to GTN.

The enzymic pathway(s) responsible for the metabolism of GTN to NO in either SMC or EC are not yet defined. In mammals, GTN denitration is mainly carried out by cytosolic hepatic glutathione-S-transferase enzymes (GST) and glutathione reductase (Needleman & Johnson, 1973). Recent

[1] Present address: Monsanto Company, 800 North Lindbergh Boulevard, St Louis, Missouri 63167, USA.
[2] Present address: Department of Preclinal and Clinical Pharmacology "M. Aiazzi Mancini", Viale G.B. Morgagni 65, 50134 Florence, Italy.
[3] Author for correspondence.

evidence in hepatic and renal cells has suggested that the cytochrome P_{450} enzyme system is involved in the formation of NO from GTN (Servent *et al.*, 1989; Schröder & Schrör, 1992).

Since SMC and EC contain the enzymes GST (Gruetter & Lemke, 1985; Steinberg *et al.*, 1989; Sakanashi *et al.*, 1991) and cytochrome P_{450} (Boeynaems *et al.*, 1987; Adeagbo *et al.*, 1990; Rosolowsky *et al.*, 1990; Pritchard *et al.*, 1990; Kauser *et al.*, 1991) we have used inhibitors to test whether these enzymes are involved in the bioconversion of GTN to NO in bovine aortic SMC or EC.

Methods

Preparation of washed platelets

Human washed platelets were prepared as described by Radomski & Moncada (1983). Indomethacin (10 μM) was added to the final platelet suspension to prevent the formation of cyclo-oxygenase products. The platelet count was adjusted to approximately $1.5-2 \times 10^8$ ml^{-1}.

Preparation of endothelial cells or smooth muscle cells

Bovine aortic EC were harvested and grown on cytodex 3 microcarrier beads (Pharmacia/LKB, Ltd.) in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories) supplemented with 4 mM L-glutamine and 10% (v/v) foetal calf serum (Gibco) as described previously (de Nucci *et al.*, 1988). The approximate cell number was 3.5×10^7 ml^{-1} of beads. Bovine aortic SMC were characterized by the presence of specific alpha actin using a Sigma kit and were prepared as previously described (Mollace *et al.*, 1991). Indomethacin (10 μM) was added to all final cell suspensions. In some experiments, GTN (600 μM) was incubated for 18 h with SMC or EC in culture (referred to as 'tolerant' cells). The cell supernatant was removed and cells washed at least 3 times with warm Krebs buffer to remove any residual GTN. The cells were then resuspended in Krebs buffer containing indomethacin (10 μM) and exposed to lower concentrations of GTN.

Platelet aggregation

A suspension of washed platelets was incubated at 37°C for 4 min in a Payton dual channel aggregometer (Born & Cross, 1963) with continuous stirring at 1,000 r.p.m. and then stimulated with thrombin (40 mu ml^{-1}) to give a submaximal aggregation (80–90%). The decrease in optical density was recorded for 5 min. After a 3 min incubation period with platelets, the inhibitor effects of GTN on platelet aggregation induced by thrombin were measured either alone or in the presence of non-tolerant or tolerant SMC or EC. When required oxyhaemoglobin (oxyHb, 10 μM) and/or N-acetylcysteine (NAC, 0.5 mM) was added to the platelet mixture for the 3 min incubation period. When using cells or oxyhaemoglobin, the calibrations were performed in the presence of these agents to compensate for possible changes in light transmission (Salvemini *et al.*, 1989). Inhibition of platelet aggregation was calculated as described previously (Salvemini *et al.*, 1989).

Measurement of cyclic nucleotides

Cyclic GMP levels were measured by radioimmunoassay (Steiner *et al.*, 1972) following prior acetylation of the samples with acetic anhydride (Harper & Brooker, 1975). Cyclic AMP levels were measured with commercially available kits (Amersham, Bucks, U.K.). A suspension (10^5 cells) of non-tolerant or tolerant SMC or EC was diluted in Krebs buffer and preincubated in an aggregometer for 3 min (37°C, 1,000 r.p.m.) in the presence or absence of GTN (200 μM), NaNp (80 μM) or atrial natriuretic factor (ANF, 10^{-7} M). 3'-Isobutyl-1-methylxanthine (IBMX, 0.1 mM) was present in the incubation mixture to inhibit phosphodiesterase activity. When required, oxyHb (10 μM) was added to cells alone or to cells in the presence of GTN for a period of 3 min. Ice cold trichloroacetic acid (TCA, final concentration 5% w/v) was then added, the samples stored at −20°C, and the cyclic nucleotides extracted from TCA with 0.5 M tri-n-octylamine dissolved in 1,1,2-trichloro-trifluoroethane. All determinations were performed in duplicate.

Nitrite analysis

Nitrite (NO_2^-) was measured using the Griess reaction. GTN (75–1200 μM), NAC (0.1–1 mM), non-tolerant or tolerant SMC (3×10^6) or a mixture of GTN together with NAC or with cells were diluted in Krebs buffer containing indomethacin (10 μM) and superoxide dismutase (SOD, 100 u ml^{-1}) and mixed for 30 min at 37°C. In some experiments, the cells were treated for 30 min in culture with SBP (0.5 mM) or metyrapone (0.5 mM). The samples were centifuged and each supernatant allowed to react with the Griess reagent (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5%H_3PO_4) to form a chromophore absorbing at 546 nm. Nitrite concentration was determined with sodium nitrite used as a standard. Results are expressed as nmol NO_2^- mg^{-1} protein as described previously (Salvemini *et al.*, 1992). That is, from the total value of NO_2^- obtained (i.e. by cells + GTN) the values obtained for NO_2^- produced by GTN alone and by cells alone are subtracted. Therefore the results give net values.

Organ bath experiments

Male New Zealand White rabbits (2.0–2.6 kg) were anaesthetized with sodium pentobarbitone (Sagatal; 30 mg kg^{-1}, i.v.) and subsequently exsanguinated. The thoracic aorta was rapidly removed, cleared of adhering periadventitial fat and cut into rings of 5 mm width. The rings were cut open, denuded of endothelium and mounted in 20 ml organ baths filled with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.17, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 5.6. Changes in isometric tension were measured by Biegestab K30 type 351 transducers (Hugo Sachs Elektronik, Germany) attached to MK II transducer coupler (Z.T.S., London, UK) and recorded on a Linearcorder mark VII WR3101 (Graphtec). The tissues were equilibrated under resting tension of 2 g for 2 h and the Krebs solution was changed every 15 min. The tissues were then washed and cumulative concentration-response curves to phenylephrine ($10^{-9}-10^{-5}$ M) were produced. When the tone of the tissue reached a plateau, the absence of an intact endothelium was confirmed by the lack of relaxation of the strips in response to acetylcholine (10^{-6} M). After 1.5 h of washing, the strips were contracted with phenylephrine ($5 \times 10^{-6}-10^{-5}$ M) to produce a 90–100% maximal contraction. When the contraction was stable, SBP, metyrapone or SKF 525A (all at 0.5 mM) were added to the baths for 30 min. A cumulative concentration-response curve to the relaxant effects of GTN ($10^{-10}-10^{-6}$ M) was then constructed. GTN-induced responses are expressed as percentage change in the phenylphrine-induced tone.

Protein determination

Protein concentrations were determined by the Lowry-method using bovine serum albumin as a standard (Lowry *et al.*, 1951). Cells in the absence or presence of drug treatment were more than 95% viable as assessed by the uptake of trypan blue.

Materials

The composition of the modified (Sneddon & Vane, 1988) Krebs bicarbonate buffer was (mM): NaCl 137, KCl 2.7, $NaHCO_3$ 11.9, NaH_2PO_4 0.3, $MgSO_4$ 0.8, glucose 5.6 and $CaCl_2$ 1. Human thrombin, haemoglobin (from bovine blood), atrial natriuretic factor, N-acetylcysteine (NAC), metyrapone (2-methyl-1,2-di-3-pyridylpropanone), superoxide dismutase (SOD, from bovine erythrocytes), indomethacin, sodium nitroprusside, sulphanilic acid, N-1-naphthylethylenediamine dihydrochloride, Na_2CO_3, sodium nitrite, and kits for characterizing alpha smooth muscle actin (procedure number S1H 903) were obtained from Sigma (Poole, U.K.). SKF-525A (proadifen) was obtained from Research Biochemicals Inc (Natick, Massachusetts, U.S.A.). Tri-n-octylamine and 3'-isobutyl-1-methylxanthine (IBMX) were obtained from Aldrich (Dorset, U.K.) and phosphoric acid from BDH Analar (Essex, U.K.). Stock solutions of IBMX were prepared in 0.1 N NaOH and then diluted in Krebs buffer as required. Kits for radioimmunoassay of adenosine 3':5'-cyclic monophosphate (cyclic AMP) were purchased from Amersham (Bucks, U.K.) as was [^{125}I]-cyclic GMP. N^G-monomethyl-L-arginine was obtained from Ultrafine Chemicals Ltd (Manchester, U.K.). Oxyhaemoglobin (oxyHb) was prepared by reduction of bovine haemoglobin with sodium hydrosulphite as described previously (Salvemini et al., 1989). Glyceryl trinitrate (Nitronal) was obtained from Lipha Pharmaceuticals Ltd (West Drayton, Middlesex, U.K.). Prostacyclin was a gift from the Wellcome Research Laboratories (Beckenham, U.K.) and the cyclic GMP-specific antibodies from Dr K. Schrör (Institute of Pharmacology, University of Düsseldorf, Germany). All other reagents were obtained from BDH.

Statistics

Results are expressed as mean ± s.e.mean for (n) experiments and for platelets each experiment was performed with blood obtained from a different donor. Student's unpaired t test was used to determine the significant difference between means, and a P value of <0.05 was taken as significant.

Results

Effects of sulphobromophthalein, metyrapone or SKF-525A on nitrite production from glyceryltrinitrate by smooth muscle cells and on the anti-platelet effects of glyceryltrinitrate in the presence of endothelial cells

A 30 min exposure of SMC to GTN (600 μM) led to the formation of 14 ± 2 nmol NO_2^- mg^{-1} protein ($n = 4$). This formation of NO_2^- from GTN by SMC was not altered when SMC were treated for 30 min beforehand with 0.5 mM SBP (10 ± 3 nmol NO_2^- mg^{-1} protein, $n = 4$), metyrapone (14 ± 2 nmol NO_2^- mg^{-1} protein, $n = 4$) or SKF-525A (12 ± 4 nmol NO_2^- mg^{-1} protein, $n = 4$) and then exposed to GTN (600 μM). Potentiation of the anti-platelet effect of GTN (44 μM) by EC (40×10^3 cells) was not affected when the cells were pretreated with SBP, metyrapone or SKF-525A (all at 0.5 mM) for 30 min (Figure 1).

Organ bath studies

Incubation of rabbit aortic strips for 30 min with SBP, metyrapone or SKF-525A (all at 0.5 mM) had no effect on the contractions produced by phenylephrine (5×10^{-6}–10^{-5} M). GTN (10^{-10}–10^{-6} M) induced a concentration-dependent relaxation (Table 1) which was not affected by SBP, metyrapone or SKF-525A (Table 1).

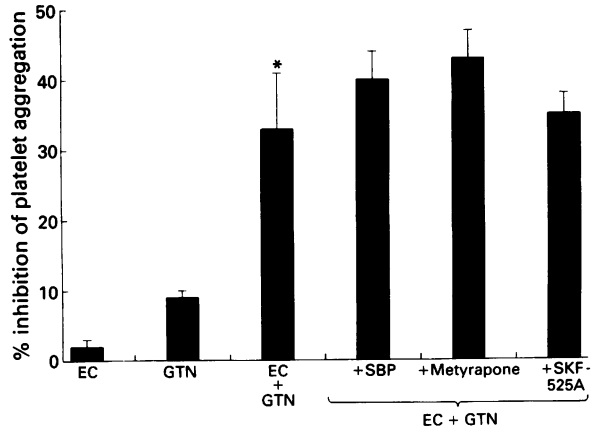


Figure 1 A 3 min incubation of a low dose of glyceryltrinitrate (GTN, 44 μM) with small numbers of endothelial cells (EC, 40×10^3 cells) enhanced the anti-platelet effects of GTN. This potentiation was not altered when the cells had been treated with sulphobromophthalein (SBP), metyrapone or SKF-525A (all at 0.5 mM) for 30 min beforehand. Results are expressed as % inhibition of platelet aggregation. Each column represents the mean of 4 experiments s.e.mean shown by vertical bars; $*P<0.01$ when compared to the value obtained with EC + GTN if the effect was an additive one.

Table 1 Lack of effects of sulphobromophthalein (SBP), metyrapone or SKF-525A on glyceryltrinitrate (GTN)-induced relaxations of rabbit aorta strips denuded of endothelium in the organ bath

GTN (M)	*Control*	*SBP*	*Metyrapone*	*SKF-525A*
10^{-10}	1 ± 1	1 ± 1	1 ± 1	0
5×10^{-10}	3 ± 1	7 ± 2	7 ± 2	2 ± 1
10^{-9}	11 ± 2	16 ± 5	17 ± 4	9 ± 1
5×10^{-9}	25 ± 3	31 ± 3	33 ± 6	23 ± 5
10^{-8}	34 ± 3	42 ± 3	42 ± 8	33 ± 4
5×10^{-8}	51 ± 4	56 ± 1	57 ± 7	50 ± 8
10^{-7}	60 ± 9	58 ± 2	60 ± 6	67 ± 9
5×10^{-7}	74 ± 8	78 ± 2	74 ± 5	85 ± 7
10^{-6}	87 ± 5	89 ± 3	86 ± 7	91 ± 6

The tissues were preconstricted with phenylephrine (5×10^{-6}–10^{-5} M) to produce approximately a 90–100% of maximal contraction and when the state of contraction of the tissue was stable a cumulative dose-response curve to GTN (10^{-10}–10^{-6} M) was constructed. The administration of SBP, metyrapone or SKF-525A (all at 0.5 mM) 30 min before GTN did not alter the relaxation of the strips in response to GTN. Results are expressed as % change in the phenylephrine-induced tone. Each value represents the mean ± s.e.mean of 4 experiments each performed with aortic strips from different rabbits (average number of observations for each point >8).

The potentiation of the anti-platelet activity of glyceryl trinitrate by smooth muscle cells or endothelial cells is lost in tolerant cells

Salvemini et al. (1992) found that the concentration of GTN required to inhibit thrombin-induced platelet aggregation by 50% (IC_{50}) is 110 ± 2 μM. This weak anti-platelet action of GTN was enhanced in the presence of small numbers of SMC or EC to give an IC_{50} of 21 ± 1 μM for SMC and an IC_{50} of 69 ± 1 μM for EC. Here, SMC (24×10^3) or EC (40×10^3) treated with a high concentration of GTN (600 μM) for 18 h beforehand and then exposed to lower concentrations of GTN (5.5–352 μM) lost their ability to enhance the anti-platelet effectiveness of GTN (Figure 2a and b). In addition, production of NO_2^- from GTN (75–600 μM) by tolerant SMC was smaller when compared to non-tolerant SMC (Figure 3).

Cyclic GMP levels

When SMC or EC (10^5) were exposed to GTN (200 μM) for 3 min there was an increase in the levels of cyclic GMP (3.7 ± 0.2 fold in SMC and 2.56 ± 0.3 fold in EC, $n = 4$) (Figure 4a and b) but not of cyclic AMP ($n = 4$, not shown). This increase in cyclic GMP is attenuated by oxyHb indicating NO formation (Salvemini *et al.*, 1992). The increase in the levels of cyclic GMP in tolerant SMC or EC by GTN (200 μM) was much smaller (Figure 4a and b). The basal levels of cyclic GMP in tolerant SMC or EC (10^5) were similar to those levels in non-tolerant cells (Figure 4a and b). NaNp (80 μM) or ANF (10^{-7} M) increased the levels of cyclic GMP in non-tolerant and in tolerant cells to the same extent (Figure 4a and b). GTN (200 μM) did not increase the levels of cyclic AMP in SMC or EC ($n = 4$, not shown).

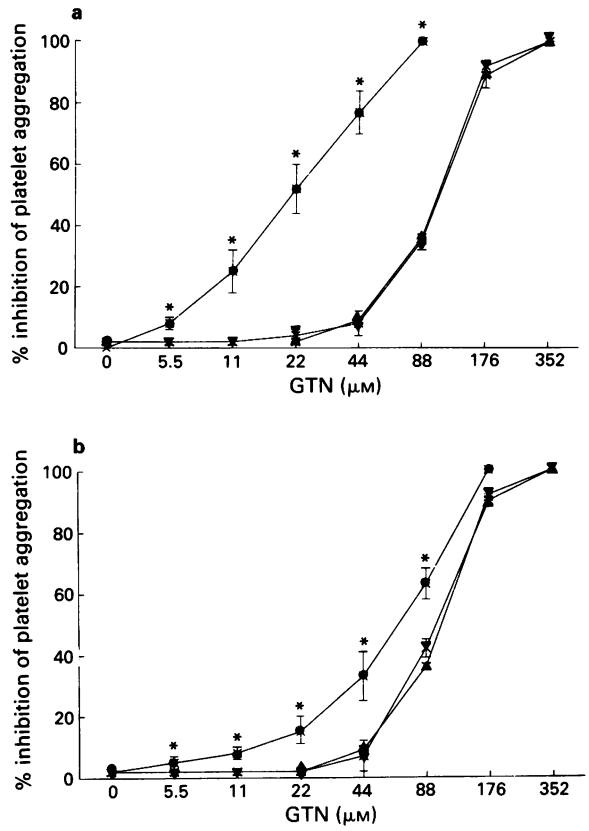


Figure 2 Inhibition of thrombin-induced platelet aggregation by glyceryl trinitrate (GTN, ▲) was substantially enhanced when GTN was incubated together with a small number of (a) smooth muscle cells (24×10^3 cells) or (b) of endothelials cells (40×10^3 cells) (●). When the cells were treated with a high concentration of GTN (600 μM) 18 h beforehand, their ability to potentiate the anti-platelet action of GTN was abolished (▼) (a and b). Results are expressed as % inhibition of platelet aggregation. Each point represents the mean ± s.e.mean (vertical bars) of 4 experiments. $^*P < 0.01$ when compared to values obtained with cells that were not pretreated with GTN for 18 h.

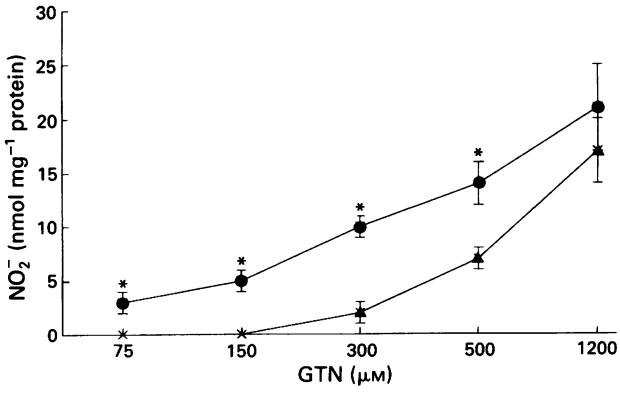


Figure 3 Exposure of smooth muscle cells to glyceryl trinitrate (GTN, 75–600 μM) for 30 min leads to a concentration-dependent increase in the levels of NO_2^- (●). When the cells were treated with GTN (600 μM) 18 h beforehand and then exposed to GTN the amounts of NO_2^- formed were less (▲). Results are expressed as nmol NO_2^- mg^{-1} protein. Each point is the mean ± s.e.mean (vertical bars) of 4 experiments. $^*P < 0.05$ when compared to values obtained with non-tolerant cells.

Effects of N-acetylcysteine

NAC (0.5 mM) when incubated for 3 min in the presence of platelets with a concentration of GTN which by itself produced very little inhibition of platelet aggregation (44 μM, promoting a 9 ± 1% inhibition of platelet aggregation) substantially increased its anti-platelet efficacy; this effect was abolished by co-incubation with oxyHb (10 μM; Figure 5). By itself NAC (0.5 mM) did not modify platelet aggregation induced by thrombin. Loss of the potentiation of the anti-platelet effect of GTN (44 μM) by tolerant SMC (24×10^3 cells) or EC (40×10^3 cells) was restored by NAC (0.5 mM;

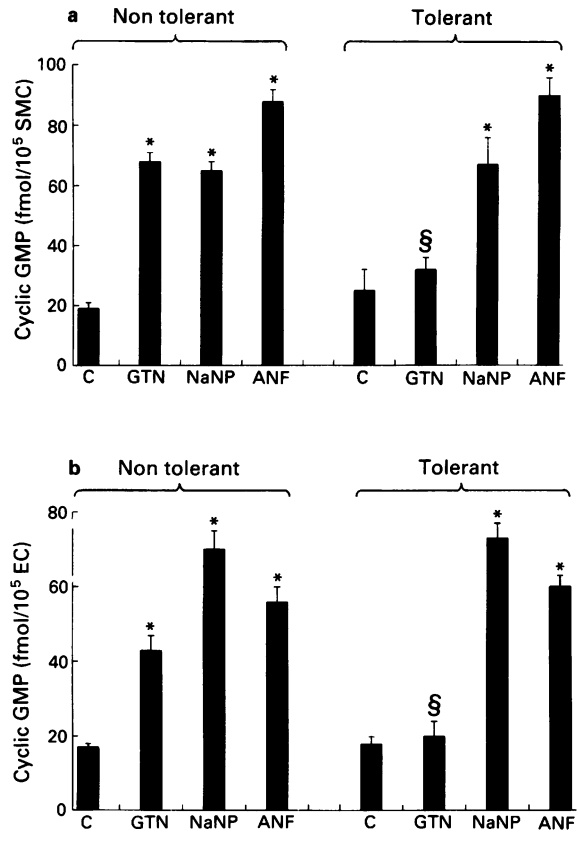


Figure 4 The effects of glyceryl trinitrate (GTN, 200 μM), sodium nitroprusside (NaNp, 80 μM) or atrial natriuretic factor (ANF, 10^{-7} M) on cyclic GMP levels in (a) smooth muscle cells (SMC) and (b) endothelial cells (EC, 10^5 cells). GTN (200 μM) increased the levels of cyclic GMP in non-tolerant SMC or EC (a and b). The increase in the levels of cyclic GMP by GTN (200 μM) was much reduced in tolerant SMC or EC (a and b). The basal levels of cyclic GMP were similar in non-tolerant or tolerant cells (a and b). NaNp or ANF increased the levels of cyclic GMP to approximately the same extent in non-tolerant or tolerant SMC or EC (a and b). Columns represent the mean ± s.e.mean (vertical bars) of 4 experiments performed in duplicate. Results are expressed as cyclic GMP fmol/10^5 cells. $^*P < 0.001$ when compared to control levels and § when compared to the value obtained in non-tolerant cells.

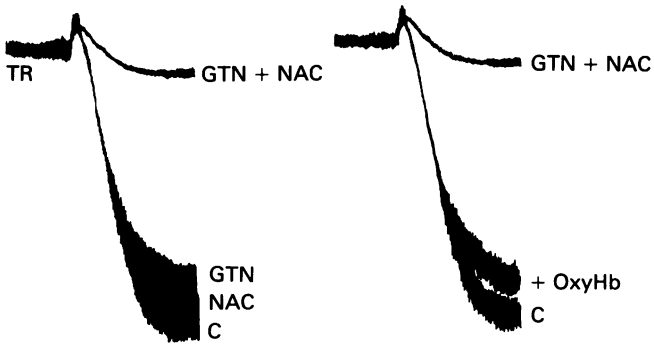


Figure 5 N-acetylcysteine (NAC, 0.5 mM) when incubated for 3 min with a small concentration of glyceryl trinitrate (GTN, 44 μM) potentiates the anti-platelet effect of GTN. These effects are abolished by co-incubation with oxyhaemoglobin (oxyHb 10 μM). Platelet aggregation was promoted by thrombin (TR) at 40 mu ml^{-1}. Tracings are representative of 3 similar experiments.

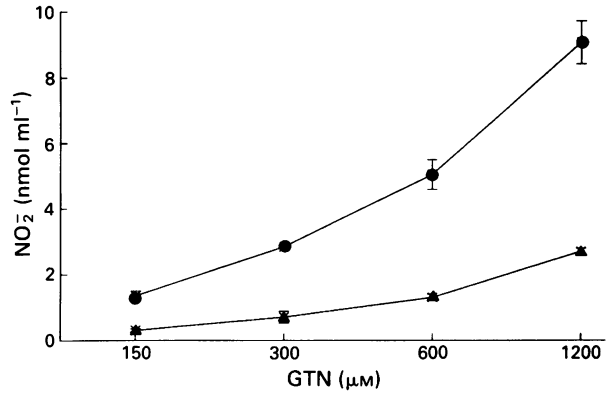


Figure 7 Exposure of glyceryl trinitrate (GTN, 75–1200 μM) to 0.1 mM (▲) or 1 mM (●) N-acetylcysteine (NAC) for 30 min at 37°C leads to a concentration-dependent increase in the formation of NO_2^-. Results are expressed in nmol NO_2^- ml^{-1}. GTN alone or NAC alone did not produce NO_2^- (see methods. Each point is the mean ± s.e.mean (vertical bars) of 4 experiments performed in duplicate.

Figure 6a and b). This was abolished by oxyHb (10 μM; Figure 6a and b). Exposure of GTN (75–1200 μM) to NAC (0.1–1 mM) for 30 min led to a concentration-dependent increase in the formation of NO_2^- (Figure 7). At these concentrations, GTN alone or NAC alone did not produce NO_2^- ($n = 4$, not shown).

Discussion

GTN dilates blood vessels and is converted to nitrite in the presence of vascular tissues (Needleman & Johnson, 1973). NO is the active intermediate in the metabolism of organic nitrates (Feelisch & Noack, 1987a) and its biological activity is destroyed by oxyHb which oxidizes NO into nitrates (Haussmann & Werringloer, 1985). Besides the well established metabolism of GTN to NO by SMC, EC can also make this conversion (Benjamin *et al.*, 1991; Feelisch & Kelm, 1991; Salvemini *et al.*, 1992). That the release of NO from GTN by SMC or EC is mediated by an enzymatic mechanism has now been substantiated. Thus we (Salvemini *et al.*, 1992) and others (Feelisch & Kelm, 1991) have demonstrated that boiled SMC or EC from bovine or porcine aortae lose about 80–90% of their capacity to form NO from GTN. Furthermore, in bovine coronary SMC the metabolic activation of GTN to NO is also enzymatic and is associated with the plasma membrane (Chong & Fung, 1990); the subcellular location for the metabolism of GTN to NO in EC is at present not known. Furthermore, the nature of the enzyme(s) and the biochemical pathway(s) responsible for the formation of NO from GTN by SMC or EC remains to be elucidated.

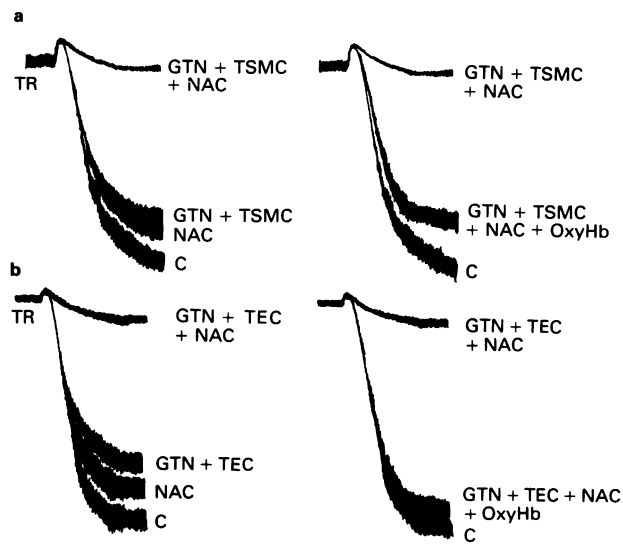


Figure 6 Tolerant (a) smooth muscle cells (TSMC, 24 × 10^3 cells) or (b) tolerant endothelial cells (TEC, 40 × 10^3 cells) do not potentiate the anti-platelet effects of glyceryl trinitrate (GTN, 44 μM). These effects were however potentiated when N-acetylcysteine (NAC, 0.5 mM) was co-incubated for 3 min with (a) TSMC and GTN or (b) TEC and GTN. This potentiation was abolished by oxyhaemoglobin (oxyHb, 10 μM). Platelet aggregation was promoted by thrombin (TR) at 40 mu ml^{-1}. Tracings are representative of 3 similar experiments.

The glutathione-S-transferase system is present in rabbit (Yeates *et al.*, 1989), bovine (Fung *et al.*, 1991) and porcine (Sakanashi *et al.*, 1991) aortic strips. This activity has been localized both in the smooth muscle and endothelium (Steinberg *et al.*, 1987; 1989). Evidence implicating the glutathione-S-transferase enzyme system in the metabolism of GTN to NO in SMC is controversial. For example, SBP a known inhibitor of GST enzymes (Yalcin *et al.*, 1983; Tahir *et al.*, 1985) has been reported by some (Yeates *et al.*, 1989) but not by others (Gruetter & Lemke, 1985; Sakanashi *et al.*, 1991) to inhibit vasorelaxation by GTN. Furthermore, in SMC of bovine coronary arteries, no changes in the intracellular levels of glutathione or glutathione-S-transferase activity have been found following GTN administration or in tissues tolerant to GTN (Gruetter & Lemke, 1985; Sakanashi *et al.*, 1991). Using SBP at a concentration 5 times greater than the one used to block GST activity and GTN-induced relaxations of rabbit aortic strips (Yeates *et al.*, 1989) we failed to inhibit the metabolism of GTN to NO as shown by a lack of effect on GTN-induced relaxations of rabbit aortic strips, NO_2^- formation from GTN by SMC or potentiation of the anti-platelet effects of GTN by EC. Our results, therefore, favour the concept that the glutathione-S-transferase system is not involved in the metabolism of GTN to NO, at least in SMC or EC.

The hepatic microsomal denitration of GTN is mediated by an NADPH-dependent cytochrome P_{450} reduction of GTN with NO formation (Servent *et al.*, 1989). A role for the cytochrome P_{450} enzyme system in the metabolism of GTN to NO has also been found in the LLC-PK_1 pig kidney epithelial cells (Schröder & Schrör, 1992). A cytochrome P_{450} system has also been described in blood vessels (Juchau *et al.*, 1976) and is present both in SMC (Serabjit-Singh *et al.*, 1988; Bornfeldt & Axelsson, 1987) and in EC (Abraham *et al.*, 1985; Pinto *et al.*, 1986). Here, the cytochrome P_{450}

enzyme system metabolizes fatty acids such as arachidonic acid (AA) into products which relax smooth muscle such as a 5,6 epoxide of AA (Carroll *et al.*, 1987). SKF-525A or metyrapone (concentrations used 1 to 10 μM for SKF-525A and 0.1 mM for metyrapone) blocked the AA-induced relaxations of rabbit pulmonary or aortic strips by blocking the cytochrome P_{450} enzyme system (Singer *et al.*, 1984; Pinto *et al.*, 1986). With SKF-525A or metyrapone at higher concentrations than those used by others to block cytochrome P_{450}-mediated responses (Singer *et al.*, 1984; Pinto *et al.*, 1986; Bornfeld & Axelson, 1987) we found that neither SKF-525A nor metyrapone altered the bioconversion of GTN to NO using the same criteria as above for GST inhibition. Thus, a cytochrome P_{450} system is not responsible for the bioconversion of GTN to NO supporting the work of Bornfeld & Axelson (1987) who demonstrated lack of effect of SKF-525A on GTN-induced relaxations of bovine mesenteric artery and vein and of bovine coronary artery.

NO is also produced endogenously from L-arginine (L-Arg) in various cells including EC (Palmer *et al.*, 1987) and SMC (Woods *et al.*, 1990; Mollace *et al.*, 1991). However, the enzymatic pathway which converts GTN into its active principle NO in SMC or EC is distinct from the one that forms NO from L-Arg and it is not regulated by NO release from L-Arg via the enzyme NO-synthase (Salvemini *et al.*, 1992).

Incubation of SMC or EC with GTN (600 μM) for 18 h led to the development of tolerance as shown by reduced pharmacological and biochemical responses upon subsequent exposure to GTN. When compared to normal cells, the potentiation of the anti-platelet effects of GTN by these cells (Figure 2) and the increase in levels of cyclic GMP in SMC or EC by GTN (Figure 4) were substantially diminished. In addition, the formation of NO_2^-, one of the breakdown products of NO, from GTN by SMC was markedly reduced (Figure 3). This suggests that tolerance to GTN is mainly due to impaired biotransformation of GTN to NO resulting from an impairment at the level of the enzyme(s) which metabolize GTN to NO. NaNp releases NO without metabolic activation (Feelisch, 1991) an observation that we have reinforced by demonstrating that the anti-platelet effects of NaNp were not potentiated by SMC or EC (Salvemini *et al.*, 1992). The increase in cyclic GMP induced by NaNp was similar in non-tolerant or tolerant SMC or EC. This indicates that a densensitization of the soluble GC during chronic exposure to GTN is unlikely to be responsible for the decreased levels of cyclic GMP observed in response to GTN in tolerant cells as suggested previously (Keith *et al.*, 1982; Kukovetz & Holzman, 1986; Mulsch *et al.*, 1988). Our experiments have also demonstrated that chronic exposure to GTN in SMC or EC does not affect responses to ANF indicating that the particulate form of the GC is not altered during tolerance.

Clearly, the enzymes responsible for the metabolism of GTN in the liver and kidney such as the GST and a cytochrome P_{450} are different from those enzymes in SMC or EC which are responsible for the vasodilator and anti-platelet effects of GTN.

The concentrations of GTN needed to inhibit platelet aggregation *in vitro* are much greater than those required *in vivo* (Schafer *et al.*, 1980; Loscalzo, 1985). Since the anti-aggregatory doses of GTN *in vitro* removed reduced glutathione (GSH) from platelets, Loscalzo (1985) postulated that at these high doses of GTN, denitration took place by GST in platelets. The thiol generated from GSH then formed a S-nitroso-thiol adduct with NO in a similar way to NAC thus potentiating the activity of the NO. Anti-aggregatory concentrations of GTN cannot be achieved pharmacologically *in vivo* and we have proposed that the increased effectiveness of GTN *in vivo* as an anti-platelet agent is due to its conversion to NO by SMC and EC (Salvemini *et al.*, 1992). The EC may be the more important cells in this respect for they can come into contact with platelets. Since EC develop tolerance to the same extent as SMC it is likely that a reduced metabolism of GTN to NO by these cells is mainly responsible for the observed reduced anti-platelet effects of GTN in patients who have become tolerant to GTN (Loscalzo & Amarante, 1989).

Thiol donors such as NAC have been used with some success to restore the decreased platelet response to GTN during tolerance (Loscalzo, 1985; Folts *et al.*, 1989) and to increase the anti-platelet effectiveness of GTN (Loscalzo, 1985). In our study the decreased ability of tolerant SMC or EC to potentiate the anti-platelet effects of GTN was restored by co-incubation with NAC. The possibilty that this restoration is due to replenishment of intracellular sulphydryl groups is unlikely, for NAC also potentiated the anti-platelet effects of GTN alone and as reported by others (Feelisch & Noack, 1987b) increased the production of NO_2^-. The potentiation by NAC of the anti-platelet effects of GTN in the absence or presence of tolerant SMC or EC was fully reversed by oxyHb (Figures 5 and 6), which traps NO but cannot enter cells (Gruetter *et al.*, 1979). This effect is therefore most likely to be the result of an extracellular interaction between GTN and NAC and subsequent NO formation. It is possible that oxyHb creates a concentration gradient of NO across the cell membrane (Salvemini *et al.*, 1990) thereby withdrawing intracellular NO. However, it is more likely that the NO is formed in the extracellular medium in this instance.

An extracellular interaction taking place either in plasma or phosphate buffer between GTN and thiols including NAC and leading to the formation of NO has been demonstrated by Feelisch & Noack (1987b) and Chong & Fung (1988; 1991). We propose that the enhancement of GTN responses or the so called 'reversal' of GTN tolerance observed with SH-donors such as NAC is not due to a replenishment of intracellular reduced SH-groups but rather to enhanced extracellular NO formation resulting from the extracellular interaction between GTN and the SH-donors. The efficacy of thiol donors in potentiating the effects of GTN or in preventing GTN tolerance will presumably depend upon their relative capacity to form NO from GTN. Thus, Chong & Fung (1991) demonstrated that amongst the aliphatic and aromatic thiols tested NAC, mercaptosuccinic acid (MPA) and thiosalicylic acid (TSA) were the most potent generators of NO from GTN in buffer and plasma with an order of potency, NAC < MPA < TSA; this correlated with their efficacy in reversing GTN tolerance (Chong & Fung, 1991). This indicates that thiol- containing compounds with higher catalylic effects with respect to NO formation may be useful for the management of GTN tolerance.

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Levamisole-activated single-channel currents from muscle of the nematode parasite *Ascaris suum*

S.J. Robertson & R.J. Martin

Dept of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, Summerhall, University of Edinburgh, Edinburgh, EH9 1QH

1 The patch-clamp technique was used to examine levamisole-activated channels in muscle vesicles from *Ascaris suum*. Cell-attached and isolated inside-out patches were used.

2 Levamisole (1–90 μM), applied to the extracellular surface, activated channels which had apparent mean open-times in the range 0.80–2.85 ms and linear I/V relationships with conductances in the range 19–46 pS. Ion-replacement experiments showed the channels to be cation selective.

3 The kinetics of the channels were analysed. Generally open- and closed-time distributions were best fitted by two, and three expotentials respectively, indicating the presence of at least two open states and at least three closed states. The distributions of burst-times were best-fitted by two exponentials.

4 Channel open- and burst-times were voltage-sensitive: at low levamisole concentrations (1–10 μM), they increased with hyperpolarization. At higher concentrations of levamisole (30 μM and 90 μM) flickering channel-block was observed at hyperpolarized potentials. Using a simple channel-block model, values for the blocking dissociation constant, K_B were determined as 123 μM at − 50 mV, 46 μM at − 75 mV and 9.4 μM at − 100 mV.

5 At the higher concentration of levamisole (30 μM and 90 μM) long closed-times separating 'clusters' of bursts were observed, at both hyperpolarized and depolarized membrane potentials and this was interpreted as desensitization.

Keywords: Levamisole; *Ascaris*; nicotinic-channels

Introduction

Nematode parasites inflict much suffering on human and animal hosts. Approximately one in five of the world's human population is infected with intestinal nematodes (Standen, 1975), while infestation in domestic animals is a major source of economic loss. Antihelminthic drugs, such as levamisole, are used to control these infestations.

Studies on the mode of action of levamisole indicate it is a selective agonist at acetylcholine receptors present on the muscle cells of parasitic nematodes. One such nematode is *Ascaris suum*. Levamisole and acetylcholine treatment of *Ascaris suum* results in depolarization and spastic paralysis of the muscle (Aceves *et al.*, 1970; Van Neuten, 1972; Coles *et al.*, 1975; Martin, 1982; Harrow & Gration, 1985; Colquhoun *et al.*, 1991).

The acetylcholine receptors of *Ascaris* muscle have been classified as nicotinic, as nicotinic agonists produce contractions antagonised by tubocurarine, whereas muscarinic agonists and antagonists have little effect on this preparation (Natoff, 1969; Rozkova *et al.*, 1980; Martin, 1982, Colquhoun *et al.*, 1991). The pharmacology of the acetycholine receptors on *Ascaris* muscle is most like that of the nicotinic ganglionic receptors of vertebrates, most notably mecamylamine is a potent antagonist at both receptor sites (Natoff, 1969; Rozkova *et al.*, 1980).

The aim of the present study was to investigate the action of levamisole at the single-channel level in *Ascaris* by use of the extrasynaptic acetylcholine receptors present in isolated muscle vesicles (Martin *et al.*, 1990; Pennington & Martin, 1990). Our results show that levamisole opens cation selective channels, but in addition, produces open channel-block and desensitization.

[1] Author for correspondence.

Methods

Single-channel recordings of levamisole-activated currents were made from *Ascaris* muscle vesicles. In general the data were analysed by the same methods as used in previous studies with acetylcholine (Pennington & Martin, 1990).

The vesicle preparation

Ascaris suum were collected from the local abattoir, maintained in Locke solution (replaced daily) at 32°C and used within 4 days. To prepare the vesicles, a 2 cm section from the anterior region of the worm was taken, cut along one of the lateral lines and pinned out to form a muscle-flap preparation. This muscle flap was treated with collagenase solution for about 10 min at 37°C, (composition as follows): mM NaCl 35, Na acetate 105, KCl 2, $MgCl_2$ 2, HEPES 10, glucose 3, ascorbic acid 2 and collagenase (1 mg ml^{-1}) pH adjusted to 7.2 with NaOH. The preparation was then kept in a maintenance solution at 37°C (composition as follows): mM NaCl 35, Na acetate 105, KCl 2, $MgCl_2$ 2, HEPES 10, glucose 3, ascorbic acid 2 and EGTA 1; pH adjusted to 7.2 with NaOH. The vesicles formed over the next hour, as outgrowths from the bag region of the muscle cells. The vesicles were used within 5 h.

Electrical recordings

The vesicles were transferred to the experimental chamber where recordings were made at room temperature (15–22°C). The experimental chamber was mounted on the stage of a Reichert-Jung Biostar inverted microscope and viewed at × 200 magnification. Vesicles were bathed in the experimental chamber in the following solution (composition mM): CsCl 35, Cs acetate 105, $MgCl_2$ 2, HEPES 10 and EGTA 1; pH adjusted to 7.2 with CsOH.

Recordings were made from either isolated inside-out pat-

ches or cell-attached patches. Patch-electrodes were made from micro-haematocrit capillary glass (Garner Glass 7052) with a resistance of 1–3 MΩ. The pipettes were coated with-Sylgard to improve frequency responses. Electrodes were filled with pipette solution (composition, mM) CsCl 140, $MgCl_2$ 2, HEPES 10 and EGTA 1; pH 7.2 with CsOH. To examine the effects of levamisole, different concentrations of levamisole were placed in the pipette solution. All of the recordings were made with Cs^+ as the main cation to avoid contamination of patch recordings with K^+ channels known to be present in the membrane. The solutions were also Ca^{2+}-free to avoid contamination of patch recordings with Ca^{2+}-activated Cl^- channels known to occur in this membrane (Thorn & Martin, 1987).

In experiments to determine ion-selectivity of the channel, the ionic composition of the bath solution was altered by diluting 50:50 with distilled H_2O. Inside-out patches were used for these experiments to ensure changes in ionic concentration at the cytoplasmic membrane surface.

Currents were recorded with a List EPC-7 and a modified Sony digital audio processor with a Betamax video recorder.

Data processing

Records were analysed with a CED 1401 interface and a DCS286 PC Computer, with PAT software kindly supplied by John Dempster, Strathclyde University. The records were filtered by an eight-pole Bessel filter, 3 dB, 1.5 kHz; the sampling time was 70 μs and the minimum detectable interval was 0.3 ms. The threshold for channel opening was set at 70% of the single channel amplitude determined from channel amplitude histograms so as to include only full openings. In some experiments a 50% threshold was also used to examine the effect on open times. Little practical difference was observed. It is pointed out that corrected P_{open} values were determined (Colquhoun & Sakmann, 1985) for levamisole concentrations between 2 and 10 μM and found to be not significantly different from apparent P_{open} values obtained from the Dempster software. Subsequently in this manuscript only apparent P_{open} values are quoted.

In these experiments, individual channel activations were interrupted with short close periods (the 'Nachschlag' phenomenon, Colquhoun & Sakmann, 1985), so bursts of openings as well as single openings were considered. Bursts were defined as groups of openings separated by gaps that are shorter than a specified length, T_{crit}. T_{crit} was determined by numerically solving the following equation:

$$1 - e^{-T_{crit}/T1} = e^{-T_{crit}/T2}$$

where T1 represents the brief closed state T1, and T2 represents the slightly longer closed state T2. The equation finds a value for T_{crit} which balances the proportion of gaps within a burst misclassified (as gaps between bursts) and gaps between a burst misclassified (as gaps within a burst) (Colquhuon & Sakmann, 1985). In general a value of 1 ms was used in these experiments. This particular value was not so important since gaps between bursts were several hundred fold larger than gaps within bursts.

Exponential curve fitting

Exponential curves were fitted to the open-, closed-, and burst-time by the method of maximum likelihood (Colquhoun & Sigworth, 1983; Martin, 1985) to a probability density function (p.d.f) of the form:

$$\text{p.d.f.} = \sum_{i=1}^{k} \frac{A_i e(^{-t}/T_i)}{T_i}$$

where A_i = the area of the ith component; T_i = the fitted time constant; t = time and K = number of exponentials fitted.

After exponential curve fitting, the data were corrected for missing events (Colquhoun & Sakmann, 1985; Pennington & Martin, 1990). This was carried out to compensate for brief events missed due to limited resolution of the recording apparatus.

The flickering channel-block seen at high levamisole concentrations was analysed by the simple channel-block scheme described by Adams (1976) and Colquhoun & Sakmann (1985):

$$C \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} O \underset{k_{-B}}{\overset{k_{+B}.X_B}{\rightleftharpoons}} B$$

where C denotes the closed state; O denotes the open state; B denotes the blocked state; X_B is the drug concentration; k_{+B} is the blocking rate constant; k_{-B} is the unblocking rate constant; α is channel closing-rate and β is the channel opening-rate. The model predicts that:

$$\text{mean open-time} = 1/(\alpha + k_{+B}.X_B),$$

$$\text{mean block-time} = 1/k_{-B},$$

$$\text{channel-block dissociation constant, } K_B = k_{-B}/k_{+B}$$

The slope of the reciprocal plot of mean durations of long openings (T2), against levamisole concentration was used to determine values for k_{+B}.

The mean block durations were determined by selecting the distinctive blocked times seen during flickering bursts greater than 0.3 ms (our limit of resolution). The method of maximum likelihood was then used to fit a single exponential to obtain a value for the mean block duration. It is pointed out that there was inevitably some overlap between the blocked time and the 'Nachschlag' closings, however this error is minimal as the interruptions due to channel block far exceed the number of interruptions due to the 'Nachschlag' closings.

Drugs

Levamisole ((−)-(5)-2,3,5,6-tetrahydro-6-phenylimadaz[2,1-b]thiazole]) was obtained as levamisole hydrochloride from Sigma chemical company, Fancy Road, Poole, Dorset, BH17 7NH. Collagenase, Sigma type 1A, was also obtained from Sigma chemical company.

Statistical analysis

The method of least squares was used to obtain the best fits for the exponential equations used to described the voltage-sensitivity of the rate constants k_{+B} and k_{-B} and the dissociation constant K_B, a Spearman correlation coefficient (r) was used to test the quality of the fits. Values are given as mean ± s.e.mean. Levels of significance were obtained by use of Student's t tests, a significance level of 0.05 was used.

Results

With levamisole in concentrations between 1–90 μM, in the patch-pipette, channel currents in 43% of 143 cell-attached and isolated inside-out patches were recorded. These channels had apparent mean open-times in the millisecond range and amplitudes of around 2.5 pA at −75 mV. Detailed analysis of channel data from a single patch recorded from both the cell-attached and the isolated inside-out configuration showed no significant differences in channel kinetics. In 20 experiments with levamisole omitted from the

patch pipette solution no such channel openings were observed. It was therefore concluded that levamisole activated these currents.

Channel conductance and current/voltage relationships

The pipette and bath-solutions had symmetrical cation concentrations but non-symmetrical anion concentrations (pipette main-ions: Cs^+ 140 mM, Cl^- 144 mM: bath main-ions: Cs^+ 140 mM, Cl^- 39 mM). Under these conditions with isolated inside-out patches, the predicted Nernst reversal potential for a cation selective channel is 0 mV, and for an anion channel is − 33 mV. In 26 out of 31 experiments the *I/V* plots of levamisole-activated channels were linear with reversal near 0 mV (Figure 1) indicating that these channels were cation-selective and could conduct Cs^+. The slopes had an average conductance of 32.9 ± 1.23 pS (mean ± s.e., $n = 26$) and ranged between 19–46 pS.

As a further test of the permeability of the channel the bath-solution was diluted 50:50 with distilled water. The predicted effect of this is to produce a shift in the Nernst reversal potential from 0 mV to + 18 mV for a cation selective channel and from − 33 mV to − 51 mV for an anion selective channel. In the experiment illustrated in Figure 1, the reversal potential shifted from 0 mV to + 13 mV.

Calculation of the relative permeability of Cs^+ and Cl^- with the Goldman constant field equation (Hille, 1984), revealed that Cs^+ was ten times more permeable than Cl^-. Similar results were obtained in three other experiments.

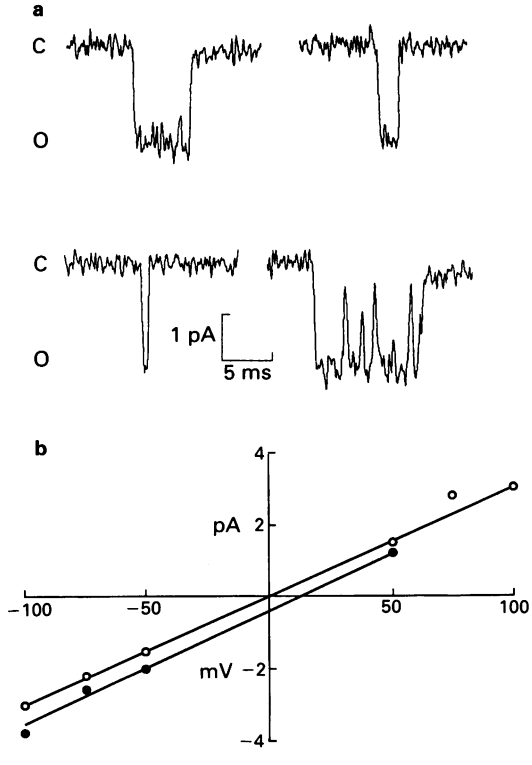


Figure 1 (a) Individual channel openings recorded from an isolated inside-out patch, at a membrane potential of − 75 mV and with levamisole 3 μM. The conductance of these channel currents was 26 pS. C: denotes the closed state. O: denotes the open state. (b) *I/V* plot of channel currents activated by 3 μM levamisole in an isolated inside-out patch. The initial plot (○) has a conductance of 31 pS and a 0 mV reversal potential. Dilution of the bath solution (●) produced a shift in the reversal potential to + 13 mV. The predicted Nernst potentials during the initial *I/V* plot were: for Cs^+, 0 mV; for Cl^- − 33 mV. The predicted Nernst potentials for the *I/V* plot obtained with the diluted solution were: for Cs^+, 18 mV; for Cl^-, − 51 mV.

Exponential curve-fitting

Channel data from 31 patches were analysed at various membrane potentials, ranging from + 150 mV to − 150 mV. The data were best-fitted by the sum of exponentials (see Methods). Files contained between 200 and 10,000 apparent open-, burst- and closed-times.

With 2–10 μM levamisole in the pipette the open- and burst-time distributions were best-fitted by two exponentials, while closed-time distributions were best-fitted by three exponentials (Table 1,2,3). Since the number of open-, burst- and closed- states must at least equal the number of exponential components fitted (Colquhoun & Hawkes, 1982), levamisole-activated channels in *Ascaris* have at least two open-states, at least two burst-states and at least three closed-states. Higher concentrations of levamisole (30 μM and 90 μM) produced substantial changes in the distribution of both the open- and closed-times, these changes are considered in the sections on channel-block and desensitisation.

Voltage-sensitivity of open- and burst-time durations

At lower concentrations of levamisole (2–10 μM) the corrected mean open-times (Figure 2) and corrected mean burst-times exhibited a degree of voltage-sensitivity, decreasing with depolarization. For example the value at − 75 mV for the corrected mean open-time was 1.47 ± 0.11 ms (mean ± s.e., $n = 10$), this value was significantly greater ($P < 0.03$) than the value for the corrected mean open-time at + 75 mV of 1.10 ± 0.25 ms ($n = 4$). The corrected mean burst duration at − 75 mV was 2.50 ± 0.28 ms ($n = 16$), this compared to a value of 1.50 ± 0.25 ms ($n = 4$) at + 75 mV where the corrected mean burst durations were also significantly reduced ($P < 0.05$).

Probability of channel opening

The probability of the channel being open (P_{open}) was concentration-dependent at depolarized potentials where P_{open} increased with concentration. However at hyperpolarized potentials concentration had no significant effect on P_{open}. Figure 3 illustrates the effect of concentration on P_{open} at ± 75 mV. Similar results were observed at ± 100 mV. It is suggested subsequently that the explanation for the failure of P_{open} to increase at − 75 mV and − 100 mV is due to the blocked state of the channel, which occurs with high concentrations of levamisole at hyperpolarized potentials, directly closing without re-entering the open-state.

At low agonist concentrations the membrane potential had little affect on P_{open}. For example, in one experiment with 3 μM levamisole, P_{open} was 0.0061 at + 75 mV compared to 0.0078 at − 75 mV; similar results were obtained in 8 other experiments with 2–10 μM levamisole. This lack of effect of membrane potential on P_{open} contrasts with the effect of membrane potential on open-times; the lack of effect on P_{open} is explained by the compensating increase in the rate of channel opening at depolarized potentials. For example in one experiment with 3 μM levamisole, the channel opening rate at − 75 mV was 8.6 channel openings per second compared to 13.6 channel openings per second at + 75 mV.

Evidence of channel block

At higher concentrations of levamisole (30 μM and 90 μM), there was a recognisable change in the kinetics of channel opening (Figure 4). At hyperpolarized potentials, channel openings were briefer than at lower concentrations and appeared as sequences of openings separated by brief closings characteristic of a 'flickering' channel-block (see, Neher & Steinbach, 1978). This behaviour may be explained by levamisole acting to produce a voltage-sensitive open channel-block, in addition to its action as an agonist. The cationic charge and large size of levamisole is consistent with

Table 1 Open-time kinetics for levamisole-activated channels

Levamisole conc. (μM)		*Open-times* (ms) A1	A2	T1	T2
Membrane potential − 75 mV					
2–10	(n = 17)	0.66 ± 0.06	0.34 ± 0.06	0.88 ± 0.10	2.30 ± 0.24
30	(n = 9)	0.78 ± 0.09	0.22 ± 0.09	0.37 ± 0.09	0.69 ± 0.08
90	(n = 12)		1.00		0.57 ± 0.06
Membrane potential − 100 mV					
2–10	(n = 13)	0.59 ± 0.08	0.41 ± 0.08	0.86 ± 0.12	2.54 ± 0.36
90	(n = 5)		1.0		0.39 ± 0.05
Membrane potential + 75 mV					
3–10	(n = 4)	0.62 ± 0.10	0.38 ± 0.10	0.71 ± 0.10	1.60 ± 0.14
30	(n = 5)	0.66 ± 0.07	0.34 ± 0.07	0.48 ± 0.07	1.22 ± 0.29
90	(n = 8)	0.66 ± 0.08	0.34 ± 0.08	0.64 ± 0.10	2.02 ± 0.28
Membrane potential + 100 mV					
3–10	(n = 4)	0.79 ± 0.12	0.21 ± 0.12	0.71 ± 0.08	2.35 ± 0.50
30	(n = 4)	0.81 ± 0.04	0.19 ± 0.04	0.50 ± 0.10	2.03 ± 0.47
90	(n = 4)	0.67 ± 0.06	0.33 ± 0.06	0.65 ± 0.07	1.88 ± 0.24

T1 and T2 are the time constants representing the two open states. The proportion of brief openings (T1) and long openings (T2) are represented by the areas of A1 and A2. Values are mean ± s.e..

Table 2 Closed time kinetics for levamisole-activated channels

Levamisole conc. (μM)		*Closed times* (ms) A1	A2	A3	A4	T1	T2	T3	T4
Membrane potential − 75 mV									
2–10	(n = 18)	0.46 ± 0.04	0.26 ± 0.05	0.28 ± 0.05		0.35 ± 0.04	237 ± 41	821 ± 127	
30	(n = 9)	0.46 ± 0.06	0.20 ± 0.04	0.28 ± 0.04	0.06 ± 0.02	0.76 ± 0.16	24 ± 10	228 ± 80	1586 ± 446
90	(n = 11)	0.55 ± 0.04	0.17 ± 0.02	0.22 ± 0.03	0.06 ± 0.02	0.63 ± 0.08	12 ± 3	251 ± 107	2528 ± 714
Membrane potential − 100 mV									
2–10	(n = 14)	0.38 ± 0.05	0.26 ± 0.04	0.34 ± 0.08		0.37 ± 0.04	224 ± 52	1081 ± 139	
90	(n = 6)	0.51 ± 0.07	0.25 ± 0.03	0.18 ± 0.06	0.07 ± 0.02	1.57 ± 0.32	20 ± 12	223 ± 131	3684 ± 2579
Membrane potential + 75 mV									
3–10	(n = 5)	0.22 ± 0.05	0.56 ± 0.09	0.21 ± 0.09		0.24 ± 0.04	132 ± 38	595 ± 115	
30	(n = 5)	0.21 ± 0.09	0.09 ± 0.05	0.57 ± 0.08	0.12 ± 0.03	0.38 ± 0.23	8 ± 4	137 ± 73	675 ± 261
90	(n = 8)	0.22 ± 0.38	0.38 ± 0.05	0.34 ± 0.06	0.05 ± 0.02	0.56 ± 0.15	32 ± 11	127 ± 30	961 ± 380
Membrane potential + 100 mV									
2–10	(n = 4)	0.31 ± 0.04	0.52 ± 0.08	0.15 ± 0.05		023 ± 0.06	146 ± 22	672 ± 189	
30	(n = 4)	0.10 ± 0.02	0.42 ± 0.26	0.34 ± 0.14	0.13 ± 0.10	0.44 ± 0.01	51 ± 38	172 ± 74	526 ± 247
90	(n = 5)	0.10 ± 0.03	0.40 ± 0.07	0.46 ± 0.65	0.04 ± 0.02	0.38 ± 0.08	16 ± 4	87 ± 36	427 ± 128

Closed states are represented by three exponentials at 2–10 μM levamisole. Brief closings (T1), intermediate closings (T2) and long closings (T3), their relative proportions are represented by the areas A1, A2 and A3. At 30–90 μM there are four closed states represented by the time constants T1, T2, T3 and T4. Their relative proportions are represented by the areas A1, A2 and A3 and A4. All values are the mean ± s.e., n is the number of experiments.

Table 3 Burst-time kinetics for levamisole-activated channels

Levamisole conc. (μM)		*Burst-times* (ms) A1	A2	T1	T2
Membrane potential − 75 mV					
2–10	(n = 19)	0.49 ± 0.07	0.51 ± 0.07	1.24 ± 0.19	3.07 ± 0.35
30	(n = 8)	0.61 ± 0.09	0.39 ± 0.09	0.59 ± 0.15	2.06 ± 0.42
90	(n = 9)	0.59 ± 0.07	0.41 ± 0.07	0.74 ± 0.19	2.23 ± 0.32
Membrane potential − 100 mV					
2–10	(n = 12)	0.56 ± 0.07	0.44 ± 0.07	1.29 ± 0.22	3.78 ± 0.50
90	(n = 4)	0.44 ± 0.08	0.56 ± 0.08	0.30 ± 0.15	2.93 ± 0.96
Membrane potential + 75 mV					
3–10	(n = 5)	0.46 ± 0.08	0.54 ± 0.08	0.69 ± 0.03	1.89 ± 0.29
30	(n = 5)	0.77 ± 0.08	0.23 ± 0.08	0.66 ± 0.19	2.33 ± 0.79
90	(n = 7)	0.73 ± 0.05	0.27 ± 0.05	0.93 ± 0.23	2.73 ± 0.64
Membrane potential + 100 mV					
2–10	(n = 5)	0.71 ± 0.06	0.29 ± 0.06	1.06 ± 0.21	2.54 ± 0.48
30	(n = 3)	0.82 ± 0.08	0.18 ± 0.08	0.69 ± 0.21	4.01 ± 1.52
90	(n = 5)	0.69 ± 0.06	0.31 ± 0.06	0.80 ± 0.22	2.33 ± 0.52

T1 and T2 are the time constants representing the two burst states. The proportion of brief bursts (T1) and the long bursts (T2) are represented by the areas A1 and A2. Values are mean ± s.e..

the direction of the voltage-sensitivity of the block. In order to describe the effect further, a simple channel-block model (Adams 1976; Colquhoun & Sakmann, 1985) was used.

The presence of open channel-block reduced the duration of the open-states at hyperpolarized potentials. Table 1 shows that this reduction in open-times at 30 μM levamisole was associated with a reduction of both the brief and longer components of the open-time distribution. At − 75 mV with 90 μM levamisole where the open-durations were also reduced, only one open-state could be detected. The loss of one component at this concentration is most likely due to the brief openings reducing to a level below the limit of resolution of the recording apparatus, as a result of channel-block. At 90 μM the corrected mean open-time at −75 mV was 0.63 ± 0.06 ms (mean ± s.e., $n = 11$), a value significantly lower than that at + 75 mV which was 1.30 ± 0.26 ms (mean ± s.e., $n = 8$), ($P < 0.02$). Thus mean open-times showed a reversed direction of voltage-sensitivity at the higher levamisole concentrations. At + 75 mV where no channel-block was observed, there was no significant reduction in the mean duration of the two open-time components compared to values at lower concentrations (2–10 μM).

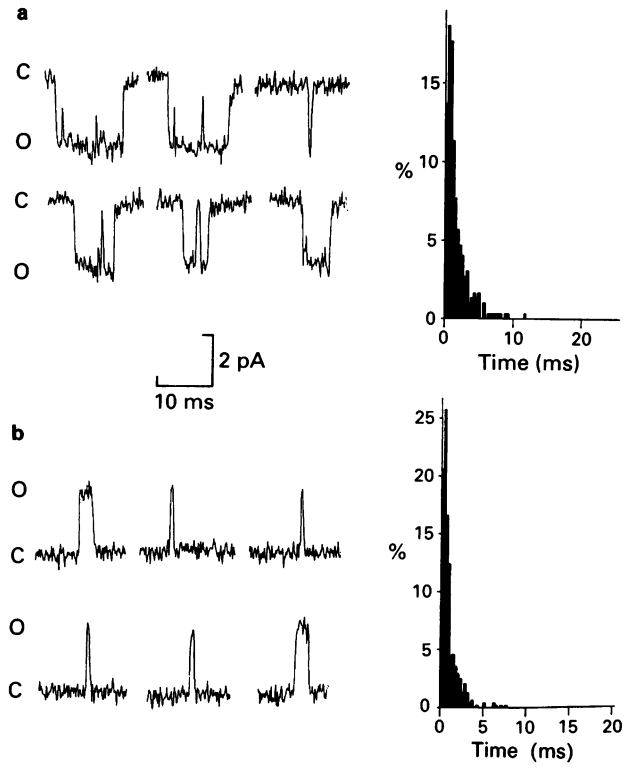


Figure 2 Levamisole (3 μM)-activated channels from a cell-attached patch illustrating the voltage-sensitivity of open-time durations. C: denotes the closed state. O: denotes the open state. (a) Individual channels recorded at − 75 mV and histogram of open-times at this potential. The open-times were fitted by two exponentials (not shown) with time constants of 0.92 ms and 2.14 ms. The corrected mean open-time was 1.59 ms. (b) Individual channels recorded at + 75 mV and histogram of open-times at this potential. The open-times were fitted by two exponentials (not shown) with time constants of 0.53 ms and 1.45 ms. The corrected mean open-time was 0.91 ms.

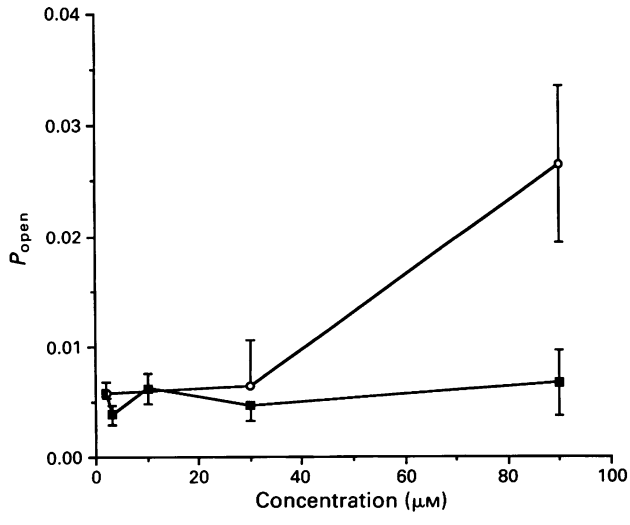


Figure 3 Graph showing P_{open} versus levamisole concentration; (■) − 75 mV membrane potential; (○) + 75 mV membrane potential. All values are mean (with vertical bars showing s.e.). $n = 4$ to $n = 9$.

Estimation of blocked-times

Mean block durations at membrane potentials of − 35 to − 100 mV for both 30 μM and 90 μM are shown in Table 4. Comparison of values at both 30 μM and 90 μM showed concentration did not affect mean block-times. The lack of effect of concentration on blocked-times is consistent with predictions of the simple channel-block model. Values obtained for mean block durations at both 30 μM and 90 μM were subsequently combined and their reciprocals used to estimate k_{-B} values (Figure 5).

Estimation of k_{+B}

The forward blocking-rate constants k_{+B} were determined from plots of the reciprocals of the mean duration of long open-times (T2) against drug concentration (Figure 6). Long open-times were used in preference to the brief open-times because they are more accurately determined, the short open durations are close to the limit of resolution of the setup and at 90 μM value for T1 appears to fall below the limit of resolution of the recording apparatus. In calculating k_{+B}, results obtained with 90 μM levamisole were not included. These points deviated from the straight line obtained with results at lower concentrations (2–30 μM). This may be due to the fact that at higher concentrations the mechanism of block may be more complex than the simple channel-block mechanism. However it is more likely that at the the higher concentration (90 μM) some of the open-time durations are lower than the limit of resolution of the recording apparatus, making the open-time duration an overestimation at this concentration. This interpretation is further supported by the fact that at 90 μM with hyperpolarized potentials open-times distributions could only be fitted by a single exponential.

Values for the forward blocking-rate constant, k_{+B} at − 50 mV, was $2.06 \times 10^7\ M^{-1}\ s^{-1}$; at −75 mV it was $3.82 \times 10^7\ M^{-1}\ s^{-1}$; and at − 100 mV it was $6.58 \times 10^7\ M^{-1}\ s^{-1}$. As predicted by the simple channel block model, k_{+B} increased with membrane hyperpolarization. The voltage sensitivity of k_{+B} was described by the equation: $k_{+B} = k_{+BO} \cdot \exp^{(E/Vk)}$. Where k_{+B} is the forward blocking rate constant at the membrane potential E, k_{+BO} is the value for k_{+B} at 0 mV and Vk is a constant. The value for k_{+BO} was $0.83 \times 10^7\ M^{-1}\ s^{-1}$ and Vk was − 35.5 mV. The correlation coefficient (r) was 0.97. Therefore k_{+B} showed an e-fold change every 36 mV. It was also possible to estimate the closing rate constants (α_2) for the long openings. This was the intercept of the plot of 1/T2 versus concentration. At − 50 mV the value for α_2 was $447\ s^{-1}$, at − 75mV it was $396\ s^{-1}$; and at − 100 mV it was $143\ s^{-1}$. As can be observed the closing rate decreases with membrane hyperpolarization, this supports earlier observations where open durations were longer at hyperpolarized potentials.

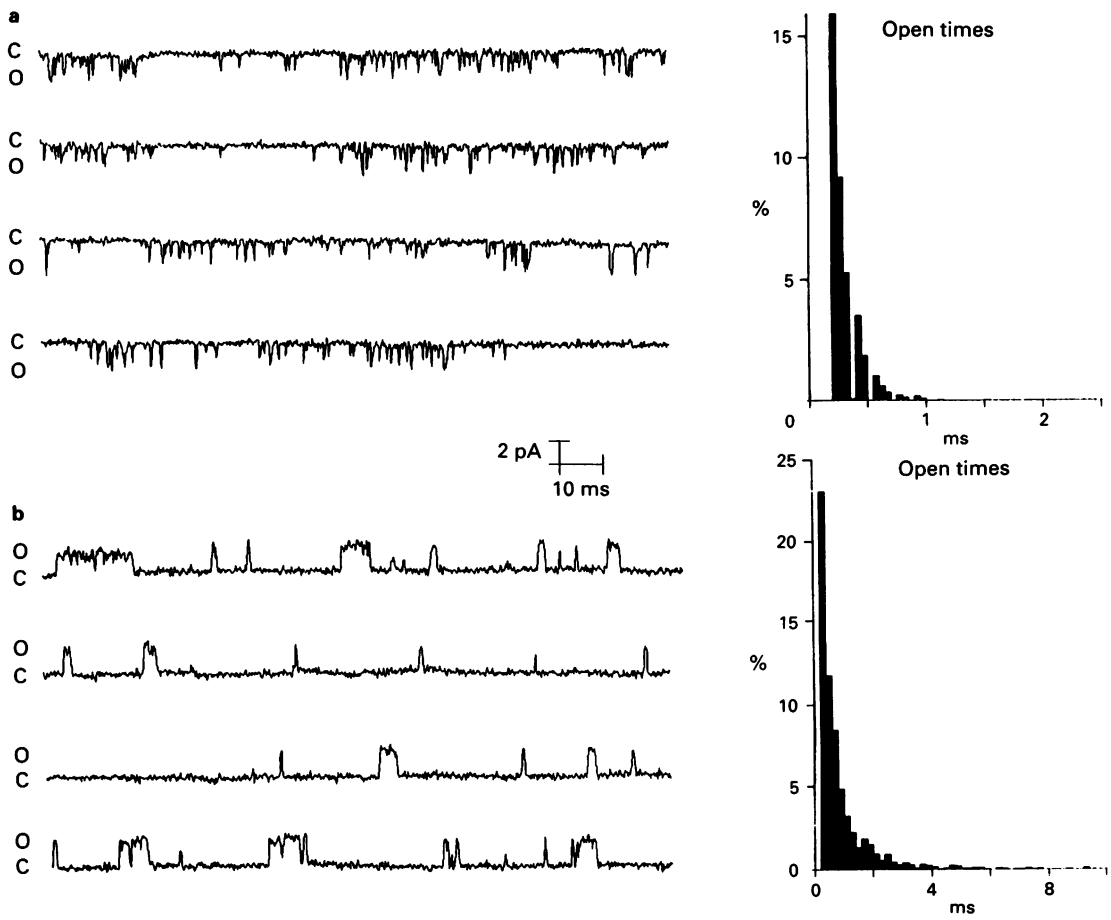


Figure 4 Levamisole-(30 μM) activated channels and open-time histograms from an isolated inside-out patch. (a) Membrane potential − 75 mV; (b) membrane potential + 75 mV. The open-times in this patch were fitted with two exponentials (not shown). (a) TI = 0.14 ms T2 = 0.69 ms; (b) T1 = 0.41 ms T2 = 1.91 ms. Note the presence of flickering open channel-block in (a).

Table 4 The effect of membrane potential on mean block time

	Mean block time (ms) *Membrane potential*			
Levamisole conc. (μM)	*− 35 mV*	*− 50 mV*	*− 75 mV*	*− 100 mV*
30		0.38 ± 0.05 (n = 5)	0.87 ± 0.18 (n = 9)	2.07 ± 0.34 (n = 4)
90	0.27 ± 0.05 (n = 4)	0.50 ± 0.07 (n = 6)	0.71 ± 0.09 (n = 11)	1.55 ± 0.18 (n = 6)
30 + 90	0.27 ± 0.05 (n = 4)	0.44 ± 0.05 (n = 11)	0.78 ± 0.10 (n = 20)	1.76 ± 0.18 (n = 10)

Values are mean ± s.e.

Determination of K_B

The values for k_{+B} and k_{-B} determined above were used to calculate the channel-block dissociation constant, K_B. At − 50 mV, K_B was $2.55 \times 10^3\ s^{-1}/2.06 \times 10^7\ M^{-1}\ s^{-1}$ or 123 μM; at − 75 mV, K_B was $1.75 \times 10^3\ s^{-1}/3.82 \times 10^7\ M^{-1}\ s^{-1}$ or 46 μM; and at − 100 mV, K_B was $0.62 \times 10^3\ s^{-1}/6.58 \times 10^7\ M^{-1}\ s^{-1}$ or 9.4 μM. The semi-log plot of K_B versus membrane potential is shown in Figure 7, this shows an e-fold change in K_B every 19.4 mV.

Burst durations

As stated earlier, the burst durations exhibit a degree of voltage-sensitivity. The bursts consisted of two components; the brief bursts (mean T1) and the longer bursts (mean T2). Values for T1 and T2, with their respective proportions A1 and A2 are listed in Table 3. The simple channel-block model predicts that the mean burst-length should increase with concentration. In order to examine this prediction the corrected mean burst lengths were determined at different levamisole concentrations. For example at − 75 mV with 2–10 μM levamisole the corrected mean burst length was 2.50 ± 0.28 ms (n = 16); with 30 μM levamisole it was 1.14 ± 0.32 ms (n = 6); and with 90 μM levamisole it was 1.40 ± 0.21 (n = 9). Thus there was no significant increase in the corrected mean burst duration. Similar results were obtained at − 100 mV. It was concluded that the mean burst durations did not increase with increasing levamisole concentration, a result not predicted by the simple channel-block model.

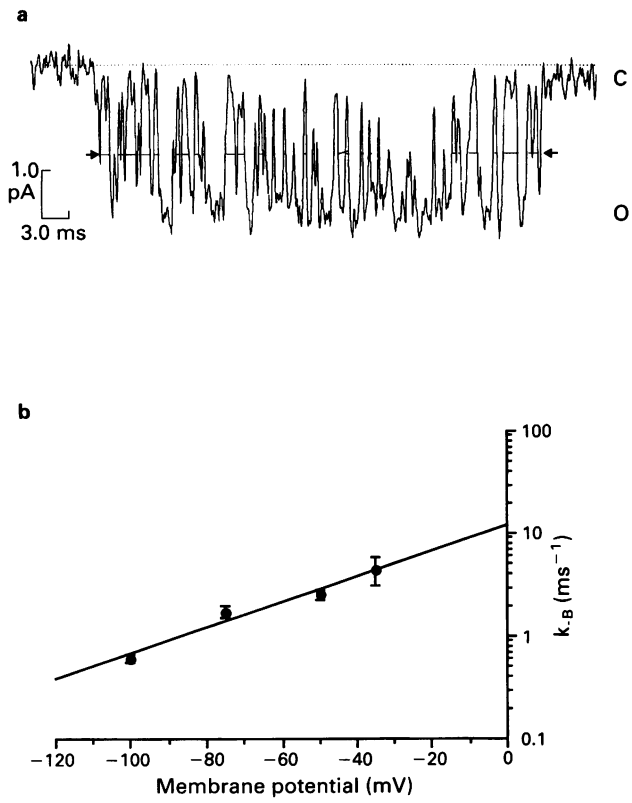


Figure 5 (a) Illustration of channel activity with 30 μM levamisole; isolated inside-out patch, − 75 mV. Fast flickering channel-block can be clearly observed. C: denotes the closed state. O: denotes the open state. The mean blocked-times were calculated from the measurements of blocked durations (indicated by the line between the arrows). In this experiment the mean blocked-time was 0.43 ms. (b) Semi-log plot of K_{-B} against membrane potential, all values are mean ± s.e.. The voltage sensitivity of K_{-B} was described by the equation: $K_{-b} = K_{-BO}.\exp^{(E/Vk)}$. Where K_{-B} is the unblocking rate constant at the membrane potential E, K_{-BO} is the value for K_{-B} at O m V and Vk is a constant. The value for K_{-BO} was 11.99 ms^{-1} and Vk was 35.02 mV.

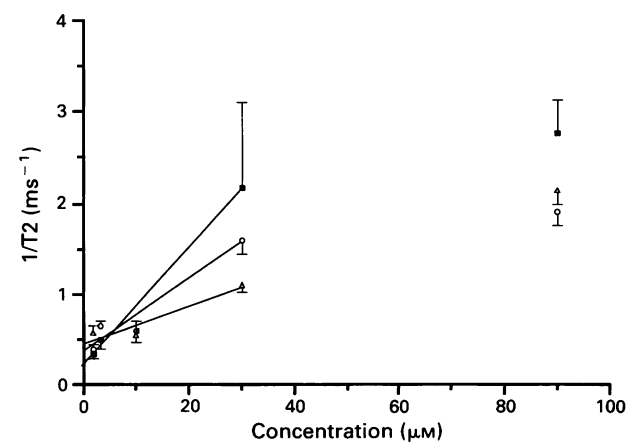


Figure 6 Reciprocal plot of open-time constant T2 against concentration, at − 50 mV (Δ), − 75 mV (O) and − 100 mV (■). The intercept represents the closing rate constant (α_2) and the slope represents the forward blocking rate (K_{+B}). Each point is the mean ± s.e. (vertical bars). Lines were fitted to 2–30 μM by least square regression without including the results at 90 μM. Values for K_{+B} were determined as − 50 mV, 2.06×10^7 M^{-1} s^{-1}; − 75 mV, 3.82×10^7 M^{-1} s^{-1}; and − 100 mV, 6.58×10^7 M^{-1} s^{-1}. The closing rate constant α_2 was also determined: − 50 mV, 447s^{-1}; − 75 mV, 396 s^{-1}; and − 100 mV, 143 s^{-1} from the intercept on the ordinate scale.

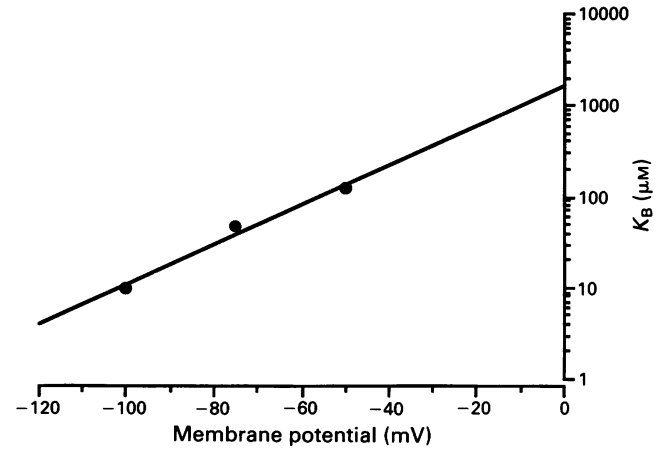


Figure 7 Semi-log plot of K_B versus membrane potential. The voltage-sensitivity of K_B was described by the equation: $K_B = K_{BO}.\exp^{(E/Vk)}$. Where K_B is the dissociation rate constant at the membrane potential E, K_{BO} is the value for K_B at 0 mV and Vk is a constant. The value for K_{BO} was 1780 μM and Vk was 19.4 mV.

Desensitization

At higher concentrations of levamisole (30 μM and 90 μM) long closed-times were seen in some patches between clusters of channel opening (Figure 8). These closed-times lasted many seconds and were observed at both depolarized and hyperpolarized potentials. The details of the closed-time components observed at higher concentrations are summarized in Table 2. These very long closed periods appear to correspond to a slow desensitization process (Cachelin & Colquhoun, 1989) and are similar to the very long closed-times observed with high concentrations of acetycholine in *Ascaris* (Pennington & Martin, 1990). In addition to the long closed durations, shorter closed times (between 10 and 50 ms) were detected at high levamisole concentrations. These might also be part of a desensitized state as they occur at both depolarized and hyperpolarized potentials but only with high concentrations.

Discussion

The simple block model

The simple channel-block model was used in this paper in order to describe quantitatively some of the effects observed

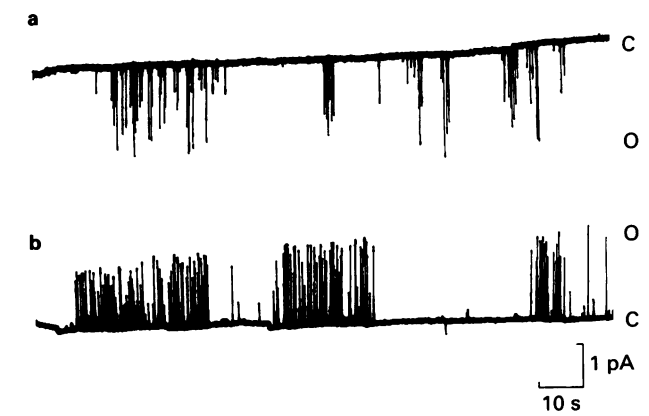


Figure 8 Channel current showing long (seconds) closed-times between clusters of openings. C: denotes closed state, O: denotes open state; 90 μM levamisole, cell attached patches. (a) − 75 mV; (b) + 75 mV. The long closed periods were interpreted as desensitization.

with levamisole. The predictions of this model, for a charged blocker, include the expectations that: the corrected mean open-times decrease with increasing levamisole concentration; burst durations increase with increasing drug concentration; the forward blocking rate constant (k_{+B}), the unblocking rate (k_{-B}), and the dissociation constant K_B all vary with membrane potential (k_{+B} and k_{-B} should have equal but opposite voltage-dependences); and the mean block duration and therefore k_{-B} are independent of drug concentration. The data presented in this report are consistent with the above predictions except that burst durations did not increase with concentration. This implies that the model used is a useful approximation but not a completely sufficient model.

We did not observe the predicted increase in the average duration of bursts, although there were frequent examples of long flickering bursts (see Figure 5). One possible explanation for the absence of increased mean burst durations could be that the channel might enter a closed state directly from the blocked state without reopening, i.e. the channel closes trapping levamisole or the blocked channel enters into a desensitised state. Limitations of the simple channel-block model are discussed further by Neher (1983).

One further difficulty in using the simple channel-block model is the presence of two open-states (O1 and O2). In our analysis it seems probable at 90 μM that the brief open-state (O1) fell below the limit of resolution of the recording set up. Thus the values for k_{-B} would be appropriate for the open-state O2 at 90 μM. Since the values for k_{-B} were not significantly different from the values for k_{-B} at 30 μM where the open-state O1 could still be resolved, it implies that the presence of O1 did not affect our estimation of k_{-B} for the open-state O2. Since the values for k_{+B} were determined from the values of the open-state O2 (see methods), our determination of K_B is likely to be an estimate of the dissociation constant for the block of the open-state O2.

Comparison with acetylcholine-activated channels in Ascaris

At the single-channel level both acetylcholine (Pennington & Martin, 1990) and levamisole activate channels in the same concentration range: acetylcholine 1–100 μM; levamisole 1–90 μM. The *I/V* relationship with both levamisole and acetylcholine are linear, both agonists showed evidence of desensitization with long closed periods appearing at higher concentrations.

Although acetylcholine and levamisole appear to activate the same channels there are some differences in the behaviour of the single-channel currents. Acetylcholine activates channels with two distinguishable conductance levels (25–35 pS and 40–50 pS) (Pennington & Martin, 1990); in contrast single-channel currents activated by levamisole had a range of detectable conductance levels between 19–46 pS with a mean of 32.9 ± 1.23 pS ($n = 26$).

The open-, closed-, and burst-time durations for levamisole or acetylcholine activated channels in this preparation show that both compounds produced two open-states and two burst-states and at least three closed-states. However the mean duration of the apparent open-times and burst-times for levamisole were shorter than for acetylcholine. The mean duration of apparent brief and long openings produced by acetylcholine (1–10 μM) was 1.18 ms and 4.89 ms, which compares with 0.80 ms and 2.30 ms for 2–10 μM levamisole under similar conditions. The mean brief and long burst durations for 1–10 μM acetylcholine were 1.17 ms and 7.62 ms compared to 1.24 ms and 3.70 ms for 2–10 μM levamisole.

A further difference between the effects of acetylcholine and levamisole is related to channel-block. Pennington & Martin (1990) did not find significant evidence for channel-block with acetylcholine on this preparation, although they did observe a reduction in the mean open-time at higher concentrations (25 μM) which might be interpreted as signs of block. Our results clearly show that levamisole blocks the channel at hyperpolarized potentials.

Comparison with previous voltage-clamp experiments in Ascaris

Harrow & Gration (1985) examined the effects, in *Ascaris*, of both acetylcholine and levamisole using two-microelectrode current-clamp and voltage-clamp. They found that the *I/V* relationship obtained from two-microelectrode voltage-clamp experiments obtained by applying levamisole by microperfusion or iontophoresis to *Ascaris* muscle had both linear (0 to −30 mV) and non-linear sections (> −30 mV). They suggested that the non linearity was due to a voltage-sensitive channel-block and/or desensitization. In our experiments we observed some desensitization at higher concentrations of levamisole, but this appeared at both depolarized and hyperpolarized membrane potentials, not just at membrane potentials greater than −30 mV. Channel-block was clearly observed in our experiments, the degree of block increased on membrane hyperpolarization. This is consistent with the voltage-sensitivity of the non linearity observed by Harrow & Gration (1985) and suggests that their observation reflects channel-block but not desensitization.

Harrow & Gration (1985) have shown that levamisole is an agonist at all concentrations (1 μM–10 mM) producing a sigmoid dose-response curve obtained under current-clamp conditions with a membrane potential of −30 mV. Our results clearly show that channel-block occurs at −35 mV and at this potential K_B was calculated at 285 μM. It is interesting to note that Harrow & Gration (1985) did not observe a reduction in the maximum conductance response with high concentrations (10 mM). This observation of Harrow & Gration (1985) is consistent with our observations at hyperpolarized potentials which showed that P_{open} failed to decrease at higher concentrations, despite the presence of channel block.

Possible therapeutic significance

Receptor desensitization produced by levamisole may have significant effects therapeutically; some *Ascaris* parasites continuously treated with higher doses of levamisole recover from the levamisole-induced paralysis (Aceves *et al.*, 1970; Coles *et al.*, 1974; 1975). This recovery may be due to desensitization since like desensitization, recovery occurs only at higher doses. Consequently it is suggested that lower doses which do not produce desensitization, potentially have a greater therapeutic effect. Also the fact that P_{open} in these experiments did not increase at hyperpolarized potentials when 2–90 μM levamisole was used suggests that high concentrations would not be therapeutically advantageous.

Therefore in conclusion, these experiments confirm that levamisole acts at the acetylcholine receptor on *Ascaris suum* muscle, both opening and blocking the channel.

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A comparison of the actions of agonists and antagonists at non-NMDA receptors of C fibres and motoneurones of the immature rat spinal cord *in vitro*

P. Pook, *F. Brugger, N.S. Hawkins, K.C. Clark, J.C. Watkins & [1]R.H. Evans

Department of Pharmacology, School of Medical Sciences, University Walk, Bristol BS8 1TD and *CIBA-GEIGY, Basle, Switzerland

1 The shift in d.c. potential in dorsal roots (EC_{50} 8.0 μM ± 0.9 s.e.mean, $n = 5$) or depression of the C elevation of the compound action potential (EC_{50} 3.0 μM ± 0.3, $n = 7$) have been used to measure the depolarizing action of kainate on dorsal root C fibres of immature (3 to 5 day old) rats. Depolarization of motoneurones was measured from the shift in d.c. potential in ventral roots.

2 6-Cyano-7-nitroquinoxaline,2-3,dione (CNQX) (pA_2 5.78 ± 0.06, $n = 8$) and 6-nitro-7-suplhamobenzo(f)quinoxaline-2,3-dione (NBQX) (pA_2 5.75 ± 0.04, $n = 7$) had similar potencies as antagonists of kainate at dorsal root fibres. The potency of NBQX as a kainate antagonist was similar also at motoneurones (pA_2 5.72 ± 0.07, $n = 3$). At motoneurones, NBQX was less potent as an antagonist of domoate (pA_2 5.29 ± 0.05) and more potent as an antagonist of S-α-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (pA_2 6.80 ± 0.09) than as an antagonist of kainate.

3 Application of L-glutamate, quisqualate and **RS**-AMPA to dorsal roots produced only short lasting depolarizations but kainate concentration-effect plots were shifted to the right in the presence of these three agonists (pA_2 5.08 ± 0.08, ($n = 3$), 5.59 ± 0.04,($n = 4$) and 4.46 ± 0.04 ($n = 4$) respectively). Slopes of dose-ratio against concentration were significantly less than one for the latter antagonism.

4 The amplitude of depolarizations induced by L-glutamate, AMPA and quisqualate were increased up to ten fold and those induced by kainate up to two fold following treatment of dorsal roots with concanavalin A. The duration of the responses was increased also by the latter treatment. Folowing 85 s applications of glutamate, quisqualate, AMPA and kainate the mean respective times (s ± s.e.mean (n)) taken for responses to decay to half the peak amplitude were increased from 63 ± 7 (10), 86 ± 17 (4), 95 ± 19 (4) and 135 ± 3 (12) to 202 ± 49 (10), 147 ± 7 (4), 160 ± 13 (6) and 163 ± 10 (10). Under similar conditions the mean decay time of γ-aminobutyric acid-induced responses was 145 ± 7 (10). This was not significantly altered by concanavalin A treatment.

5 Application to dorsal roots of L-aspartate at concentrations up to 5 mM (with or without concanavalin A treatment), the selective metabotropic agonist 1**S**,3**R**-*trans*-1-aminocyclopentane-1,3-dicarboxylate (1 mM,) and D-serine (20 μM) in the presence or absence of N-methyl-D-aspartate (NMDA, 500 μM) neither depolarized the preparations nor shifted the kainate concentration-effect plot.

6 It is concluded that primary afferent C fibres possess only one type of non-NMDA receptor which is activated strongly by domoate or kainate but only weakly by AMPA. This receptor is readily desensitized by glutamate, quisqualate or AMPA and it is less readily desensitized by kainate.

Keywords: Kainate; C fibres; motoneurones; quinoxalinediones

Introduction

Non-N-methyl-D-aspartate (NMDA) ionotropic excitatory amino acid receptors can be activated by the selective agonists α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), quisqualate or kainate. The non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline,2-3,dione (CNQX) (Honoré *et al.*, 1988) has been shown to be five times more effective at displacing bound AMPA than bound kainate from rat brain membranes (Honoré *et al.*, 1988) and a higher selectivity (30 fold) in the same direction has been reported for 6-nitro-7-sulphamobenzo(f)quinoxaline-2,3-dione (NBQX) (Sheardown *et al.*, 1990). In a functional test at neonate rat motoneurones little selectivity was found between the antagonism of quisqualate or kainate by CNQX (Long *et al.*, 1990; Watkins *et al.*, 1990). Therefore it appeared likely that the depolarizations of motoneurones produced by kainate and quisqualate are mediated mainly by the same population of non-NMDA receptors.

Somatic primary afferent C fibres are selectively sensitive to kainate, apparently lacking sensitivity to quisqualate, AMPA or NMDA (Agrawal & Evans, 1986). This kainate-sensitive site is suitable therefore to examine the antagonist selectivity of the quioxalinediones in a functional test. In the present study the isolated dorsal roots of immature rats have been used for this purpose. Potencies of the antagonists at primary afferent C fibres have been compared with measurements from motoneurones, recorded in ventral roots. The results suggest that two subtypes of non-NMDA receptor are present on motoneurones.

A voltage clamp study of primary afferent neurones (Huettner, 1990) revealed a population which showed sensitivity to kainate analogous to that observed previously in dorsal root C fibres (Agrawal & Evans, 1986). In these neurones AMPA evoked inward current responses which desensitized rapidly and the desensitization was diminished by concanavalin A treatment of the neurones (Huettner, 1990). The effects of concanavalin A have been investigated on dorsal root C fibres in the present study and the findings are consistent with the previous observations on dorsal root ganglion neurones.

[1]Author for correspondence.

Methods

Dorsal roots and hemisected spinal cords were isolated from rats of 2 to 5 days post partum age. Depolarizing responses of C fibres were measured either from depression of the C fibre component of the compound action potential recorded via a suction electrode or from the d.c. shift in a dorsal root placed across a grease gap (Agrawal & Evans, 1986). The compound action potential was evoked by 0.5 ms pulses applied at 0.1 Hz at eight times threshold. Depolarization of motoneurones was measured from d.c. shifts in ventral root polarity (Long *et al.*, 1990). Dose-ratios for antagonism and equipotent molar ratios for agonists were measured as described by Evans *et al.* (1987). Dose-ratios for the non-parallel shifts as occurred with agonist-induced desensitization were estimated at the mid-point of the control concentration-effect range (at two to five times threshold concentration).

The bathing medium for both preparations contained (mM): NaCl 118, KCl 3, $CaCl_2$ 1.5, $MgSO_4$ 0.75, $NaHCO_3$ 24 and dextrose 12. The medium and recording site were maintained at 25°C and the medium was superfused over the preparations at a rate of 2 ml min^{-1}. For the recordings of motoneuronal depolarization the medium contained 0.1 μM tetrodotoxin.

Concanavalin A type III was applied to isolated dorsal roots dissolved (1 to 10 mg ml^{-1}) in bathing medium containing no dextrose. The normal dextrose containing medium was restored following up to 2 h incubation in concanavalin A.

Concanavalin A (Type III) was obtained from Sigma (Poole, U.K.) and CNQX, (S)-AMPA and (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) from Tocris Neuramin (Bristol, U.K.). (RS)-AMPA and NBQX were kindly provided by Prof. P. Krogsgaard-Larsen and Dr T. Honoré respectively.

Values are given as mean ± s.e.mean (*n*).

Results

Since it was reported that kainate-sensitive C fibres are insensitive to NMDA (Agrawal & Evans, 1986), it has been determined that glycine has an obligatory role at the NMDA receptor/ionophore (Henderson *et al.*, 1990). Also NMDA has been reported to depolarize neurones of the nodose ganglion (Lovinger & Weight, 1988). Thus it was possible that the failure to observe any effect of NMDA in the previous study may have been due to a requirement for co-agonism at the NMDA-linked glycine site (see Thomson, 1990). Therefore in the present experiments, NMDA was tested on dorsal root C fibres in the presence and absence of D-serine which is a selective agonist for this glycine site (see Thomson, 1990). As previously observed (Agrawal & Evans, 1986) kainate (EC_{50} 3.0 ± 0.3 μM, $n = 7$) reversibly depressed the C wave of the compound action potential but NMDA at concentrations up to 1 mM failed to depolarize dorsal root C fibres in the presence or absence of D-serine (20 μM) (Figure 1).

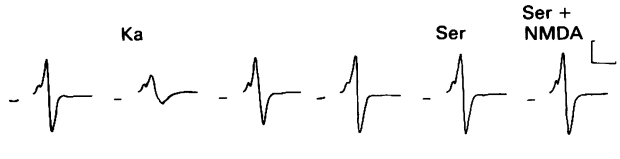


Figure 1 Failure of 100 μM N-methyl-D-aspartate (NMDA) and/or 20 μM D-serine (Ser) to modify the C fibre compound action potential recorded from an immature dorsal root. The potential was depressed by 25 μM kainate (Ka). Traces are shown 8 min following application of agonists. Scale bar 10 ms, 1 mV.

Also as reported previously (Agrawal & Evans, 1986) L-glutamate depolarized C fibres and depolarizations induced by concentrations higher than 50 μM contained an initial fast-decaying component (see Figure 4). AMPA and quis-

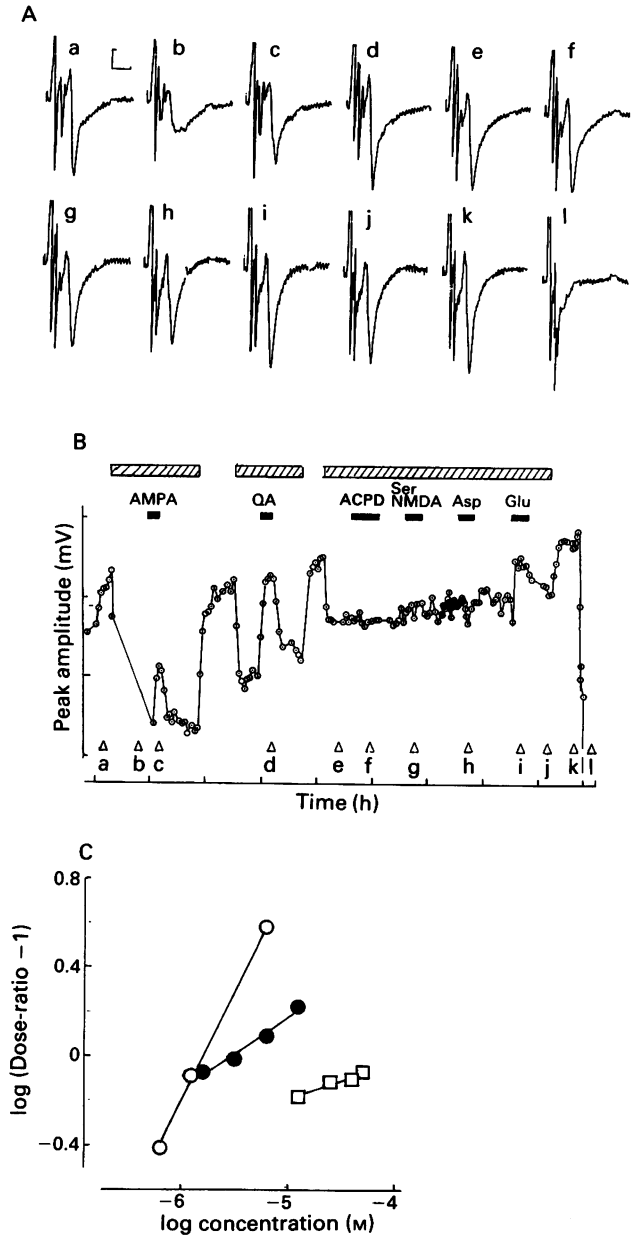


Figure 2 Effect of excitatory amino acid agonists on kainate-induced depression of the C-fibre compound action potential. The twelve oscillographic records in (A) (scale bar 0.2 mV, 10 ms) were made at the points indicated on the time-course plotted in (B). The points on the time course are the means of four consecutive recordings, standard errors of the mean all fall within the width of the symbol. Kainate (25 μM) was present during the periods indicated by the hatched bar above. S-α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA, 0.2 mM), quisqualate (0.2 mM) (QA), (1S, 3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), D-serine (Ser, 20 μM) + N-methyl-D-aspartate (NMDA, 1 mM), L-aspartate (Asp, 2.5 mM) and L-glutamate (Glu, 0.2 mM) were introduced as indicated by the solid bars. It can be seen that introduction of kainate depressed the peak amplitude and this depression was reversed by AMPA, quisqualate and glutamate but not by ACPD, NMDA or aspartate. The large depression near the end of the experiment was due to introduction of capsaicin (4 μM) which abolished the C-fibre potential (trace l). (C) Gaddum-Schild plot for the antagonism of kainate produced by 6-cyano-7-nitroquinoxaline,2,3-dione (CNQX) (○), AMPA (□) and quisqualate (●).

qualate at appropriate concentrations also produced fast decaying depolarizations similar to those produced by glutamate. At the lower range of concentration, responses induced by AMPA, although smaller in amplitude, were similar in profile to those produced by kainate (see Figure 5). L-Glutamate (pA_2 5.06 ± 0.09, $n = 3$), AMPA (pA_2 4.53 ± 0.05, $n = 4$) and quisqualate (pA_2 5.38 ± 0.08, $n = 4$), but not ACPD or NMDA or aspartate, were able to antagonize the depolarizing action of kainate. This is illustrated by the reversal of the depressant action of kainate on the C fibre compound action potential shown in Figure 2. Preparations showed a slow loss of sensitivity to kainate which developed over a period of several hours. This is illustrated in Figure 2 by the progressive reduction in the depressant action of kainate which occurred over a period of almost 8 h application of 25 μM kainate (cf. recordings (b) and (j)).

It appears likely that, as described for dorsal root ganglion neurones (Huettner, 1990), the antagonism of kainate produced by glutamate, AMPA and quisqualate represents receptor desensitization rather than competitive antagonism. The slopes of the plots in Figure 2C illustrate the non-competitive nature of the antagonism produced by quisqualate and AMPA. CNQX at concentrations from 3 to 50 μM (pA_2 5.78 ± 0.06, $n = 8$) however, produced a plot of dose-ratio versus concentration consistent with competitive antagonism of kainate. NBQX at concentrations of 1 to 10 μM gave a mean pA_2 of 5.75 ± 0.04 ($n = 7$) for antagonism of the kainate-induced depolarization of C fibres. NBQX was tested also against non-NMDA agonist-induced depolarization of motoneurones. The pA_2 (n) values obtained with domoate, kainate, quisqualate and (S)-AMPA were respectively 5.29 ± 0.05 (3), 5.72 ± 0.07 (3), 6.12 ± 0.08 (3) and 6.80 ± 0.09 (4). Thus for this series of four agonists a differential of NBQX on motoneurones was most evident when domoate (least affected) and AMPA (most affected) were tested.

Concanavalin A prevented desensitization of AMPA-induced responses of dorsal root ganglion neurones (Huettner, 1990). In the present study concanavalin A (1 to 10 mg ml^{-1}) was applied to dorsal roots for 15 to 20 min in the absence of glucose. Concanavalin A-containing and glucose-free medium produced reversible depolarization of grease-gap mounted dorsal root preparations. In four preparations the mean concanavalin A-induced depolarization was 1.62 ± 0.24 mV. The depolarizing effect of glucose-free medium developed slowly and became clearly evident because of the hyperpolarization which developed over a few minutes on reintroduction of glucose. In four preparations the mean hyperpolarizing effect of reintroduction of glucose was 1.06 ± 0.32 mV. Following treatment of dorsal roots with concanavalin A the effect of L-glutamate was enhanced considerably such that the C fibre compound action potential was permanently depressed in the presence of glutamate (Figure 3). Figure 4a illustrates the effect, on responses to glutamate, of a 15 min application of each concentration of the lectin. It can be seen that 10 mg ml^{-1} was the most effective concentration and that the effect once established persisted for long after the tissue was returned to lectin-free medium. In a similar manner to glutamate-induced depolarizations, depolarizations of dorsal root C fibres induced by AMPA, kainate or quisqualate were also increased in amplitude relative to control responses. As illustrated in Figure 4b, L-aspartate was still found to be inactive on dorsal roots following concanavalin A treatment. In concanavalin A-treated nerves, mean equipotent molar potency ratios for depolarizations induced by L-glutamate, quisqualate and AMPA (relative to kainate = 1) were 0.065 ± 0.01 (5), 0.061 ± 0.01 (4) and 0.029 ± 0.004 (6) respectively. The responses induced by kainate were less affected by concanavalin A treatment than those induced by the other three agonists.

In eight preparations the leftward shift of the kainate concentration-effect plot induced by concanavalin A was equivalent to a mean dose-ratio of 2.69 ± 0.36. The corres-

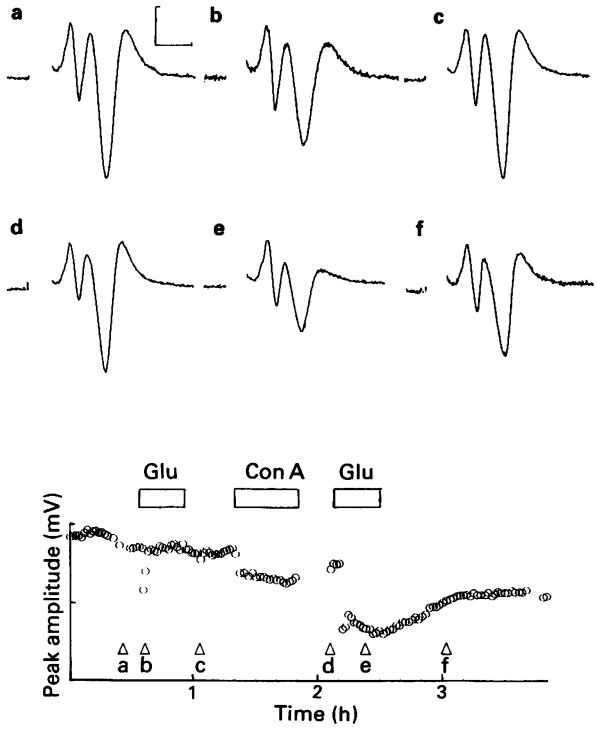


Figure 3 Effect of concanavalin A (Con A) on glutamate-induced depression of C-fibre compound action potential. The oscillographic traces (scale bar 0.25 mV, 10 ms) (a) to (f) were recorded at the points indicated below the time-course. L-Glutamate (Glu, 0.2 mM) and concanavalin A (1 mg ml^{-1}) were applied as indicated above the time-course. The preparation was superfused with glucose-free medium for 15 min before application of concanavalin A which was applied in glucose-free medium. The glutamate-induced depression was very brief but following concanavalin A treatment, glutamate caused a persistent depression.

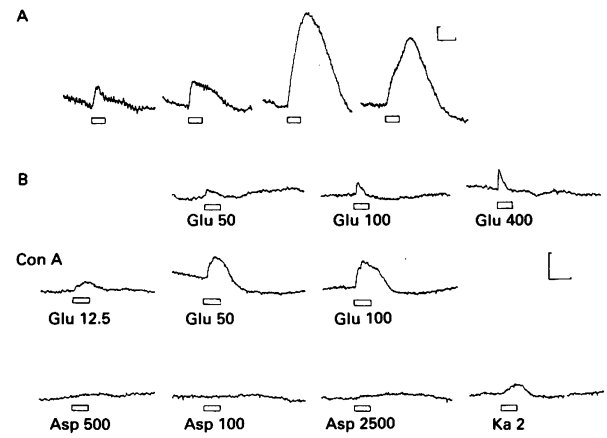


Figure 4 Effect of concanavalin A (Con A) treatment on the grease-gap recorded agonist-induced depolarization of dorsal root fibres. (A) Glutamate (200 μM) was applied as indicated by the bars below the traces. The lectin was applied at 1 mg ml^{-1} during the interval between the first and second trace and at 10 mg ml^{-1} between the second and third trace. The last trace was recorded 4 h after the third trace. (B) Glutamate (Glu) and aspartate (Asp) were applied as indicated (μM) below the traces. The second and third row of traces were obtained following 15 min incubation of the dorsal root in concanavalin A (10 mg ml^{-1}) containing medium. Fading responses to glutamate can be seen before the lectin was applied and the lectin-treated nerve failed to respond to aspartate but was still sensitive to low concentrations of kainate. Vertical scale bar 0.1 mV, horizontal scale bar 2 min.

ponding value for GABA-induced responses was 0.77 ± 0.15. The concentration-effect plots for AMPA, glutamate and quisqualate were shifted in a non-parallel manner following concanavalin A treatment. Consequently it was not possible to estimate mean values for the leftward shift. However, the effect of concanavalin A (illustrated in Figures 4 and 5) is characterized by the mean times taken for the responses to decay to half the peak depolarization from the start of an 85 s application of agonist. These values (s) for glutamate, quisqualate, AMPA and kainate were 63 ± 7 (10), 86 ± 17 (4), 95 ± 19 (4) and 135 ± 3 (12) s respectively. Following concanavalin A treatment these were increased to 202 ± 49 (10), 147 ± 7 (4), 160 ± 13 (6) and 163 ± 10 (10) respectively. As illustrated in Figure 5 depolarizations induced by γ-aminobutyric acid (GABA) in ten preparations were not significantly affected by concanavalin A treatment with the time to half decay being 145 ± 7 s under control conditions and 149 ± 12 s following treatment with concanavalin A.

It is possible that the potentiating effect of concanavalin A is produced through unmasking of fast desensitizing AMPA sites on C fibres. CNQX shows a five fold higher affinity for AMPA- than for kainate-binding sites (Sheardown *et al.*, 1990). Thus it is possible that a changed proportion of AMPA/kainate sites could be reflected by an alteration in potency of CNQX. Thus dose-ratios for the antagonism by CNQX (10 μM) of kainate-induced responses were measured before and after concanavalin A treatment. In four preparations the mean pA_2 value following concanavalin A treatment was 5.69 ± 0.10. This is not significantly different ($P > 0.3$) from the value above obtained in the absence of concanavalin A.

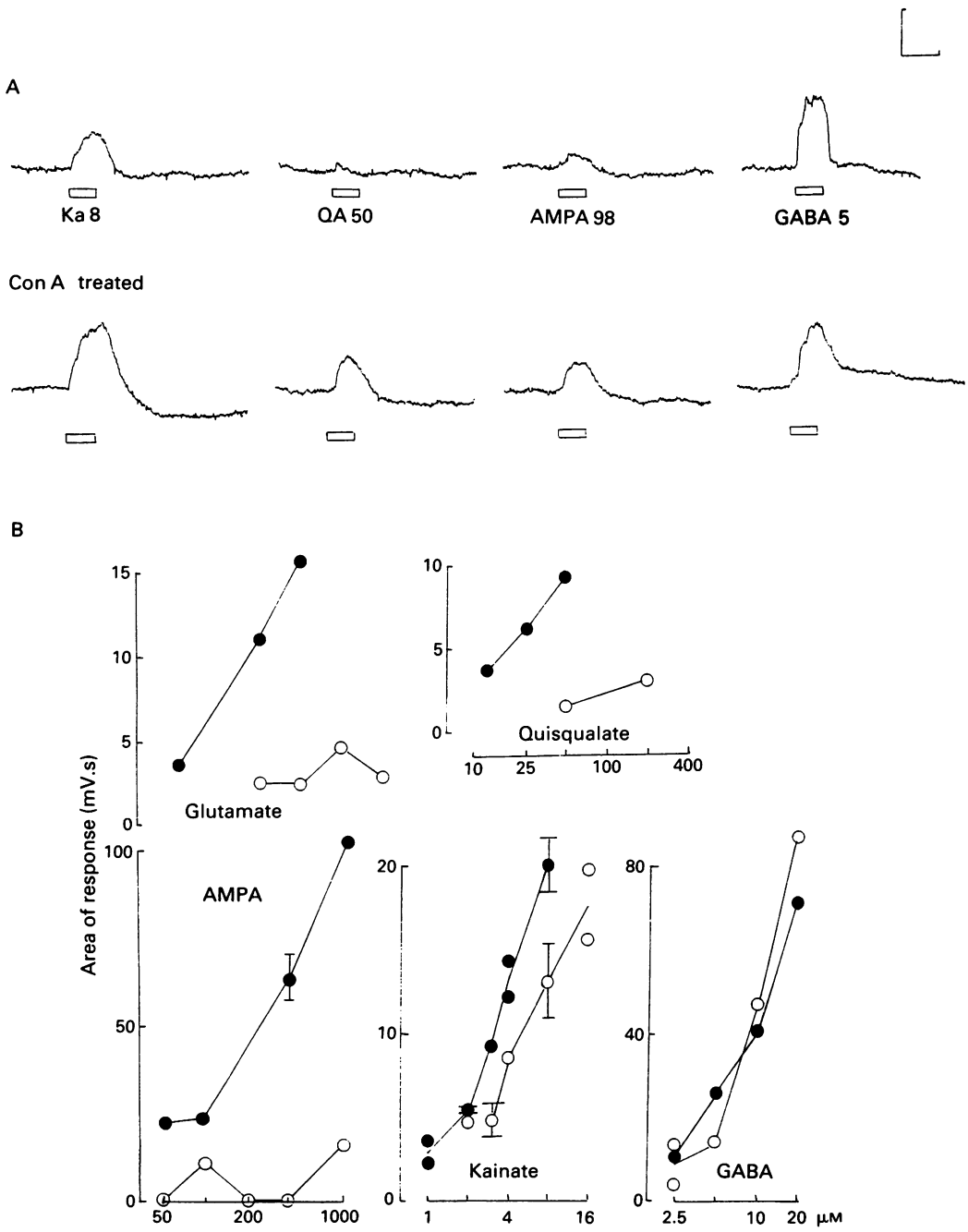


Figure 5 Effect of concanavalin A (Con A) treatment on agonist-induced depolarization of dorsal root fibres. The second row of traces was obtained following concanavalin A treatment. Integrated areas of the response profiles (mV s) are plotted in (B) against μmolar concentration of agonist. All except S-α-amino-3-hydroxy-5-methyl-4-inoxazole propionic acid (AMPA) are from the same preparation; (●) after concanavalin A treatment. Vertical bars are standard errors of three or four responses. In (A) the vertical scale bar 0.1 mV, horizontal scale bar 2 min. Other details as for Figure 4.

Discussion

The EC_{50} of 8 μM for kainate-induced depolarization of C fibres is consistent with previous studies (Agrawal & Evans, 1986; Evans *et al.*, 1987). The EC_{50} of 3 μM obtained for depression of the C elevation of the compound action potential in the present study is significantly lower than the former value, presumably because the action potential of individual C fibres is fully depressed at submaximal levels of depolarization.

It has been established in the present study that the lack of functional receptors for NMDA on primary afferent C neurones (Evans & Agrawal, 1986; Huettner, 1990) is unlikely to be due to absence of a co-agonist for the NMDA-linked glycine receptor. The absence of any effect of ACPD suggests also that C fibres either lack metabotropic glutamate receptors or that their action on the membrane electrical properties of C fibres induces no measurable change under the present experimental conditions.

The antagonism of kainate produced by CNQX, and by analogy that produced by NBQX, was probably competitive as suggested by the slope of Figure 2c. The plots of dose-ratio against concentration for the antagonism produced by AMPA, glutamate and quisqualate (Figure 2c) suggest a non-competitive antagonism of kainate by these ligands. The antagonism produced by quisqualate, AMPA and glutamate was likely to have been caused by receptor desensitization (Huettner, 1990). The potencies of quisqualate, AMPA and glutamate for occluding kainate-induced whole cell current responses at cultured striatal neurones (Tse *et al.*, 1991) were similar to those of the present study on C fibres. The desensitization induced by the non-NMDA receptor agonists was prevented following concanavalin A treatment (Huettner, 1990). Thus these agonists might be expected to lose their antagonistic properties following concanavalin A treatment. This possibility was not tested during the present experiments because of interference by the persistent depolarizing action which the agonists had after concanavalin A treatment (Figure 3).

There was, as illustrated in Figure 2 and as previously observed (Agrawal & Evans, 1986; Evans *et al.*, 1987), little evidence of fast desensitization caused by kainate on the present preparation. However, there was a very slow decrease in sensitivity to kainate which developed over periods of exposure greater than 60 min. Concanavalin A treatment produced a consistent two fold displacement of the kainate-induced concentration-effect plot which was independent of the period of exposure to kainate. Thus it is unlikely that reversal of the slow loss in sensitivity to kainate was responsible for the effect of concanavalin A on kainate-induced responses. This very slowly developing loss in sensitivity may have been due to rectification of the membrane current induced by kainate. The depolarizing effect of kainate and the other agonists on C fibres measured in the present study is likely to correspond to the steady state currents described by Huettner (1990) at dorsal root ganglion neurones. The rank order of potency of the agonists used in the present study is similar to that obtained in Huettner's study. However it can be estimated that glutamate and quisqualate were only four times less active than kainate in Huettner's experiments on dorsal root ganglion somata whereas these two compounds and AMPA were more than thirty fold less active than kainate in the present study on dorsal root fibres. The present values correspond closely to previous molar potency ratios estimated at immature dorsal roots (Agrawal & Evans, 1986; Ishida & Shinozaki, 1991). The latter studies were on preparations untreated with concanavalin A. Thus the more than twenty fold lower potency of AMPA relative to kainate at this site is unlikely to be a consequence of AMPA-induced desensitization.

The present effects of concanavalin A were consistent with the two to four fold increase in agonist-induced responses of cultured hippocampal neurones (Mayer & Vycklicky, 1989) and a two fold increase at dorsal root ganglion cells (Huettner, 1990). In the present study concanavalin A treatment distinguished between responses induced by glutamate, quisqualate or AMPA and induced by kainate because, following such treatment, the time-course of responses to the former three agonists was prolonged significantly. This effect may correspond to an effect of concanavalin A on the slower phase of desensitization described by Huettner (1990) since the present method of application of agonists did not allow resolution of processes with time constants in the millisecond range. It is possible that desensitization involves interaction with a binding site which is separate from that which mediates the depolarizing action of kainate. Or it may be, as others have proposed (Mayer & Vycklicky, 1989; Huettner, 1990), that AMP, quisqualate and glutamate have higher affinities for a desensitized form of the receptor analogous to the model proposed for desensitization at the nicotinic receptor (Katz & Thesleff, 1957). Presumably in concanavalin A-treated nerves, both forms of the receptor are expressed in the depolarizing response.

The present pA_2 of 5.84 for the antagonism of kainate-induced depolarization of C fibres by CNQX is similar to values obtained previously at motoneurones (5.8 from Long *et al.* (1990) and 5.7 calculated from the data of Ishida & Shinozaki (1991)). In the present study NBQX gave pA_2 values not significantly different from those above for antagonism of kainate-induced depolarization at motoneurones and C fibres. A similar value was obtained for the antagonism of kainate-induced responses of cerebrocortical neurones (Lodge *et al.*, 1991). Thus in these functional tests the two quinoxalinediones appear to have similar affinities for the kainate receptor. This contrasts with the six fold greater potency of NBQX relative to CNQX for the displacement of AMPA from cerebrocortical membranes (Sheardown *et al.*, 1990; Lodge *et al.*, 1991). Thus the present C fibre site represents a 'kainate' rather than AMPA subtype of non-NMDA receptor.

The selectivity of CNQX or NBQX for AMPA/kainate binding sites should allow the dose-ratio for antagonism to be used as an index for the relative proportions of AMPA and kainate sites involved in generation of responses. CNQX has a five fold higher affinity for AMPA- compared to kainate-binding sites (Sheardown *et al.*, 1990). In the present study, due to availability, CNQX rather than NBQX was used for this purpose in order to show if concanavalin A treatment caused any significant change in the receptors which generate the kainate-induced depolarization of C fibres. There was no significant difference between pA_2 values obtained before and after treatment with concanavalin A. This suggests that concanavalin A did not affect the interaction between CNQX and the non-NMDA receptors. CNQX has been found to cause a reduction in desensitization at non-NMDA receptors (Rossier *et al.*, 1991). Similar effects which cause a paradoxical enhancement of responses by antagonists have been observed at $GABA_A$ (Simmonds, 1983) and strychnine-sensitive glycine (Long *et al.*, 1989) receptors. The latter phenomenon would be expected to result in an increase in the potency of CNQX following concanavalin A treatment.

The use of domoate and AMPA as agonists revealed a degree of selectivity of NBQX (approximately thirty fold) which was not apparent with kainate and quisqualate (Long *et al.*, 1990; Watkins *et al.*, 1990). A similar but smaller difference has been observed with CNQX (Watkins *et al.*, 1990; Ishida & Shinozaki, 1991) and it suggests that domoate and AMPA are more selective agonists for sub-types of non-NMDA receptors than either kainate or quisqualate and that NBQX discriminates better between these receptors than does CNQX (Sheardown *et al.*, 1990). In particular the 'kainate' receptor appears to be characterized by high agonist sensitivity to domoate (Agrawal & Evans, 1986) and the weak activity of AMPA at C fibres is likely to be due to a relatively low affinity and/or efficacy of this agonist for

kainate receptors in this tissue. L-Aspartate and NMDA would appear to be devoid of affinity and efficacy at C fibre kainate receptors.

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Effect of bradykinin and prostaglandins on the release of calcitonin gene-related peptide-like immunoreactivity from the rat spinal cord *in vitro*

[1]L. Andreeva & [2]H.P. Rang

Sandoz Institute for Medical Research, Gower Place, London WC1E 6BN

1 The release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) from the dorsal horn of the rat spinal cord *in vitro* in response to dorsal root stimulation was measured by radioimmunoassay.

2 Stimulation of the dorsal roots (3 or 4 roots on each side) at 10 Hz for 5 min evoked a mean release (R_1) of 134.3 ± 17.5 ($n = 10$) fmol CGRP-LI; the release (R_2) evoked by a second stimulation period 30 min later under control conditions was 77 ± 10% ($n = 10$) of R_1. Test compounds were applied to the preparation following release R_1, and their effect calculated from the value of R_2/R_1.

3 Bradykinin (0.01–10 μM) had no significant effect on the basal release of CGRP-LI, but at 0.1–10 μM it increased 2–3 fold the release evoked by dorsal root stimulation.

4 This effect of bradykinin was prevented by indomethacin (10 μM), or by the B_2-receptor antagonist, Hoe140 (1–10 μM). In the presence of Hoe140, bradykinin significantly reduced R_2/R_1; the explanation for this is not clear.

5 The B_1-receptor agonist, Des-Arg^9-bradykinin (10 μM), did not affect CGRP-LI release nor was the effect of bradykinin blocked by the B_1-receptor antagonist, Des-Arg^9-Leu^8-bradykinin (10 μM).

6 Various prostaglandins were found to mimic the effect of bradykinin on CGRP-LI release. Their approximate order of potency was prostaglandin D_2 (PGD_2) = PGE_1 > $PGF_{2\alpha}$ = PGE_2; PGI_2 was ineffective at 10 μM.

7 Forskolin (30 μM) and 3-isobutyl 1-methylxanthine (IBMX; 10 μM) also increased the evoked release of CGRP-LI.

8 It is concluded that bradykinin acts on B_2-receptors in the spinal cord, causing the formation of prostanoids, which in turn cause an enhancement of neuropeptide release from primary afferent nerve terminals in the dorsal horn. This effect may be secondary to activation of adenylate cyclase. Because B_2-receptors are mainly associated with primary afferent nerve terminals, it is likely that prostanoid production is also a function of these structures. Whether this action of bradykinin has any physiological function in nociceptive transmission remains unclear.

Keywords: Rat spinal cord; CGRP release; bradykinin; B_2-receptors; prostaglandins; cyclic AMP

Introduction

Bradykinin is an inflammatory mediator, produced in tissues when kallikrein is activated. It exerts many peripheral effects, including vasodilatation, increased vascular permeability, smooth muscle contraction, stimulation of epithelial ion transport and excitation of nociceptive nerve terminals (see reviews by Regoli & Barabe, 1980, Proud & Kaplan, 1988).

The excitatory effect of bradykinin on peripheral nociceptive afferent terminals has been described by many authors (Regoli & Barabe, 1980; Braas *et al.*, 1988; Steranka *et al.*, 1988; Dray *et al.*, 1988). There is also some evidence to suggest that bradykinin may exert central effects. Thus the presence of both bradykinin-like immunoreactivity (Correa *et al.*, 1979; Perry & Snyder, 1984) and bradykinin binding sites (Steranka *et al.*, 1988; Fujiwara *et al.*, 1988; Sharif & Whiting, 1991; Privitera *et al.*, 1992), has been demonstrated in the mammalian central nervous system. Furthermore, bradykinin injected intrathecally in conscious rats affects nociceptive transmission in a complex way, causing an initial aversive response followed by an antinociceptive effect, which may be due to stimulation of catecholamine release from the terminals of inhibitory bulbospinal neurones in the dorsal horn. If this adrenergic component is blocked, bradykinin causes enhancement of nociceptive transmission (Laneuville *et al.*, 1989). Studies on the neonatal rat spinal cord *in vitro* (Dray *et al.*, 1988; Dunn & Rang, 1990) show that bradykinin causes depolarization of capsaicin-sensitive primary afferent nerve terminals in the dorsal horn, leading to synaptically-mediated depolarization of ventral horn neurones. These data suggest that bradykinin might affect spinal cord transmission by evoking release of neuropeptides from primary afferent terminals.

The purpose of the present study was to test this directly by measuring the effect of bradykinin on the release of a neuropeptide, calcitonin gene-related peptide (CGRP), which is believed to facilitate nociceptive transmission (Kuraishi *et al.*, 1988) in the rat spinal cord. Contrary to our expectations, bradykinin was not effective in evoking the release of CGRP-like immunoreactivity (CGRP-LI) directly, but produced a marked increase in the amount of peptide released by stimulation of the dorsal roots. This effect of bradykinin is mediated by B_2 receptors, depends on the release of prostaglandins, and may be mediated by adenosine 3′:5′-cyclic monophosphate (cyclic-AMP).

[1] Present address: Department of Biochemistry, University College London, Gower Street, London, WC1E 6BT.
[2] Author for correspondence.

Methods

Experimental protocol

Sprague-Dawley rats of either sex, 2–4 weeks old were killed by halothane inhalation, and the spinal cords were dissected with attached dorsal roots. The spinal cords were placed in ice-cold physiological salt solution (Krebs solution), containing: (mM): NaCl 119, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ $7H_2O$ 1.2, $NaHCO_3$ 2.5, $CaCl_2$ 2.5 and D-glucose 11. The Krebs solution was bubbled with 95% O_2:5% CO_2. The cords were cleared of connective tissue and a horizontal slice comprising approximately the dorsal third of the cord was cut from the lumbar region with a vibratome. The slice was pinned out, ventral surface uppermost, in a rectangular chamber (approx 0.5 ml) moulded from Sylgard resin. Three or 4 dorsal roots on each side were laid on platinum wire stimulating electrodes in side-compartments of the main perfusion chamber, so that the 6–8 intact roots were stimulated simultaneously. Silicone grease was used to cover the dorsal roots and keep them in contact with the stimulating electrodes, and the roof of the chamber was sealed with a plastic lid. The chamber was perfused at a rate of 1 ml min^{-1} with oxygenated Krebs solution at 37°C containing 0.1% bovine serum albumin, which improved the recovery of CGRP-LI, presumably by reducing its adsorption to the tubing. After equilibration for about 1 h, collection of 5 min fractions was begun. The collection tubes contained 50 µl glacial acetic acid to give 0.5 M final concentration. Control high performance liquid chromatography (h.p.l.c.) experiments showed that this concentration of acetic acid prevented peptide degradation. No peptidase inhibitors were used. Stimulation at 10 Hz for 5 min was used routinely to assess drug effects. Preliminary experiments showed that the use of lower stimulation frequencies (1 or 3 Hz) gave small and variable responses.

After an initial 1 h equilibration period, the basal level of CGRP-LI release was determined for 15 min, (Samples 1–3). The dorsal roots were then stimulated (10 Hz, 20 V, 1 ms pulses) for 5 min during collection of Sample 4. After a 15 min recovery period (Samples 5–7), the test drug was added to the perfusion medium, and after a further 20 min (Samples 8–11), the dorsal roots were stimulated for 5 min (Sample 12) in the presence of the test drug. The run was terminated after Sample 15. In experiments designed to determine the effect of inhibitors, such as indomethacin or bradykinin receptor antagonists, on the response to bradykinin, the inhibitors were present throughout the experimental run.

Sample preparation and radioimmunoassay

Each perfusate fraction was purified and concentrated on 100 mg Sep-Pak C_{18} cartridges (Waters) as described by Saria *et al.* (1988). Before each run the cartridges were conditioned with 1 ml of methanol, and washed with 3 ml of TFA-saline (0.1% trifluoracetic acid in 60 mM NaCl). The perfusate fraction (5 ml) was then applied to the cartridge, which was washed first with 2 ml TFA-saline, then with 2 ml of 60% TFA-saline:40% methanol. The CGRP was eluted with 0.5 ml methanol. The methanol extracts were dried under nitrogen, and the samples reconstituted in 0.5 ml radioimmunoassay (RIA) buffer, containing (mM): sodium phosphate buffer (pH 7.0) 60; sodium azide 7.7; di-sodium EDTA 9.0 and bovine serum albumin 1 mg ml^{-1}.

Control experiments with [^{125}I]-CGRP showed that this procedure extracted approximately 85% of the CGRP in the sample. The extraction procedure was done automatically on an ASPEC sample preparation machine (Gilson). In control experiments we found that this procedure resulted in the recovery of 60.6 ± 3.6% ($n = 9$) of CGRP added to the perfusion chamber. We made no correction for this.

Radioimmunoassay for CGRP-LI was carried out by the scintillation proximity bead technique (Amersham). The assay mixture contained: concentrated sample (100 µl; antibody solution (50 µl); labelled CGRP (50 µl); suspension of scintillation proximity beads (50 µl). These components were placed in a 96-well plastic tray designed for counting in a Betaplate 1205 scintillation counter (Pharmacia). All samples were run in duplicate. The trays were sealed and placed on a rotary shaker for 24–48 h at room temperature before being counted. With this assay system, the Protein-A-coated beads, which contain the scintillant, specifically bind the antibody, bringing the antibody-bound labelled CGRP, but not the free labelled CGRP, in direct contact with the scintillant, thus making a physical separation step unnecessary.

A standard curve was run with each batch of assays. Non-radioactive CGRP was made up in a series of dilutions in RIA buffer, usually ranging from 30 pmol l^{-1} to 3 nmol l^{-1}, 100 µl being added to each standard well in place of the sample. The sensitivity limit for the assay (10% displacement) was 1–3 fmol/sample.

Holzer *et al.* (1990) have shown that, with a similar extraction procedure and the same antibody that we used, the immunoreactivity of samples derived from rat stomach perfusates behaves on h.p.l.c. as a single peak coeluting with human CGRP.

The readout from the counter was stored on a computer disc and analysed by means of a spreadsheet programme (Excel) and a curve-fitting programme (Grafit) running under Microsoft Windows, the mean count rate for the duplicate assay wells being converted to an estimate of CGRP-LI by reference to the fitted standard curve.

Calculation and statistical analysis

Of the total evoked release 88–90% was collected in the three 5 min samples collected during and immediately after the stimulation period (Figure 1). For the purposes of calculation, the evoked release, R, was expressed as the total CGRP-LI collected in these three samples, corrected for the basal release averaged from the samples immediately before and after the release period.

For each experimental run, the release (R_2) evoked by the second stimulation was expressed relative to that evoked by the first stimulation (R_1). Under control conditions with no test drug (Figure 1) R_2/R_1 was 0.77 ± 0.10 (mean ± s.e.mean; $n = 10$). Trials of test drugs were made on 3–6 preparations, and the mean value of R_2/R_1 compared with the control value by means of an unpaired t test.

Drugs and materials

Drugs and chemicals were obtained from the following sources: methanol and trifluoracetic acid (Romil Chemicals, h.p.l.c. grade); Rabbit anti-CGRP (human) serum (Peninsula Laboratories, Inc); 2[^{125}I]iodohistidyl^{10}-CGRP (human; ~2000 Ci $mmol^{-1}$; Amersham); protein-A-coated scintillation proximity beads (Amersham) suspended in RIA buffer, as directed; bradykinin (BK), Des-Arg^9-bradykinin, Leu^8,Des-Arg^9-bradykinin (Bachem); bovine serum albumin, forskolin, indomethacin, 3-isobutyl 1-methylxanthine (IBMX), prostaglandin E_1 (PGE_1), PGE_2, PGI_2, $PGF_{2\alpha}$, PGD_2 (Sigma). Hoe140 (D-Arg-Arg-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg (Thi = L-3-(2-thienyl)alanine; D-Tic = D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Oic = L-(3aS,7aS)-octahydroindol-2-carboxylic acid)) was synthesized by Dr A. Hallett, Sandoz Institute for Medical Research. The drugs were made up as stock solutions in distilled water, and stored frozen, fresh dilutions being prepared for each experiment.

For the radioimmunoassay, the antibody (standard vial) was diluted to 50 ml in 0.1% Triton X-100, and stored frozen in aliquots. The labelled CGRP was diluted in RIA buffer to give about 10,000 d.p.m. in 50 µl.

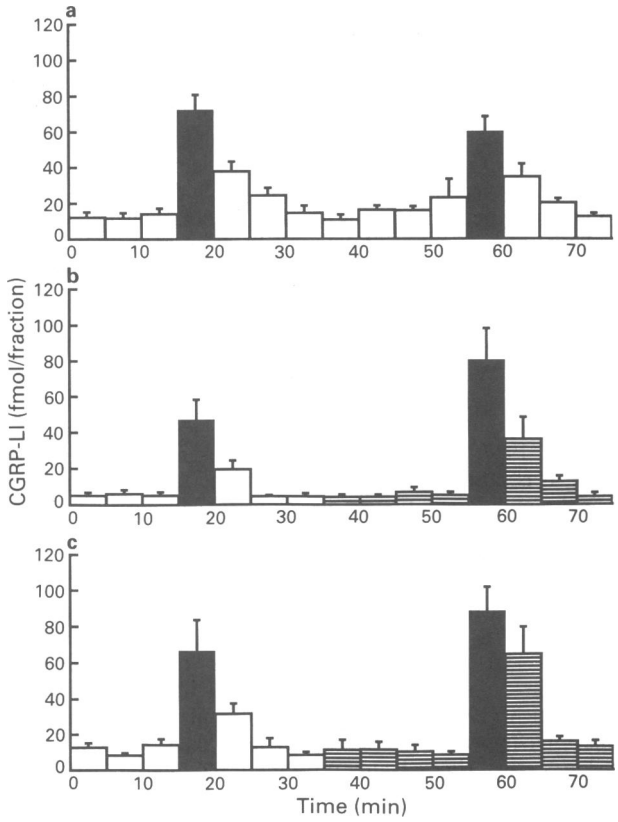


Figure 1 Release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) from slices of rat spinal cord in response to dorsal root stimulation. The CGRP-LI content (uncorrected for % recovery) of successive 5 min fractions of perfusate is shown. At 15–20 min, and at 55–60 min (solid columns), the dorsal roots were stimulated (20 V, 1 ms pulses at 10 Hz) for the 5 min collection period. (a) Control experiments in normal Krebs solution, $n = 10$; (b) bradykinin (10 μM) added after 7th sample (hatched columns, $n = 6$); (c) prostaglandin E_2 (PGE_2, 10 μM) added after 7th sample (hatched columns, $n = 5$). Columns show mean values with s.e.mean indicated by vertical bars.

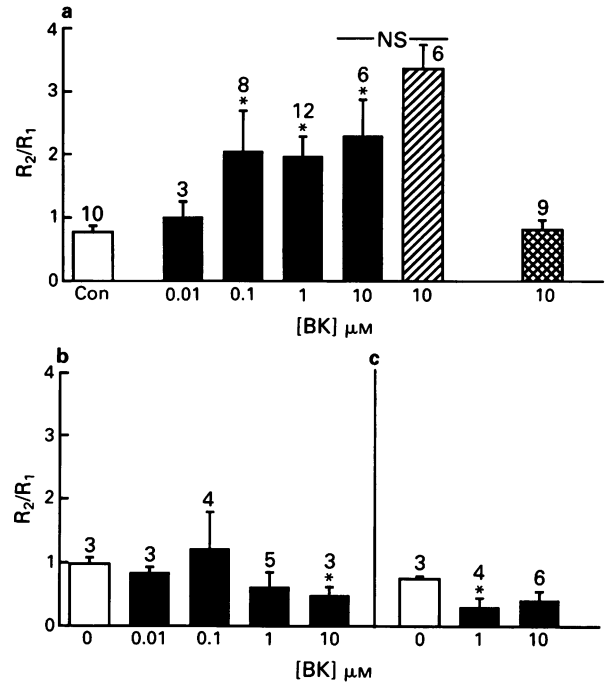


Figure 2 The effect of bradykinin, related peptides and indomethacin on the evoked release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI). The ratio shown (R_2/R_1) is a measure of the effect of the test compound. Numbers over each column are the number of measurements. The open columns show results obtained in the absence of bradykinin. The solid columns show the effect of various concentrations of bradykinin applied to the preparation before the second stimulation period. Columns marked with an asterisk indicate a significant ($P < 0.05$) difference from the control value (open columns). (a) Effect of increasing concentrations of bradykinin (solid columns); effect of bradykinin (10 μM) in the presence of the B_1-receptor antagonist Des-Arg^9,Leu^8-bradykinin (10 μM) (diagonal-hatched column); effect of the B_1-receptor agonist, Des-Arg^9-bradykinin (10 μM), (cross-hatched column). (b) Effect of indomethacin (10 μM) and (c) the B_2-receptor antagonist, Hoe140, 10 μM, on the response to bradykinin. The inhibitor, indomethacin or Hoe140, was present throughout the experiment, the controls (open columns) being the ratio R_2/R_1 measured in the presence of the inhibitor, but without bradykinin addition. Columns show mean values with s.e.mean indicated by vertical bars.

Results

The stimulation-evoked release of CGRP-LI declined to the basal level in about 15 min (Figure 1). In normal Krebs solution the mean total release evoked by R_1 was 134 ± 17.5 fmol ($n = 10$), and the ratio R_2/R_1 was 0.77 ± 0.10 ($n = 10$).

Addition of bradykinin (0.01–10 μM) after R_1 produced no significant change in the basal release of CGRP-LI, but significantly increased the evoked release (Figures 1 and 2). This effect was maximal ($R_2/R_1 = 2.05 \pm 0.64$; $n = 8$) at 0.1 μM bradykinin The B_1-receptor agonist, Des-Arg^9-bradykinin, tested at 10 μM, had no effect on CGRP-LI release. Furthermore, the B_1-receptor antagonist, Des-Arg^9,Leu^8-bradykinin (10 μM) did not reduce the response to bradykinin (Figure 2), suggesting that the effect of bradykinin was not mediated through B_1-receptors. The selective B_2-receptor antagonist, Hoe140 (Hock *et al.*, 1991), on the other hand, tested at 1 or 10 μM, prevented the enhancement of CGRP-LI release by bradykinin (Figure 2). Indeed, in the presence of Hoe140 (10 μM), addition of bradykinin significantly reduced R_2/R_1 below the value measured in the presence of Hoe140 alone (Figure 2).

Indomethacin (10 μM) on its own did not affect the resting or evoked release of CGRP-LI, but abolished the action of bradykinin (Figure 2). This suggested that eicosanoid production might be responsible for the bradykinin-evoked enhancement of CGRP-LI release, so we tested various prostaglandins for their ability to mimic the effect of bradykinin. In the presence of indomethacin, as with Hoe140, 10 μM bradykinin significantly reduced the evoked release of CGRP-LI.

PGD_2 and PGE_1 (0.1–10 μM) enhanced CGRP-LI release in a similar way to bradykinin (Figure 3). PGD_2 was more effective than PGE_1 at 0.1 μM, but less effective than PGE_1 at 1 μM, so a simple potency comparison was not possible. Both agents were, however, clearly more potent than PGE_2 or $PGF_{2\alpha}$, which were effective only at 10 μM (Figure 3). PGI_2 (1 μM) was ineffective. No effect on the resting release of CGRP-LI was seen with any of the prostaglandins.

Since many effects of prostanoids are mediated through activation of adenylate cyclase (see Halushka *et al.*, 1989), we tested the effects of forskolin (30 μM), which activates adenylate cyclase directly, and the phosphodiesterase inhibitor, IBMX (10 μM). Neither of these agents directly elicited any release of CGRP-LI, but both enhanced the evoked release of CGRP-LI to a similar extent to that seen with bradykinin and prostanoids (Figure 3).

Discussion

In this study we have shown that bradykinin and various prostaglandins enhanced the release of CGRP-LI evoked by stimulation of the dorsal roots. The stimulation-frequency

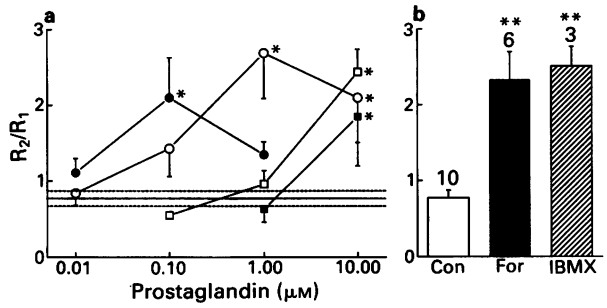


Figure 3 (a) Effects of various prostaglandins on calcitonin gene-related peptide (CGRP) release from the spinal cord, expressed as the ratio R_2/R_1 ($n = 3$ to 5). The horizontal lines show the control value of R_2/R_1 (mean ± s.e.mean; $n = 10$). The symbols are: (●) prostaglandin D_2 (PGD_2); (○) PGE_1; (■) PGE_2; (□)$PGF_{2\alpha}$. Symbols show mean values with s.e.mean indicated by vertical bars. (b) Effect of forskolin, 30 μM (For; $n = 6$) and IBMX, 10 μM ($n = 3$), compared with controls (Con; $n = 10$). $^*P < 0.01$; $^{**}P < 0.01$.

needed to evoke a readily measurable release of CGRP-LI (10 Hz) is in the upper range of the physiological discharge frequency of nociceptive afferent fibres (see Campbell *et al.*, 1989), and is consistent with findings reported in other studies of peptide release (Otsuka & Konishi, 1976; Saria *et al.*, 1988; Santicolli *et al.*, 1992). CGRP in the dorsal horn is contained exclusively in primary afferent nerve terminals (Chung *et al.*, 1988), so the enhanced release must reflect an action on these terminals. Though indirect effects of bradykinin and prostaglandins acting on interneurones which synapse on primary afferent terminals cannot be ruled out, it is most likely that these substances act directly on the terminals, which are known to possess bradykinin receptors (Steranka *et al.*, 1988). Suprisingly, since bradykinin causes depolarization of capsaicin-sensitive primary afferent nerve terminals in the dorsal horn (Dunn & Rang, 1990), it did not by itself evoke any measurable release of CGRP-LI. In contrast, both bradykinin and prostanoids have been shown to evoke neuropeptide release from sensory nerve terminals in various peripheral tissues (Amann *et al.*, 1989; Franco-Cereceda *et al.*, 1989; Geppetti *et al.*, 1990a,b; 1991; Mapp *et al.*, 1991).

Involvement of B_2-receptors

The selective B_1-receptor agonist, Des-Arg^9-bradykinin (see Regoli & Barabe, 1980) did not mimic the effect of bradykinin, nor did the selective B_1-receptor antagonist, Des-Arg^9-,Leu^8-bradykinin inhibit it. On the other hand, the B_2-receptor antagonist, Hoe140 (Hock *et al.*, 1991) inhibited the action of bradykinin, indicating that the facilitation of CGRP-LI release is mediated by B_2-receptors, as are all of the hitherto reported actions of bradykinin on sensory neurones (see Steranka *et al.*, 1988; Dray *et al.*, 1988; Burgess *et al.*, 1989a; Dunn & Rang, 1990). A surprising finding was that, in the presence of Hoe140, bradykinin significantly inhibited the evoked release of CGRP-LI. Since no inhibition was seen with Des-Arg^9-bradykinin, it is unlikely that B_1-receptor activation can account for this. The cause of this inhibitory action of bradykinin remains unclear at present.

Involvement of prostaglandins

Indomethacin completely inhibited the increase of CGRP-LI release produced by bradykinin. Other studies have similarly shown that indomethacin inhibited the action of bradykinin on sensory neurones (Juan & Lembeck, 1974; Weinreich, 1986; Manzini *et al.*, 1989; Geppetti *et al.*, 1990a; 1991) and that bradykinin, acting via B_2-receptors, evoked the release of prostaglandins from various tissues and cell lines (Lembeck *et al.*, 1976; Sametz & Juan, 1982; Bareis *et al.*, 1983; Gammon *et al.*, 1989). The effect of indomethacin in our studies, and the ability of various prostaglandins to mimic the action of bradykinin, suggests that the effect of bradykinin was secondary to prostaglandin release. The origin of the prostaglandins could be the sensory neurone terminals themselves, or other neurones or glial cells. The fact that bradykinin receptors are confined to the superficial laminae of the dorsal horn (Steranka *et al.*, 1988) suggests that bradykinin may act only on the sensory neurones. Furthermore, Gammon *et al.* (1989) showed that bradykinin stimulated arachidonic acid release from sensory neurones in culture, free of non-neuronal cells. In our limited investigation PGD_2 and PGE_1 were the most active prostaglandins, roughly 100 fold more potent than PGE_2 or $PGF_{2\alpha}$; PGI_2 was inactive. Other comparative studies of the effects of prostaglandins on sensory neurones (e.g. Moncada *et al.*, 1975; Coleridge *et al.*, 1976; Longhurst & Dittman, 1987; Weinreich & Wonderlin, 1987; Mizumura *et al.*, 1991) show a broadly consistent pattern, the main difference being that PGI_2 has been found to be active in some tests (Longhurst & Dittman, 1987; Mizumura *et al.*, 1991) in contrast to our results. It is possible that differences in the rate of degradation of PGI_2 in different tissues accounts for this.

Mechanism of enhanced CGRP-LI release

The biochemical and electrophysiological processes linking the activation of B_2-receptors to enhanced transmitter release are not yet clear from published reports on the effects of bradykinin on neurones and related cell lines.

Weinreich (1986) showed that bradykinin inhibits the slow spike after-hyperpolarization (sAHP) in vagal afferent C-fibre neurones, this effect being prevented by indomethacin, and mimicked by various agents that activate adenylate cyclase, such as PGD_2, PGE_2, histamine and forskolin. Inhibition of the sAHP has the effect of allowing the cell to fire at a higher frequency than it can normally follow. Our experiments were carried out at a stimulation frequency of 10 Hz. Since stimulation at lower frequencies evoked much less CGRP-LI release, we conclude that the C-fibres were able to follow at 10 Hz in the absence of bradykinin, so inhibition of a sAHP cannot account for the effect of bradykinin.

Other studies on the cellular actions of bradykinin on sensory neurones, which have focused mainly on the role of inositol phosphates, calcium and protein kinase C in neuronal cell cultures (see Thayer *et al.*, 1988; Burgess *et al.*, 1989a; Gammon *et al.*, 1989; Ewald *et al.*, 1989; Boland *et al.*, 1991), do not provide a ready explanation for the effect described here. At the membrane level, bradykinin causes depolarization, associated with increased sodium conductance, which is partly due to activation of protein kinase-C (Burgess *et al.*, 1989a); it has also been reported to inhibit voltage-activated calcium currents, which has also been ascribed to protein kinase C (Ewald *et al.*, 1989; Boland *et al.*, 1991), which would be expected to reduce, rather than increase, the evoked release of peptides. However, in neuronal cultures, bradykinin does not lead to an increase of cyclic AMP (Burgess *et al.*, 1989b), possibly because the cells responsible for prostanoid production in tissues are absent from the cultures, so the calcium current inhibition reported in cultured neurones may not reflect what happens in intact tissues. A rise in intracellular calcium occurs in the soma and dendrites of sensory neurones in culture in response to bradykinin, mainly due to the release of intracellular calcium secondary to inositol tris-phosphate production (Thayer *et al.*, 1988; Naruse *et al.*, 1992); if this occurs also in the central terminals of neurones *in situ*, it is presumably insufficient to evoke neuropeptide release directly, but might bring the intracellular calcium concentration closer to the release threshold, and thus enhance the stimulation-evoked release.

Our results with forskolin and IBMX suggest that increased cyclic AMP was able to mimic the effect of bradykinin, and this mechanism could account for the action of bradykinin. Increased cyclic AMP formation is implicated in many of the actions of prostaglandins (see Halushka *et al.*, 1989), but the nature of the linkage between intracellular cyclic AMP and

peptide release remains unclear. Dolphin *et al.* (1989) reported that increased intracellular cyclic AMP caused an increase of the sustained voltage-activated calcium current in rat sensory neurones, and Docherty *et al.* (1989) reported a similar effect with PGD_2. On the other hand, Gross & MacDonald (1989) found that agents raising intracellular cyclic AMP reduce the transient N-type calcium current in mouse sensory neurones, and there is evidence that this, rather than the sustained current, is responsible for the calcium entry associated with peptide release (Lou *et al.*, 1991; Santicolli *et al.*, 1992). Correlated studies of peptide release and calcium currents in cultured neurones would help to resolve these discrepancies.

Recent studies on peripheral mechanisms of hyperalgesia (Taiwo *et al.*, 1989; Taiwo & Levine, 1991) have implicated a rise in intracellular cyclic AMP as a factor in the sensitization of peripheral nociceptive neurones, but the ionic mechanism underlying this is not known. Enhancement of neuropeptide release in the periphery, leading to increased neurogenic inflammation, could be a factor.

There is so far no evidence to suggest that the effect of bradykinin in the spinal cord is involved in the physiological modulation of pain transmission, though this is clearly a possibilty. The antinociceptive effect of nonsteroidal anti-inflammatory drugs given intrathecally (see Taiwo & Levine, 1988) suggests a role for endogenous prostaglandins in the dorsal horn. Studies on the effect of bradykinin antagonists administered intrathecally may be expected to provide further evidence on the possible role of bradykinin.

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Lack of effect of sumatriptan and UK-14,304 on capsaicin-induced relaxation of guinea-pig isolated basilar artery

[1]C.T. O'Shaughnessy, G.J. Waldron & H.E. Connor

Department of Neuropharmacology, Glaxo Group Research Ltd, Park Road, Ware, Herts, SG12 0DP

1 The objectives of this study were to assess the effects of sensory neuropeptide antagonists and presynaptically acting receptor agonists on capsaicin-induced relaxations of guinea-pig isolated basilar artery (GPBA).

2 Capsaicin, human α-calcitonin gene-related peptide (CGRP) and substance P (SP) caused concentration-related relaxations of GPBA which had been pre-contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Responses to capsaicin were not modified by the peptidase inhibitors, phosphoramidon (1 μM) and bestatin (100 μM).

3 The relaxant responses to capsaicin were blocked in a selective manner by ruthenium red (3 μM) and by the CGRP antagonist, $CGRP_{8-37}$ (1 μM). $CGRP_{8-37}$ also selectively inhibited the relaxant effects of CGRP.

4 The selective NK_1 receptor antagonist, GR82334 (10 μM), inhibited SP-induced relaxations but had little effect on capsaicin-induced relaxations.

5 The 5-HT_1 receptor agonist, sumatriptan, produced small contractions of GPBA under conditions of resting tone. In the presence of $PGF_{2\alpha}$, sumatriptan had no further contractile effect. Sumatriptan (0.3 and 3 μM) did not modify capsaicin-induced relaxations of GPBA.

6 The α_2-adrenoceptor agonist, UK-14,304 (0.1 μM), had no effect on basal or $PGF_{2\alpha}$-induced tone. UK-14,304 did not modify capsaicin-induced relaxations.

7 These results suggest that capsaicin causes relaxation of GPBA via a release of CGRP. This process is amenable to blockade by $CGRP_{8-37}$ and ruthenium red, but not to modulation by either sumatriptan or UK-14,304.

Keywords: Basilar artery of guinea-pig; sumatriptan; α_2-adrenoceptor agonist; calcitonin gene-related peptide; substance P

Introduction

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) releases substance P (SP) and calcitonin gene-related peptide (CGRP) from trigeminal sensory nerves resulting in intracranial vasodilatation (Edvinsson *et al.*, 1990; Jansen *et al.*, 1990) and plasma protein extravasation (Buzzi & Moskowitz, 1990). Sterile neurogenic inflammation in dural blood vessels may contribute to migraine headache (Moskowitz *et al.*, 1989). The selective 5-HT_1 receptor agonist, sumatriptan, is a novel, highly effective treatment for migraine headache (Cady *et al.*, 1991; Ensink, 1991; Ferrari, 1991). Its efficacy has been attributed to its ability to constrict selectively dilated, oedematous intracranial blood vessels via activation of 5-HT_1 receptors on the vascular smooth muscle (Humphrey *et al.*, 1990; Humphrey & Feniuk, 1991). Alternatively, or additionally, sumatriptan may directly inhibit the release of sensory peptides from trigeminal sensory nerve endings innervating cranial blood vessels, thus blocking the development of vasodilatation and oedema (Buzzi & Moskowitz, 1990).

Sumatriptan and the selective α_2-adrenoceptor agonist, UK-14,304 (Cambridge, 1981), inhibit plasma protein extravasation in dura following either electrical stimulation of the trigeminal ganglion or intravenous administration of capsaicin. It has been suggested that the mechanism of this inhibition is a prejunctionally-mediated inhibition of SP and CGRP release from trigeminal sensory nerve endings (Buzzi & Moskowitz, 1990; Moskowitz, personal communication). In order to investigate further presynaptic mechanisms which may modulate sensory neurotransmission in the cranial vasculature, we have studied the effect of sumatriptan and UK-14,304 *in vitro*, using the responses to capsaicin in the guinea-pig isolated basilar artery (GPBA) as a model of trigeminal sensory nerve activation. Capsaicin induces relaxation in the pre-contracted GPBA, which has been suggested to be due to neuropeptide release from trigeminal sensory afferent nerves (Jansen *et al.*, 1990). A preliminary account of some of these findings has been presented to the British Pharmacological Society (Waldron *et al.*, 1992).

Methods

Male Dunkin-Hartley guinea-pigs (300–400 g) were exsanguinated under pentobarbitone anaesthesia and their brains removed. Basilar arteries were dissected free under a microscope and placed in oxygenated Krebs-Henseleit solution (Apperley *et al.*, 1976). Two artery segments (2–3 mm) were obtained from each animal and these were paired, one segment acting as the control preparation for the other. The segments were mounted between two parallel L-shaped wires (100 μM diameter) in 10 ml tissue baths at 37°C under a resting tension of 0.2 g for measurement of isometric tension changes. Tissues were allowed to stabilize under this tone for 90 min.

Effects of neuropeptide agonists and capsaicin

After the 90 min equilibration period, tissues were exposed to a submaximal concentration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1–30 μM); this produced an increase in tone of 0.1–0.3 g (equivalent to about 70% of the maximum concentration to $PGF_{2\alpha}$) which was well-maintained over 15–20 min. Capsaicin, SP or human α-CGRP (0.1–100 nM) were applied in cumulative concentrations to the pre-contracted vessels, until

[1] Author for correspondence.

a maximum relaxant response was obtained. Only one concentration-response curve to capsaicin, SP or CGRP was constructed in each tissue.

Effect of ruthenium red and neuropeptide antagonists

Tissues were incubated for 10 min in the presence of ruthenium red (3 μM), the selective neurokinin NK_1 receptor antagonist, GR82334 (10 μM), or the CGRP antagonist, $CGRP_{8-37}$ (1 μM), before contraction was induced with $PGF_{2\alpha}$. Capsaicin, SP or CGRP were then applied cumulatively.

Tissues from the same animal were paired; a single concentration of ruthenium red or neuropeptide antagonist was added to one of the pair, whilst the other served as the time-matched control.

Effect of presynaptically acting agonists

Two protocols were used to test the effects of sumatriptan and UK-14,304. In initial experiments, sumatriptan (0.3–3 μM) was added to the isolated arteries under conditions of resting tone (as above). As small contractile effects of sumatriptan were observed under these conditions, in subsequent experiments sumatriptan was added to the isolated tissues after induction of stable tone by $PGF_{2\alpha}$. Ten minutes later a cumulative concentration-response curve to capsaicin was constructed.

UK-14,304 (0.1 μM) was initially added to GPBA before the tissues were contracted with $PGF_{2\alpha}$ and 10 min before exposure to capsaicin (as for the neuropeptide antagonists). In subsequent experiments, UK-14,304 was added to tissues pre-contracted with $PGF_{2\alpha}$ (as for sumatriptan).

Data analysis

Relaxant responses are presented as percentage maximum relaxation (estimated at the end of each experiment with 200 μM papaverine). Mean pD_2 values (± s.e.mean) were determined to assess agonist potency. Antagonist potency, estimated at the agonist pD_2 level, was expressed in terms of apparent pK_B values (± s.e.mean) calculated from individual shifts in paired preparations, assuming competitive antagonism. Student's paired t test was used for single comparisons between mean values. The level of significance was set at $P<0.05$.

Drugs and solutions

The following were used: acetylcholine, papaverine, ruthenium red, capsaicin, phosphoramidon, bestatin (all Sigma), substance P, human α-calcitonin gene-related peptide (CGRP), human α-$CGRP_{8-37}$ (Peninsula), prostaglandin $F_{2\alpha}$ (dinoprost tromethamine, Upjohn), UK-14,304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline, Pfizer, UK). GR82334 ([D-Pro^9 [Spiro-γ-lactam] Leu^{10}, Trp^{11}]PHYS(1-11)) and sumatriptan were synthesized by the Medicinal Chemistry Department, Glaxo Group Research, Ware. Stock solutions of peptides and peptide antagonists (approximately 1 mM) were prepared in 1 mM acetic acid and frozen until use. Capsaicin was prepared freshly in absolute ethanol at 10 mM. All drug dilutions were made in Krebs-Henseleit solution and appropriate solvent controls were tested in parallel. All concentrations quoted below are the final molar concentration in the tissue bath during the experiments.

Results

Effects of neuropeptide agonists and capsaicin

Capsaicin, SP and CGRP produced concentration-related relaxations of pre-contracted GPBA (Figure 1a). Capsaicin

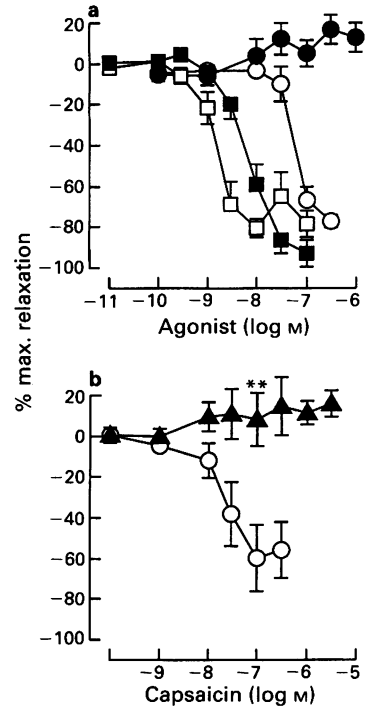


Figure 1 (a) Concentration-effect curves to capsaicin (1st application ○; 2nd application 60 min later ●), substance P (■) and calcitonin gene-related peptide (□) in guinea-pig basilar artery pre-contracted with prostaglandin $F_{2\alpha}$ (1–30 μM). Responses are expressed as % papaverine (200 μM)-induced relaxation. Values are means with s.e.mean shown by vertical bars, $n=6-14$. (b) Concentration-effect curves to capsaicin in the absence (○) and presence (▲) of ruthenium red, $n=6$. $^{**}P<0.01$, (treated versus control).

vehicle (0.003% ethanol) had no effect. A second exposure of GPBA to capsaicin resulted in no relaxation, indicating that desensitization had occurred (Figure 1a). The peptidase inhibitors, phosphoramidon (1 μM) and bestatin (100 μM) did not modify responses to capsaicin (pD_2 in the absence and presence of peptidase inhibitors: 7.4 ± 0.1; 7.5 ± 0.1 respectively, $P\geqslant 0.05$, $n=4$).

Effect of ruthenium red and neuropeptide antagonists

Ruthenium red (3 μM) had no effect on basal or $PGF_{2\alpha}$-induced tone, but blocked the relaxant effect of capsaicin on GPBA (Figure 1b). The response to CGRP was unaffected by ruthenium red pretreatment (pD_2; CGRP alone = 8.1 ± 0.3; CGRP + ruthenium red = 8.2 ± 0.2, $n=4$, $P\geqslant 0.05$). The response to SP was also unaffected by ruthenium red pretreatment (pD_2; SP alone = 8.3 ± 0.1; SP + ruthenium red = 8.6 ± 0.0, $n=3$, $P\geqslant 0.05$).

$CGRP_{8-37}$ (1 μM) had no effect on basal tone and it had no significant effect on the subsequent $PGF_{2\alpha}$-induced tone (105.4 ± 29.2 mg and 95.0 ± 26.7 mg in the absence and presence of $CGRP_{8-37}$, 1 μM, $n=5$, respectively). $CGRP_{8-37}$ (1 μM) blocked the effects of capsaicin (Figure 2a) and also inhibited the relaxations induced by CGRP (Figure 2b; $pK_B = 7.0 \pm 0.2$, $n=4$). The specificity of this antagonism was demonstrated by the lack of effect of $CGRP_{8-37}$ (1 μM) when tested against SP-induced relaxations (pD_2: SP alone = 8.4 ± 0.6; SP + $CGRP_{8-37}$ = 8.3 ± 0.2, $n=3$, $P\geqslant 0.05$).

The NK_1 receptor antagonist, GR82334 (10 μM), had no effect on basal or $PGF_{2\alpha}$-induced tone. GR82334 produced a non-significant rightward displacement of the capsaicin-induced relaxation curve (Figure 2c) whilst significantly inhibiting SP-induced relaxations (Figure 2d; $pK_B = 7.1 \pm 0.1$, $n=3$). GR82334 (10 μM) had no effect on CGRP-induced

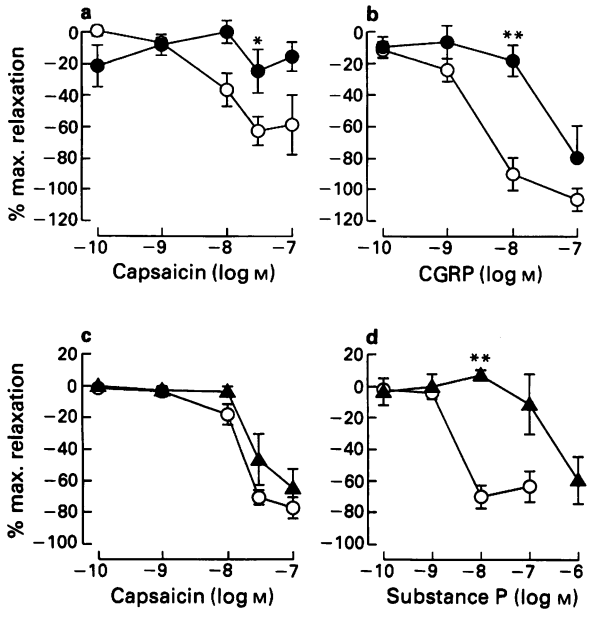


Figure 2 Effect of (a and b) $CGRP_{8-37}$ (1 μM, ●) on relaxant responses to capsaicin ($n = 5$) and calcitonin gene-related peptide (CGRP) ($n = 4$) respectively. Effect of (c and d) GR82334 (10 μM, ▲) on capsaicin- and substance P-induced relaxations ($n = 4$); (○) no antagonist present. $^*P < 0.05$; $^{**}P < 0.02$.

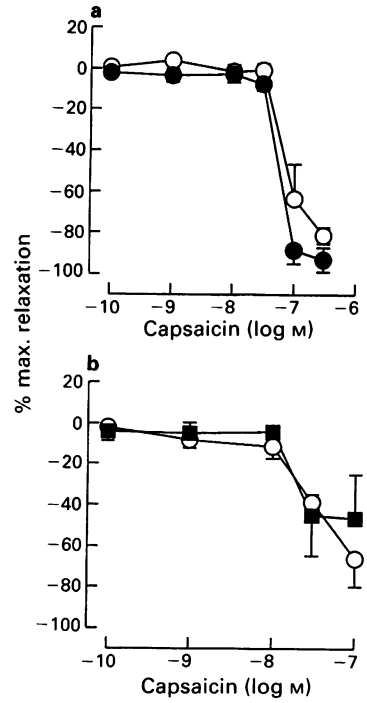


Figure 3 Effect of (a) sumatriptan (0.3 μM) and (b) UK-14,304 (0.1 μM) on relaxant responses to capsaicin in pre-contracted guinea-pig isolated basilar artery: (○) capsaicin alone; (●) in the presence of sumatriptan or (■) UK-14,304. Values are mean ± s.e.mean (vertical bars). ($n = 3$).

relaxations (pD_2: CGRP alone = 8.4 ± 0.0; CGRP + GR82-334 = 8.4 ± 0.1, $n = 4$, $P \geqslant 0.05$).

Sumatriptan and UK-14,304

Sumatriptan produced small contractions of GPBA under conditions of resting tone (21 ± 8 mg at 3 μM, $n = 12$). However, sumatriptan (0.3 and 3 μM) had no significant effect either on $PGF_{2\alpha}$-induced tone or on capsaicin-induced relaxations (Figure 3a shows effects of 0.3 μM sumatriptan). Lowering the calcium concentration in the Krebs solution (from 1.3 mM to 0.65 mM Ca^{2+}) did not reduce the contractile response to $PGF_{2\alpha}$ nor did it unmask any effects of sumatriptan on the relaxant responses to capsaicin (results not shown).

The α_2-adrenoceptor agonist, UK-14,304 (0.1 μM), had no effect on basal or $PGF_{2\alpha}$-induced tone. Capsaicin-induced relaxations of GPBA were unaffected by UK-14,304 (0.1 μM), regardless of whether the α_2-adrenoceptor agonist was added before or after induction of $PGF_{2\alpha}$ tone. Figure 3b shows results obtained when UK-14304 was added prior to $PGF_{2\alpha}$.

Discussion

Capsaicin is a potent vasodilator of intracranial blood vessels, both *in vitro* and *in situ* (Edvinsson *et al.*, 1990; Jansen *et al.*, 1990). Its mechanism of action appears to be through a release of neuropeptides from perivascular trigeminal sensory nerve fibres. We have confirmed the findings of Jansen *et al.* (1990) that capsaicin causes relaxation of GPBA and that desensitization to this stimulus occurs rapidly. It has been suggested that CGRP, rather than SP, is a more important mediator of capsaicin-induced relaxation in feline and guinea-pig cerebrovasculature (Edvinsson *et al.*, 1990; Jansen *et al.*, 1990). The results of the present study confirm this suggestion; the CGRP antagonist, $CGRP_{8-37}$, blocked the relaxant response to capsaicin and also antagonized the relaxant effects of CGRP with a pK_B of 7.0. This value is similar to pA_2 values obtained for antagonism of CGRP by $CGRP_{8-37}$ in GPBA ($pA_2 = 7.11$; Jansen, 1992) and in guinea-pig isolated left atria ($pA_2 = 6.89$) (Maggi *et al.*, 1991).

Neurokinin receptors are also present in the GPBA and this study confirms earlier findings (Edvinsson & Jansen, 1987) that SP-induced relaxation in this tissue is mediated by NK_1 receptors. Thus the recently identified, highly selective NK_1 receptor antagonist, GR82334 (Hagan *et al.*, 1991), blocked the relaxant effects of SP with a pK_B value of 7.1. This value is similar to that obtained by Hagan *et al.* (1991) for GR82334 against NK_1 agonists in guinea-pig ileum (7.64) and guinea-pig trachea (7.23). Neurokinin A (NKA) is derived from the same gene as SP and co-exists with SP and CGRP in trigeminal nerves (Edvinsson *et al.*, 1988). The relative potency of the tachykinins in mediating relaxation of guinea-pig basilar artery is reported to be SP >>NKA> >NKB (Jansen *et al.*, 1991). This rank order of potency suggests that NK_1 receptors predominate in GPBA (Regoli *et al.*, 1987). This finding is also in agreement with recent studies in dog isolated middle cerebral arteries where, by use of selective neurokinin agonists and antagonists, the neurokinin receptor present was shown to be NK_1 (Stubbs *et al.*, 1992). In the present study, the NK_1 receptor antagonist GR82334 had little or no effect on capsaicin-induced relaxations of GPBA. This supports the suggestion that neither SP nor NKA plays a major role in capsaicin-mediated relaxations of GPBA.

The primary aim of this study was to investigate whether the relaxant effects of capsaicin in cranial blood vessels, which have been established as being due to sensory nerve activation, are susceptible to presynaptic receptor modulation. Buzzi & Moskowitz (1990) have demonstrated that the $5\text{-}HT_1$ receptor agonist, sumatriptan, inhibits capsaicin-induced plasma protein extravasation in the dural circulation in anaesthetized rats and guinea-pigs. They have postulated that this action is attributable to an inhibition of neuropeptide release from trigeminal sensory nerve terminals, via activation of pre-junctional $5\text{-}HT_1$ receptors. This has led to the suggestion that the anti-migraine effects of sumatriptan

may result from a prejunctionally-mediated inhibition of neuropeptide release in intracranial blood vessels which are dilated, oedematous and pain-sensitive during a migraine headache. Interestingly, sumatriptan had no effect on plasma protein extravasation in extracranial tissues (Buzzi & Moskowitz, 1990), suggesting that such 5-HT_1 receptors may be restricted to the intracranial vasculature. Thus in the present study, the GPBA was used as an example of an intracranial artery. 5-HT_1 receptors that mediate inhibition of neurogenic contraction are present on sympathetic nerve terminals in dog saphenous vein (Humphrey *et al.*, 1988) although their localization on trigeminal nerves remains to be definitively established.

There is much evidence to show that α_2-adrenoceptors are located on sensory nerve terminals and receptor activation reduces sensory nerve mediated responses (Lundberg *et al.*, 1991). Indeed, we have recently found that α_2-adrenoceptor activation markedly inhibits sensory nerve-mediated relaxations of guinea-pig pulmonary artery (Butler *et al.*, 1992). Also α_2-adrenoceptor activation inhibits plasma protein extravasation from dural blood vessels following electrical stimulation of the trigeminal ganglion (Moskowitz, personal communication). In the present study, however, neither sumatriptan nor UK-14,304, at concentrations which should maximally activate presynaptic 5-HT_1 receptors (Humphrey *et al.*, 1988) or α_2-adrenoceptors (Cambridge, 1981), modified capsaicin-induced relaxations in the GPBA *in vitro*. Lowering extracellular calcium, a method which can be used to enhance detection of presynaptic mechanisms (Feniuk *et al.*, 1979) failed to unmask any inhibitory effect of sumatriptan.

Ruthenium red, which inhibits capsaicin-induced CGRP release in peripheral tissues (Maggi *et al.*, 1988b), blocked capsaicin-induced relaxation in GPBA. This effect was specific since ruthenium red had no effect on CGRP and SP-induced relaxations in this study. Capsaicin activates cation-specific membrane ion channels which are permeable to a number of cations including calcium (Marsh *et al.*, 1987; Docherty *et al.*, 1991) and are insensitive to the sodium channel blocker, tetrodotoxin (Marsh *et al.*, 1987). Ruthenium red acts by inhibiting such transmembrane calcium fluxes (Tapia *et al.*, 1985). Capsaicin-induced relaxations of GPBA are insensitive to tetrodotoxin (O'Shaughnessy, unpublished observations) which is in keeping with reports in isolated vagal sensory C fibres and cell bodies (Marsh *et al.*, 1987).

It is possible that capsaicin-induced release of neuropeptides *in vitro* is not susceptible to modulation by any prejunctionally acting agonists. Selectivity of opioid agonists for inhibiting responses to electrical field stimulation but not those to capsaicin has been reported in guinea-pig bronchus (Barthó, 1987) and guinea-pig left atrium (Giuliani *et al.*, 1990). Similar selective modulation of electrical but not capsaicin-mediated responses has also been observed for galanin (Giuliani *et al.*, 1989a) and neuropeptide Y (Giuliani *et al.*, 1989b). However, capsaicin-induced release of CGRP from guinea-pig heart and lung is inhibited by prejunctional α_2-adrenoceptor activation (Lundberg *et al.*, 1991). Also, capsaicin-induced SP release from rat spinal primary afferents is enhanced by μ-opioid agonists and inhibited by δ-opioid agonists (Pohl *et al.*, 1989). Thus, although the present study shows that neither sumatriptan nor UK-14,304 modulated capsaicin-induced responses in guinea-pig isolated cerebral arteries, the possibility that other pre-junctionally acting agonists may have a modulatory effect cannot be ruled out.

In conclusion, this study provides further support for the proposal that capsaicin-induced relaxations of GPBA are mediated via CGRP released from sensory nerves. However, there is no evidence to suggest that the 5-HT_1 receptor agonist, sumatriptan, or the α_2-adrenoceptor agonist, UK-14,304, modulate capsaicin-induced relaxations in this cranial vascular preparation. Whether or not these agents can influence neuropeptide release following electrical stimulation of trigeminal sensory nerves remains to be established.

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Histamine H_1-receptor-mediated inositol phospholipid hydrolysis in DDT_1MF-2 cells: agonist and antagonist properties

Teresa E. White, John M. Dickenson & [1]Stephen J. Hill

Department of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham NG7 2UH

1 The effect of histamine H_1-receptor stimulation on inositol phospholipid hydrolysis has been investigated in the hamster vas deferens smooth muscle cell line, DDT_1MF-2.

2 Histamine (EC_{50} = 27 μM) stimulated the accumulation of [^{3}H]-inositol phosphates in DDT_1MF-2 cells prelabelled with [^{3}H]-*myo*-inositol. 2-Thiazolylethylamine (EC_{50} 42 μM) produced a maximal response of similar magnitude to histamine while the maximal response obtained with N^{α}-methylhistamine (EC_{50} = 72 μM) and 2-pyridylethylamine (EC_{50} = 85 μM) were much lower (*circa* 65%, histamine = 100%).

3 The H_1-selective agonists 2-(3-fluorophenyl)-histamine (2-FPH) and 2-(3-chlorophenyl)-histamine (2-CPH) both appeared to act as partial H_1-agonists in this system. Both compounds produced maximal responses of only 30% (with respect to histamine = 100) and were able to antagonize the inositol phosphate response to histamine (estimated K_p = 10.4 and 18.9 μM for 2-FPH and 2-CPH respectively).

4 The response to histamine was antagonized by the H_1-antagonists, mepyramine (K_D 0.4 nM), (+)-chlorpheniramine (K_D 1.2 nM) and promethazine (K_D 0.3 nM). Furthermore, the (−)-isomer of chlorpheniramine was approx. three orders of magnitude less potent than the corresponding (+)-isomer.

5 The response to histamine (0.1 mM) was not altered by prior treatment of cells with pertussis toxin (100 ng ml^{-1}; 24 h) whereas the inositol phosphate response to adenosine A_1-receptor stimulation in this cell line was significantly attenuated under these conditions.

6 These data indicate that histamine-stimulated inositol phospholipid hydrolysis in DDT_1MF-2 cells is mediated via a classical H_1-receptor. Furthermore, the results also suggest that histamine H_1- and adenosine A_1-receptors activate phospholipase C in DDT_1MF-2 cells via two different G-protein-coupled pathways.

Keywords: Histamine; H_1-receptor; inositol phospholipids; DDT_1MF-2 cells; smooth muscle

Introduction

Histamine H_1-receptors belong to the family of G-protein-coupled receptors which possess the characteristic seven-transmembrane domains (Yamashita *et al.*, 1991). H_1-receptors are coupled to inositol phospholipid hydrolysis and mediate functional responses in many tissues as a consequence of intracellular calcium mobilization (via the intermediacy of inositol-1,4,5-trisphosphate) and calcium influx (Hill, 1990; 1992; Hill & Donaldson, 1992). A notable feature of both binding and functional studies of the H_1-receptor is the striking species and tissue variations in the potencies of H_1-receptor antagonists (O'Neill & Patil, 1975; Chang *et al.*, 1979; Hill & Young, 1980; Al-Gadi & Hill, 1985; Bielkiewicz & Cook, 1985; Driver & Mustafe, 1987; Hill, 1990). The most likely explanation for this is that these tissue and species differences in the H_1-receptor are attributable to variations in either the H_1-receptor protein sequence or the extent of post-translational modifications such as glycosylation (Garbarg *et al.*, 1985; Mitsuhashi & Payan, 1989a). However, studies of soluble H_1-receptors isolated from rat and guinea-pig brain provide no physicochemical evidence for the difference in H_1-receptor properties between these two species (Ruat & Schwartz, 1989). A unique H_1-receptor isoform (68 kDa) has been isolated from guinea-pig atria but this cardiac isoform cannot be distinguished pharmacologically from the isoform present in guinea-pig brain (56 kDa) (Ruat *et al.*, 1991).

The hamster vas-deferens smooth muscle line, DDT_1MF-2, is of particular interest in this respect because the dissociation constants derived for a wide range of H_1-receptor antagonists from ligand-binding studies with [^{3}H]-mepyramine are at least two orders of magnitude greater than those obtained in guinea-pig tissues (Mitsuhashi & Payan, 1988; 1989b). Furthemore, this low-affinity [^{3}H]-mepyramine binding site has now been purified to homogeneity (38–40 kDa; Mitsuhashi & Payan, 1989b). Support for the suggestion that this binding site may represent a functional H_1-receptor sub-type has been provided by studies of histamine-induced calcium mobilization and actin polymerization in DDT_1MF-2 cells where high concentrations of the H_1-antagonist (±)-chlorpheniramine (100 nM to 1 μM) are required for significant antagonism of these responses (Mitsuhashi & Payan, 1988). High concentrations of the H_1-antagonist, diphenhydramine (100 nM to 1 μM) were also required to inhibit histamine-stimulated phosphatidylinositol turnover in this cell line (Cowlen *et al.*, 1990). In marked contrast, however, we have previously shown that histamine-stimulated increases in intracellular calcium in DDT_1MF-2 cells can be antagonized by low concentrations of the selective H_1-antagonist, mepyramine (K_D 1 nM; Dickenson & Hill, 1991). These latter data are more consistent with the involvement of a 'classical' H_1-receptor such as that found in guinea-pig tissues (Hill, 1990).

The aim of the present study was to evaluate the functional characteristics of the effect of histamine on inositol phospholipid hydrolysis in DDT_1MF-2 cells. We describe here the effect of a range of H_1-receptor ligands (both antagonists and agonists) on this response, including two 2-phenylhistamines which have recently been reported to be highly selective H_1-agonists (Zingel *et al.*, 1990).

[1] Author for correspondence.

Methods

Cell culture

DDT_1MF-2 cells (European Cell Collection, Porton Down) were cultured at 37°C in a humidified air/CO_2 (90:10) atmosphere in 75 cm^3 flasks (Costar). The growth medium was Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum (FCS). Cells were passaged twice a week (1:5 split ratio) by vigorous shaking of the flask and fed with fresh growth medium every 48 h. Cells for inositol phospholipid hydrolysis were finally grown in 24 well cluster dishes. All experiments were performed on confluent monolayers (passages 4–17, number assigned after receiving the cell line).

Inositol phospholipid hydrolysis

At confluence, monolayer cultures were loaded with [^{3}H]-*myo*-inositol (37 KBq/well) for 24 h in 24 well cluster dishes in inositol-free DMEM. Prelabelled cells were then washed twice with 1 ml/well Hanks/HEPES (composition mM: NaCl 137, KCl 5.4, Na_2HPO_4 0.03, KH_2PO_4 0.4, $CaCl_2$ 1.3, $MgSO_4$ 0.8, D-glucose 5.5, HEPES 20 and phenol red sodium salt 10 mg ml^{-1}) buffer pH 7.4 and incubated at 37°C for 30 min in the presence of 20 mM LiCl. Where appropriate, antagonists were added at the beginning of this incubation period. Agonists were then added in 10 µl medium and the incubation continued for 10 min. Incubations were terminated by aspiration of the incubation medium and the addition of 1 ml cold (−20°C) methanol/0.12 M HCl (1:1 v/v). Cells were left a minimum of 2 h at −20°C before isolation of total [^{3}H]-inositol phosphates in the supernatant of the disrupted cell monolayers by anion exchange chromatography (Alexander *et al.*, 1989). Briefly, 800 µl of the supernatant was neutralized by the addition of 135 µl 0.5 M NaOH, 1 ml Tris-HCl (pH 7) and 3.1 ml of water and added to columns of Dowex 1 anion exchange resin (X8, 100–200 mesh, chloride form). [^{3}H]-inositol and [^{3}H]-glycerophosphoinositol were removed with 20 ml of distilled water and 10 ml of 25 mM ammonium formate respectively. Total [^{3}H]-inositol phosphates were then finally eluted with 3 ml of 1 M HCl. Radioactivity was quantified by scintillation counting in the gel phase (scintillator 299, Packard).

Data analysis

Agonist concentration-response curves were fitted to a logistic equation by the non-linear regression programme GraphPad (ISI) as described previously (Ruck *et al.*, 1990). The equation fitted was:

$$\text{Response} = \frac{E_{max} \times A^n}{(EC_{50})^n + A^n}$$

where E_{max} is the maximal response, A is the agonist concentration and n is the Hill coefficient.

Antagonist dissociation constants (K_d) were estimated by a modification of the null method described by Lazareno & Roberts (1987). Briefly, a concentration-response curve to histamine was generated and a concentration (C; usually 100 µM) of histamine was chosen which gave a response greater than 50% of the maximum agonist response. The concentration of antagonist (IC_{50}) required to reduce the response of this concentration (C) of histamine by 50% was then determined. The histamine concentration-response curve was fitted to a logistic equation as described above and a concentration of histamine identified which yielded a response equivalent to 50% of that produced by concentration C (in the absence of antagonist). The apparent K_d was then determined from the relationship:

$$C/C' = IC_{50}/K_d + 1$$

Dissociation constants (K_p) for the partial agonists 2-(3-fluorophenyl)-histamine and 2-(3-chlorophenyl)-histamine were estimated from their interactions with histamine based on the method of Stephenson (1956) as modified by Kenakin & Black (1978). Stephenson's method was based on the assumption that there is a large receptor reserve for the full agonist and since this seems unlikely for histamine in DDT_1MF-2 cells (see Discussion), the calculations of Kenakin & Black (1978) were used which do not assume such a receptor reserve. Briefly, a concentration-response curve was obtained for histamine in the presence and absence of a fixed concentration (P; 100 µM) of partial agonist. The concentrations of histamine that produced equal responses in the absence (A1) and presence (A2) of the partial agonist were then determined. K_p was then determined from the linear regression of the expression:

$$A1 = \frac{A2}{1 + (1 - e_p/e_A)P/K_p} + C \qquad (1)$$

where P is the concentration of the partial agonist, e_p and e_A represent the efficacies of the partial agonist and histamine respectively and C is a constant (see Kenakin & Black, 1978).

An estimate (K_p[est]) of K_p can then be obtained from the slope (s) produced by linear regression analysis of equation 1 where $K_p[est] = P \times s/(1-s)$. In this case K_p[est] will underestimate the true K_p by a factor of $(1 - e_p/e_A)$ which approaches unity if $e_A >> e_p$.

Statistical analysis was performed by paired or unpaired *t* tests. *n* in the text refers to the number of separate individual experiments.

Chemicals

Histamine, bradykinin, noradrenaline, 2-chloroadenosine, mepyramine, promethazine, N^6-cyclopentyladenosine, indomethacin and adenosine-5'-triphosphate were purchased from Sigma. Pertussis toxin (batch 160) was obtained from Porton Products Ltd. [2-^{3}H]-*myo*-inositol (529 GBq $mmol^{-1}$) was purchased from New England Nuclear. Gifts of AA861 (2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadynyl)1,4-benzoquinone (Takeda Chemical Industries, Osaka, Japan), 2-(3-chlorophenyl-histamine and 2-(3-fluorophenyl)-histamine (Prof. W. Schunack, Institute of Pharmacy, University of Berlin), N^{α}-methylhistamine, 2-thiazolylethylamine and 2-pyridylethylamine (Smith Kline Beecham), and (+)- and (−)-chlorpheniramine (Schering) are gratefully acknowledged.

Results

Histamine-stimulated inositol phosphate accumulation

Histamine (0.1 mM) produced a rapid and significant increase in total [^{3}H]-inositol phosphates in DDT_1MF-2 cells after 10 min stimulation in the presence of 20 mM LiCl (3.3 ± 0.3 fold, $n = 10$, $P < 0.05$; Figure 1). This response to histamine (10 min stimulation) was concentration-dependent (Figure 2) and the mean EC_{50} obtained in 12 separate experiments was 27 ± 14 µM. At longer incubation times (> 10 min) the response to histamine significantly declined such that after 40 min the response to 0.1 mM histamine was only 59 ± 4% of that measured at 10 min ($P < 0.01$; paired *t* test; $n = 11$). These data were in marked contrast to the results obtained with either noradrenaline (0.1 mM) or the adenosine receptor agonist, 2-chloroadenosine (10 µM) in DDT_1MF-2 cells where [^{3}H]-inositol phosphates continued to accumulate over the full 40 min period (Figure 3).

The reduction in the level of [^{3}H]-inositol phosphates following prolonged histamine receptor stimulation provides evidence that inositol monophosphate metabolism has not been completely attenuated by 20 mM LiCl in this cell line. This hypothesis is supported by studies performed in the

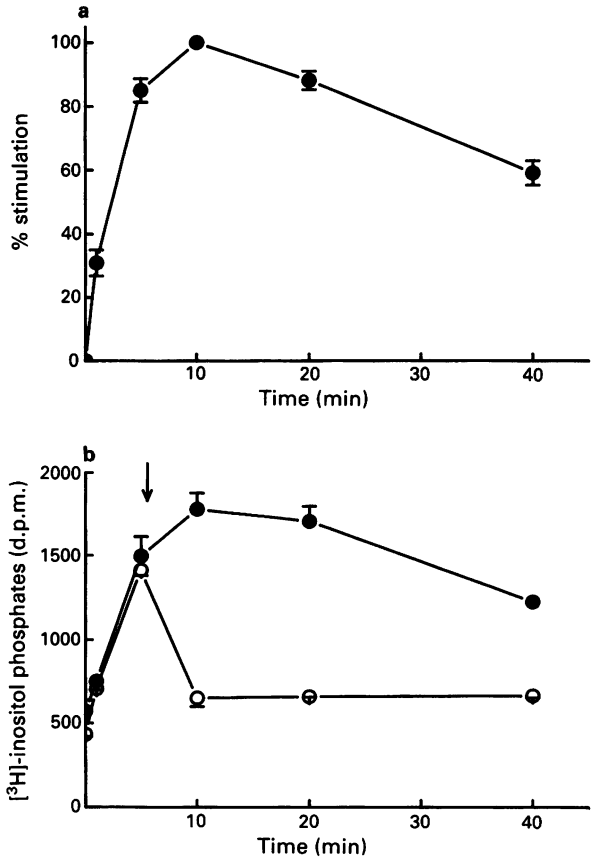


Figure 1 Time course of the inositol phosphate response to histamine (0.1 mM) in DDT_1MF-2 cells. (a) Combined data from 11 separate experiments, each conducted in quadruplicate. Values represent mean ± s.e.mean (vertical bars) of data expressed as a percentage of the [^{3}H]-inositol phosphate response (after subtraction of control basal values) measured following 10 min stimulation with histamine (0.1 mM) in each experiment. (b) Effect of mepyramine addition (100 nM; O; added at the arrow) 5 min after histamine (0.1 mM; added at time zero). The control responses obtained with histamine (in the absence of mepyramine) are indicated by (●). Values represent mean ± s.e.mean (vertical bars) of quadruplicate determinations in a single experiment. Similar data were obtained in three further experiments.

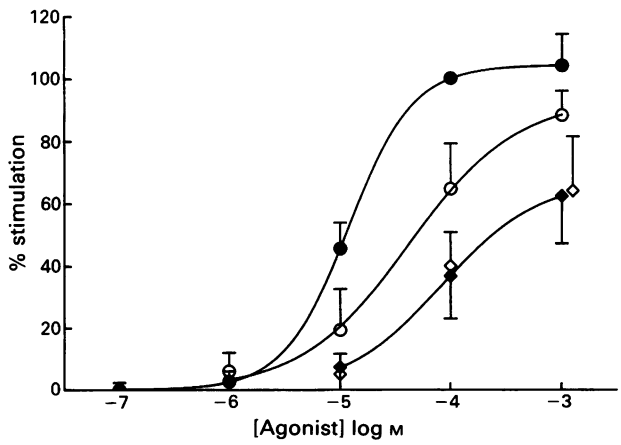


Figure 2 Stimulation of [^{3}H]-inositol phosphate accumulation by H_1-agonists in DDT_1MF-2 cells. (●) Histamine; (O) 2-thiazolylethylamine; (◆) N^{α}-methylhistamine; (◇) 2-pyridylethylamine. To normalize responses from different experiments, data are expressed as a percentage of the response to 0.1 mM histamine which was measured in every experiment. Each point represents the mean ± s.e.mean (vertical bars) from 12 (histamine) and 4 (other agonists) separate experiments. The curves are best fit lines to the Hill equation obtained as described under Methods.

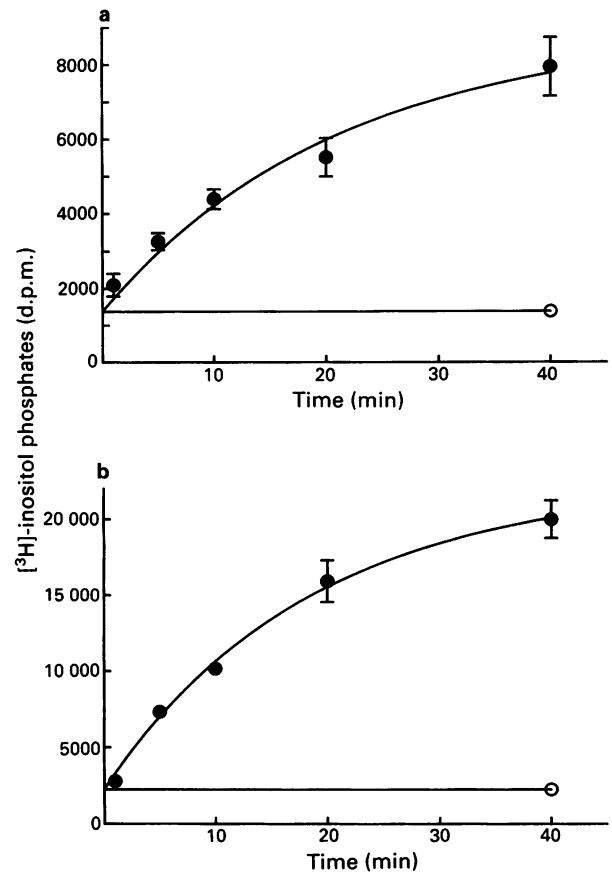


Figure 3 Time course of inositol phosphate responses to (a) 2-chloroadenosine (10 μM) and (b) noradrenaline (0.1 mM). (●) Agonist added at time zero; (O) unstimulated control. Values represent mean ± s.e.mean (vertical bars) from quadruplicate determinations in a single experiment. Similar data were obtained with each agonist on three other occasions.

presence of 10, 20 and 40 mM LiCl. In the presence of 40 mM LiCl the response to 0.1 mM histamine (6.6 ± 0.9 fold over basal levels, $n = 4$) was much greater than that obtained in the presence of 20 mM (3.0 ± 0.2 fold, $n = 12$) or 10 mM (1.6 ± 0.1 fold) LiCl. Furthermore, addition of the H_1-receptor antagonist mepyramine (0.1 μM), immediately following a 5 min period of stimulation with 0.1 mM histamine in media containing 20 mM LiCl, produced a marked reduction in the levels of [^{3}H]-inositol phosphates (in the continued presence of histamine and Li^+ ions) over the next 5 min ($n = 3$; Figure 1b). The response to 0.1 mM histamine (10 min stimulation, 20 mM LiCl), was antagonized by low concentrations of mepyramine, promethazine and (+)-chlorpheniramine (Figure 4; Table 1). Furthermore, the (+)-stereoisomer of chlorpheniramine was three orders of magnitude more potent than the corresponding (−)-isomer (Figure 4). The apparent dissociation constants obtained for mepyramine, promethazine and (+)-chlorpheniramine assuming competitive antagonism, agreed well with those obtained in guinea-pig brain and were much lower than those reported for inhibition of [^{3}H]-mepyramine binding in DDT_1MF-2 membranes (Table 1).

H_1-agonist properties

The H_1-agonists, N^{α}-methylhistamine, 2-thiazolylethylamine and 2-pyridylethylamine were also able to stimulate [^{3}H]-inositol phosphate accumulation in DDT_1MF-2 cells (Figure 2). The relative potencies (histamine = 100) obtained for these three compounds were 17%, 28% and 14% respectively (Table 2). A notable feature of these data is that N^{α}-methylhistamine and 2-pyridylethylamine did not produce the

Table 1 Antagonism of histamine-stimulated inositol phosphate accumulation in DDT_1MF-2 cells

Antagonist	*Inositol phosphate hydrolysis* IC_{50} (nM)	K_D (nM)		*[^{3}H]-mepyramine binding (K_D, nM)* *G-pig brain**	*DDT_1MF-2†*
Mepyramine	2.9 ± 0.6	0.36 ± 0.08	(4)	0.8	288
(+)-Chlorpheniramine	9.9 ± 2.3	1.22 ± 0.29	(4)	0.8	1,400
(−)-Chlorpheniramine	> 10,000	> 10,000	(4)	200	10,500
Promethazine	2.7 ± 0.8	0.33 ± 0.10	(5)	1.4	

Values for inositol phosphate accumulation represent mean ± s.e.mean of 4–5 experiments (n given in parentheses) of antagonist IC_{50} values obtained from inhibition of histamine-stimulated (100 μM) total [^{3}H]-inositol phosphate accumulation in DDT_1MF-2 cells. Apparent K_D values were calculated from these IC_{50} values as described under Methods. * Data from Hill *et al.* (1978). †Data from Mitsuhashi & Payan (1989b).

Table 2 Characteristics of H_1-agonists as stimulants of [^{3}H]-inositol phosphate accumulation in DDT_1MF-2 cells

Agonist	E_{max}	log (EC_{50}, M)	n_H	*RP*	n
Histamine	104.2 ± 0.1	− 4.92 ± 0.01	1.48 ± 0.02	100	12
2-Thiazolylethylamine	93.8 ± 3.8	− 4.37 ± 0.07	0.88 ± 0.09	28	4
N^{α}-methylhistamine	66.0 ± 2.0	− 4.14 ± 0.04	1.28 ± 0.16	17	4
2-pyridylethylamine	67.7 ± 0.4	− 4.07 ± 0.01	0.98 ± 0.01	14	4
2-FPH	31.3 ± 4.5	− 5.18 ± 0.22	0.99 ± 0.39	182	8
2-CPH	29.9 ± 5.7	− 5.00 ± 0.35	0.63 ± 0.17	120	8

Best-fit values ± estimated s.e.mean of log EC_{50} (concentration of agonist producing half-maximal stimulation), n_H (Hill coefficient) and E_{max} (maximal response; expressed as a percentage of the response to 0.1 nM histamine) were obtained from non-linear regression analysis of concentration-response data as described under Methods. n is the number of individual concentration-response curves which were combined for the analysis. RP is the relative agonist potency determined from the ratio EC_{50} [histamine]/EC_{50} [agonist] × 100. 2-FPH is 2-(3-fluorophenyl)-histamine and 2-CPH is 2-(3-chlorophenyl)-histamine.

same maximal response as histamine (Figure 2). In order to make a more quantitative comparison of the maximal responses, concentration-response curves were fitted to a logistic equation as described under Methods. The best-fit values obtained for EC_{50}, Hill coefficient and maximum response (relative to that obtained with 0.1 mM histamine) are presented in Table 2. Both N^{α}-methylhistamine and 2-pyridylethylamine produced maximum responses which were 66–68% of that produced by 0.1 mM histamine. Hill coefficients were generally close to unity with the exception of histamine (1.48) which was slightly steeper than the other agonists.

Partial agonist activity was also noted with the H_1-selective agonists, 2-(3-fluorphenyl)-histamine (2-FPH) and 2-(3-chlorophenyl)-histamine (2-CPH) (Zingel *et al.*, 1990) (Figure 5). Both compounds produced maxima (31% and 30% respectively; histamine = 100) which were markedly lower than that produced by histamine (Figure 5; Table 2). However, the EC_{50} values obtained for both compounds were lower than that obtained for histamine (Table 2). The large errors on the best-fit Hill coefficients obtained for both 2-FPH and 2-CPH are indicative, however, of the difficulty in obtaining best-fit parameters for such small responses. Consequently, the dissociation constants (K_p) for these two partial agonists were determined from partial agonist/full agonist interactions rather than from analysis of partial agonist concentration-response curves. Concentration-response curves for histamine were therefore determined in the presence and absence of either 2-FPH (100 μM) or 2-CPH (100 μM). Both compounds were able to antagonize the response to high concentrations of histamine (e.g. Figure 6) yielding estimates for K_p of 10.4 ± 3.2 μM (n = 4) and 18.9 ± 8.3 μM (n = 4) for 2-FPH and 2-CPH respectively.

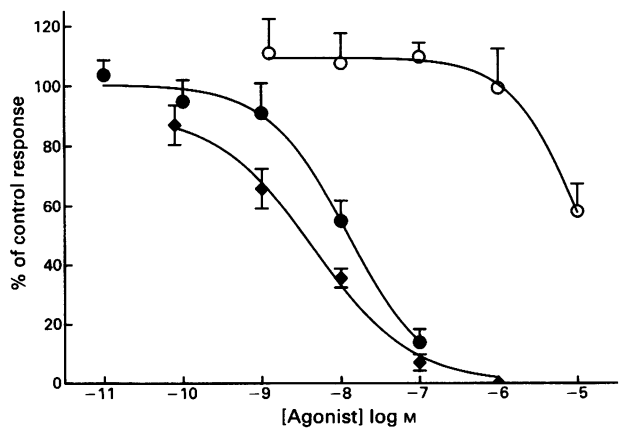


Figure 4 Antagonism by mepyramine and the stereoisomers of chlorpheniramine of inositol phosphate responses to 0.1 mM histamine in DDT_1MF-2 cells. (◆) Mepyramine; (●) (+)-chlorpheniramine; (○) (−)-chlorpheniramine. Values represent the combined mean ± s.e.mean (vertical bars) from quadruplicate determinations in 4 separate experiments.

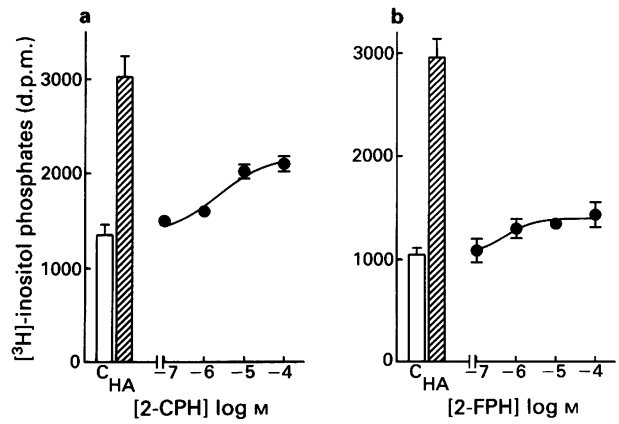


Figure 5 Effect of (a) 2-(3-chlorophenyl)-histamine (2-CPH) and (b) 2-(3-fluorophenyl)-histamine (2-FPH) on [^{3}H]-inositol phosphate accumulation in DDT_1MF-2 cells. Values represent mean ± s.e.mean (vertical bars) of quadruplicate determinations in a single experiment. The histograms marked C and HA respectively represent the basal and histamine-stimulated (0.1 mM) inositol phosphate accumulation measured in the same experiment. Similar data were obtained in seven other experiments with each agonist. Concentration-response parameters for all eight experiments are summarized in Table 2.

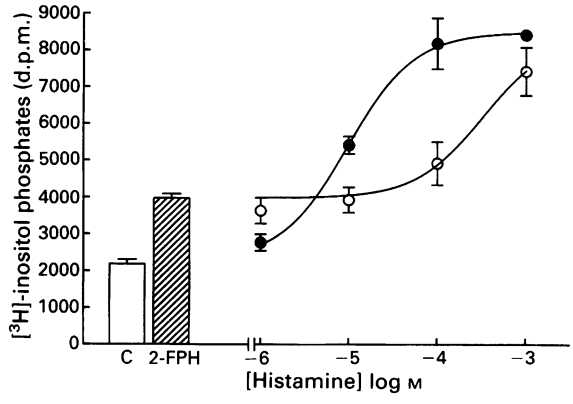


Figure 6 Antagonism of histamine-stimulated inositol phospholipid hydrolysis by 2-(3-fluorophenyl)-histamine in DDT_1MF-2 cells. (●) Histamine added alone; (○) histamine added simultaneously with 0.1 mM 2-(3-fluorphenyl)-histamine. The basal accumulation of [^{3}H]-inositol phosphates and the response to 0.1 mM 2-(3-fluorophenyl)-histamine alone are shown by the histograms marked C and 2-FPH respectively. Values represent mean ± s.e.mean (vertical bars) of quadruplicate determinations in a single experiment. Similar data were obtained in three other experiments and the combined data are presented in the text.

Role of extracellular calcium, arachidonic acid metabolites and pertussis toxin-sensitive G proteins

Previous studies of H_1-receptor-mediated inositol phospholipid hydrolysis in mammalian brain slices have indicated that the response to histamine is very dependent upon extracellular calcium ion concentration ($[Ca^{2+}]_e$) and increases linearly with $[Ca^{2+}]_e$ up to 4 mM (Kendall & Nahorski, 1984; Alexander *et al.*, 1990a,b). However, in DDT_1MF-2 cells the response to histamine was much less dependent upon $[Ca^{2+}]_e$. Thus, in the presence of 0.6 mM and 1.2 mM $[Ca^{2+}]_e$ the inositol phosphate response to 0.1 mM histamine was 61 ± 6% ($n = 4$) and 78 ± 8% ($n = 4$) of that obtained in the presence of 2.4 mM Ca^{2+} (Figure 7). Furthermore, in the absence of added extracellular calcium a significant accumulation of [^{3}H]-inositol phosphates (25 ± 7% of that obtained in presence of 2.4 mM Ca^{2+}, $n = 4$; $P < 0.05$) was still evident.

We have previously shown that the inositol phospholipid response to adenosine A_1-receptor stimulation in DDT_1MF-2 cells is sensitive to inhibition by the 5-lipoxygenase inhibitor, AA861 (White *et al.*, 1992). Consequently, the effect of histamine (100 μM) on [^{3}H]-inositol phosphate accumulation in DDT_1MF-2 cells has been investigated in the presence of

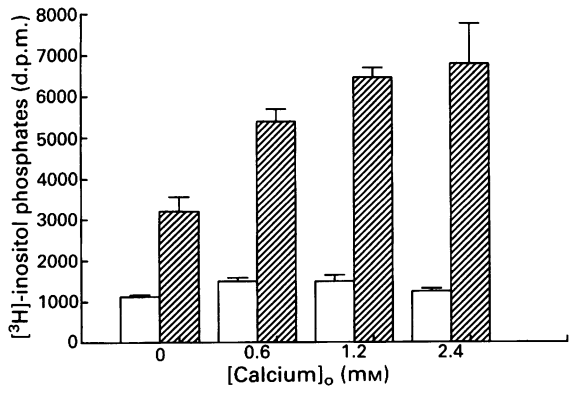


Figure 7 Influence of extracellular Ca^{2+} concentration on histamine H_1-receptor-mediated inositol phospholipid hydrolysis in DDT_1MF-2 cells. Measurements were made in the absence (open columns) or presence (hatched columns) of 0.1 mM histamine. Data represent mean ± s.e.mean (vertical bars) of quadruplicate determinations in a single experiment. Similar data were obtained in three other experiments and the combined data are summarized in the text.

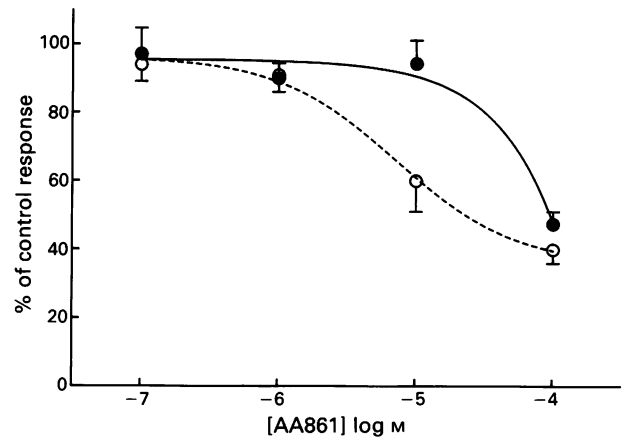


Figure 8 Effect of AA861 on histamine- (0.1 mM) and 2-chloroadenosine- (10 μM) stimulated [^{3}H]-inositol phosphate accumulation in DDT_1MF-2 cells. Values represent combined mean ± s.e.mean (vertical bars) of quadruplicate determinations in six (histamine) or four (2-chloroadenosine) separate experiments. Data are expressed as a percentage of the control response to 0.1 mM histamine or 10 μM 2-chloroadenosine (after subtraction of basal levels). (●) Histamine; (○) 2-chloroadenosine. Data for 2-chloroadenosine are taken from White *et al.* (1992).

either indomethacin (an inhibitor of cyclo-oxygenase) or AA861 (Figure 8). The response obtained in the presence of 1 μM indomethacin was not significantly different (98.7 ± 7.3% of control values, $n = 4$) from that obtained in its absence. Furthermore, no significant reduction in the response to histamine was obtained at concentrations of AA861 up to 10 μM (Figure 8). This concentration (10 μM) significantly inhibited the response to 2-chloroadenosine (Figure 8; White *et al.*, 1992). A significant attenuation of the histamine response was, however, produced at 100 μM AA861 (Figure 8).

Another difference between histamine H_1- and adenosine A_1-receptor-mediated inositol phospholipid hydrolysis in DDT_1MF-2 cells is their sensitivity to pertussis toxin. Pretreatment of cells with pertussis toxin (100 ng ml^{-1}; 24 h) significantly attenuated the response to the A_1-agonist, cyclopentyladenosine (0.1 μM; $P < 0.05$), but was without significant effect on the responses to ATP (0.1 mM), noradrenaline (0.1 mM), histamine (0.1 mM) and bradykinin (0.1 μM) (Figure 9).

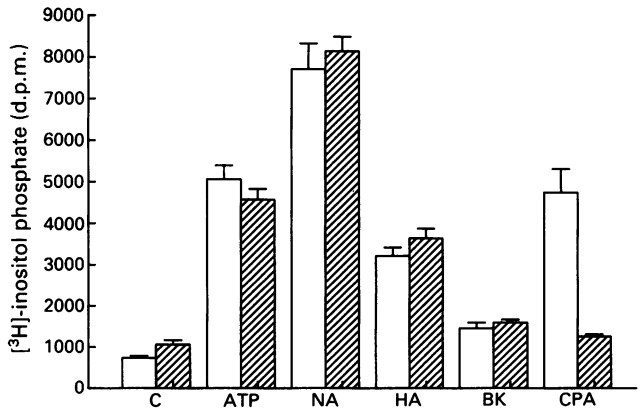


Figure 9 Effect of pertussis toxin pretreatment on agonist-induced inositol phospholipid hydrolysis in DDT_1MF-2 cells. Data are mean ± s.e.mean (vertical bars) of quadruplicate determinations obtained in control cells (open columns) or cells pretreated for 24 h with 100 ng ml^{-1} pertussis toxin (hatched columns). C, control; ATP (0.1 mM): NA, noradrenaline (0.1 mM); HA, histamine (0.1 mM); BK, bradykinin, 100 nM; CPA, cyclopentyladenosine, 100 nM. Data are from a single experiment. Similar results were obtained in three further experiments.

Discussion

A 38–40 kDa [^{3}H]-mepyramine binding protein which has been solubilized and purified to homogeneity from the hamster vas deferens smooth muscle cell line DDT_1MF-2 , has been postulated to represent the histamine H_1-receptor (Mitsuhashi & Payan, 1989b). A striking feature of this protein in both solubilized and membrane preparations is the relatively low affinity of H_1-receptor antagonists for the [^{3}H]-mepyramine binding domain of the protein (Mitsuhashi & Payan, 1988; 1989b). A major aim of the present study was therefore to determine the potencies of several H_1-antagonists as inhibitors of functional H_1-receptor-mediated response (inositol phospholipid hydrolysis) in this cell line.

Mepyramine, (+)-chlorpheniramine and promethazine were all potent antagonists of histamine-stimulated [^{3}H]-inositol phosphate accumulation in DDT_1MF-2 cells with dissociation constants in close agreement with those reported for the H_1-receptor in guinea-pig brain (Hill *et al.*, 1978; Table 1) and ileal smooth muscle (Hill & Young, 1981). However, these values are at least two orders of magnitude lower than those obtained from studies of ligand binding to the 38–40 kDa protein from DDT_1MF-2 cells (Table 1). These data strongly suggest that the low affinity 38–40 kDa [^{3}H]-mepyramine binding protein does not represent a functional H_1-receptor. Furthermore, the dissociation constant obtained in the present study for mepyramine (0.36 nM, Table 1) agreed well with the value that we have obtained previously for this antagonist (1 nM) from inhibition of histamine-stimulated calcium mobilization in DDT_1MF-2 cells (Dickenson & Hill, 1991). (+)-Chlorpheniramine was also three orders of magnitude more potent than the (−)-isomer in studies of inositol phospholipid hydrolysis in DDT_1MF-2 cells. A similar potency ratio has been reported for these stereo-isomers from studies of H_1-receptor function in guinea-pig brain and ileum (Roth & Govier, 1958; Hill *et al.*, 1981).

SDS-PAGE analysis of the guinea-pig H_1-receptor protein following photoaffinity labelling of brain membranes indicates that molecular weight of the H_1-receptor is 56 kDa (Ruat *et al.*, 1988). The mepyramine-binding protein from DDT_1MF-2 cells is considerably smaller than this and is closer in size to the 47 kDa proteolytic product detected in guinea-pig brain (Ruat *et al.*, 1988). Interestingly, antiserum raised against the purified 38–40 kDa protein from DDT_1MF-2 cells recognizes a 57 kDa protein from whole DDT_1MF-2 cell lysates (Mitsuhashi & Payan, 1989b) which may represent the functional H_1-receptor detected in the present study. It is possible, therefore that the 38–40 kDa protein isolated from DDT_1MF-2 cells represents a fragment of the full H_1-receptor or a related protein with an altered antagonist-binding domain.

The inositol phosphate response to histamine in DDT_1MF-2 cells was short-lived and declined from a peak at 10 min to significantly lower levels after 40 min (Figure 1) despite the presence of Li^+ ions to inhibit inositol monophosphatase (Hallcher & Sherman, 1980; Berridge *et al.*, 1982). These data suggest that the metabolism of inositol monophosphate in DDT_1MF-2 cells is much less sensitive to inhibition by Li^+ ions than in brain slices where maximal effects of Li^+ are obtained at 5–10 mM (Brown *et al.*, 1984; Whitworth & Kendall, 1988). A similar low sensitivity to Li^+ has been reported in C6 glioma and HeLa cells (Ananth *et al.*, 1987; Bristow *et al.*, 1991).

A comparison of the time courses of inositol phosphate accumulation obtained with histamine, 2-chloroadenosine and noradrenaline (cf. Figures 1 and 3) suggests that the H_1-receptor-mediated response is more susceptible to desensitization than the two other receptor-mediated effects. Thus, the rapid decline in the responsiveness to histamine in the presence of 20 mM Li^+ is indicative of a marked decrease in the synthesis of [^{3}H]-inositol phosphates after 10 min stimulation. A similar preferential induction of H_1-receptor desensitization (compared with muscarinic receptors) has been observed in 1321N1 cells and in smooth muscle from guinea-pig jejunum and taenia coli where histamine induces homologous desensitization of the H_1-receptor (Nakahata & Harden, 1987; Hishinuma & Uchida, 1989; Leurs *et al.*, 1990). Interestingly, previous studies of histamine-stimulated [^{3}H]-inositol phospholipid turnover in DDT_1MF-2 cells have shown that prior treatment with histamine can induce a rapid and selective desensitization of this response (Cowlen *et al.*, 1990).

Studies of H_1-agonist activity in the present study have provided some interesting data. Both 2-pyridylethylamine and N^{α}-methylhistamine produced lower maximal inositol phosphate responses in DDT_1MF-2 cells than that produced by histamine itself. Similar low efficacies have been detected for 2-pyridylethylamine and N^{α},N^{α}-dimethylhistamine in functional responses in guinea-pig cerebral cortical slices where the H_1-spare receptor reserve appears to be low (Daum *et al.*, 1982; 1984; Donaldson & Hill, 1986). The data obtained with the selective H_1-agonists 2-FPH and 2-CPH (Zingel *et al.*, 1990) in DDT_1MF-2 cells would also be consistent with a low receptor reserve in DDT_1MF-2 cells. Both of these compounds are low efficacy agonists and can act as partial agonists in these cells (e.g. Figure 7). The dissociation constant for both compounds is *circa* 10 μM. In guinea-pig ileum, however, where the spare H_1-receptor reserve is relatively large (Reasbeck & Young, 1973), both compounds are full agonists and have EC_{50} values around 0.2 μM (Zingel *et al.*, 1990).

The inositol phosphate response to histamine in DDT_1MF-2 cells appears to be much less dependent upon extracellular Ca^{2+} than the response to H_1-receptor stimulation in mammalian brain slices (Alexander *et al.*, 1990a,b; Kendall & Nahorski, 1984). A significant stimulation of inositol phospholipid hydrolysis is still evident under calcium-free conditions in DDT_1MF-2 cells and these data are consistent with our previous observations that histamine can mobilize Ca^{2+} ions from intracellular stores in the absence of extracellular calcium (Dickenson & Hill, 1991; 1992). The inositol phospholipid response to histamine did not appear to be mediated by prostaglandins or leukotrienes since it was not affected by the cyclo-oxygenase inhibitor, indomethacin (Higgs & Vane, 1983; 1 μM) and was much less sensitive to the 5-lipoxygenase inhibitor AA861 (Yoshimoto *et al.*, 1982; Ashida *et al.*, 1983) than the response mediated by adenosine A_1-receptor-stimulation in this cell line (see Figure 8; White *et al.*, 1992).

The response to histamine was unaffected by pertussis toxin treatment indicating that the inositol phospholipid response to H_1-receptor stimulation is probably mediated by G_Q which lacks pertussis toxin-sensitive ribosylation sites (Taylor *et al.*, 1991). The responses to noradrenaline, ATP and bradykinin were similarly insensitive to pertussis toxin in DDT_1MF-2 cells (Figure 9). In marked contrast, the response to the adenosine A_1-receptor-selective agonist, cyclopentyadenosine, was completely inhibited by 24 h treatment with 100 ng ml^{-1} pertussis toxin. These data suggest that A_1-receptor mediates [^{3}H]-inositol phosphate accumulation via one of the pertussis toxin-sensitive G-proteins (G_{i2} or G_{i3}) present in DDT_1MF-2 cells (Gerwins & Fredholm, 1991; 1992).

In summary, the present study shows that histamine-stimulated inositol phospholipid hydrolysis in DDT_1MF-2 cells is mediated via a 'classical' H_1-receptor with pharmacological characteristics very similar to those of the H_1-receptor coupled to inositol phospholipid hydrolysis in guinea-pig cerebral cortex. We also provide evidence that histamine and cyclopentyladenosine can activate phospholipase C activity in DDT_1MF-2 cells via two different G-protein-coupled pathways, only one of which is sensitive to inhibition by pertussis toxin.

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Renal selective N-acetyl-L-γ-glutamyl prodrugs: studies on the selectivity of some model prodrugs

[1]J.C. Drieman, H.H.W. Thijssen & H.A.J. Struyker-Boudier

Department of Pharmacology, University of Limburg, 6200 MD Maastricht, The Netherlands

1 In this study, a number of structurally different N-acetyl-L-γ-glutamyl prodrugs were investigated with respect to selective uptake by the kidney in male Wistar rats.

2 All prodrugs were tested *in vitro* in rat kidney slices and kidney homogenate to study their uptake and conversion. It was found that the prodrugs of *para*-nitroaniline (agPNA), aminophenyl acetic acid (agAFA), sulphamethoxazole (agSM), sulphadimethoxine (agSDM), propranolol (agPP) and metoprolol (agMP) were accumulated by a probenecid-sensitive carrier. The prodrug of 4'-aminoantipyrine (agAAP) was not accumulated by a probenecid- or buthionine sulphoximine-sensitive carrier. Unlike all other prodrugs, agAAP and agMP were not, or only a very limited extent converted to the parent compound *in vitro*.

3 agPNA, agAFA and agPP were also investigated *in vivo*. The tissue distribution of the prodrugs and the parent drugs was established, as was their urinary excretion and pharmacokinetic behaviour. agPNA and agAFA showed selective uptake by the kidney, in contrast to agPP which accumulated in the liver. The distribution of the parent compounds following prodrug administration was as follows: agPNA was found in kidney and plasma: agAFA in kidney only; agPP in liver only.

4 The factors which determine the selectivity of N-acetyl-L-γ-glutamyl prodrugs are discussed. The main factors are: the transport into the kidney, the conversion rate, the residence time of the prodrug in the kidney and the presence or absence of competition for uptake and conversation by other tissues, e.g. the liver. It is concluded that this prodrug approach offers the possibility of delivering drugs selectively to the kidney, but also that it is not universally applicable.

Keywords: Renal transport; drug targeting; kidney

Introduction

Drug targeting to a well-defined compartment may be achieved via different routes. Macromolecular carriers coupled to antibodies (Sezaki & Hashida, 1984), liposomes (Ranade, 1989; Fielding, 1991), low molecular weight proteins (Franssen *et al.*, 1991) and magnetic microspheres (Papisov *et al.*, 1987) are some of the vectors used to achieve drug targeting. Another strategy is the design of prodrugs that are selectively activated and retained at the target site. Gastric proton pump inhibitors such as omeprazole (Wallmark, 1986; Bohnenkamp *et al.*, 1987) and redox delivery systems for the brain (Boder, 1985; Gogu *et al.*, 1989) are examples of this approach.

Targeted delivery of drugs in the kidney offers new possibilities for treatment of hypertension, renal cancer, (acute) renal failure, focal glomerulosclerosis or renal allograft rejection. A promising system to achieve drug targeting to the kidney via prodrugs is the use of N-acetyl-L-γ-glutamyl derivatives. Several N-acetyl-L-γ-glutamyl prodrugs that are kidney-selective have been described (Orlowski *et al.*, 1980; Smits & Struyker Boudier, 1985; Koepke *et al.*, 1991). Recently, we investigated the mechanism of the renal selectivity of these prodrugs (Drieman *et al.*, 1990a,b). Carrier-mediated transport and intracellular conversion were found to be the main determinants of kidney selectivity.

This study was undertaken to investigate how universally applicable the N-acetyl-L-γ-glutamyl prodrug approach is for drug targeting to the kidney. Seven prodrugs were tested for kidney-selectivity. The uptake of the prodrugs by rat kidney slices, the effect of two transport inhibitors on this transport and the conversion by rat kidney homogenate was chosen as an *in vitro* system to screen prodrugs on their potential selectivity and to investigate the involvement of anion transporters. Based on these results, three of these prodrugs were chosen to be evaluated *in vivo*.

[1] Author for correspondence.

Methods

Animals

Male Wistar rats weighing 200–250 g (Winkelman, Borchen, Germany) were used throughout the study.

In vitro *experiments*

Kidney slice uptake studies Rat kidney slices containing both cortex and medulla were prepared as described by Drieman *et al.* (1990b). The ability of the slices to accumulate organic anions was studied by the method described by Smith *et al.* (1982). Briefly, renal slices (75–150 mg) were incubated in 4 ml of a phosphate buffer (pH 7.4), containing (mM); phosphate 7.4, NaCl 96.7, KCl 40, $CaCl_2$ 0.74, lactate 10 and *para*-aminohippuric acid 75 μM or prodrug 100 μM. The mixtures were incubated at 25°C for 90 min under 95% O_2/5%CO_2 and agitated at 100 cycle min^{-1}. At the end of the incubation, the kidney slices were removed, gently blotted dry on filter paper and weighed.

Slices, used for the determination of *para*-aminohippuric acid (PAH) accumulation were homogenized in 3 ml of 10% trichloroacetic acid. Subsequently, the volume was adjusted to 10 ml with distilled water. This mixture was centrifuged and 2 ml was analysed for PAH by the method of Smith *et al.* (1945). The incubation medium (1 ml) was treated with 4 ml of 3.75% trichloroacetic acid; 1 ml was analysed for PAH. Slices, used for the determination of prodrug uptake were homogenized in 2 ml of acetonitrile; subsequently the volume was adjusted to 4 ml with distilled water, mixed and centrifuged. The mixture was then analysed for the amount

of prodrug present. One ml of the incubation medium was treated likewise and also analysed for prodrug.

Prodrug conversion Kidney homogenates were prepared in 3 volumes of ice-cold 0.05 M Tris-HCl buffer (pH 8). The *in vitro* conversion of the prodrugs was measured in incubations containing 1 mM substrate and 0.5 mM Tris-HCl buffer (pH 8) in a total volume of 200 µl. The mixtures were preincubated at 37°C and the incubations were started by addition of 50 µl of homogenate. Reactions were terminated at 20 min by heat denaturation (2 min at 100°C). The appropriate denatured tissue fractions served as controls. Each incubation was performed in triplicate. Protein was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

In vivo *experiments*

Tissue levels Under light ether anaesthesia, the left femoral vein of the rats was catheterised (Smits & Struyker Boudier, 1985). After a recovery of at least 24 h the animals were injected i.v. with 10 mg kg^{-1} prodrug. At 20 min a plasma sample was taken and kidney and liver were removed and homogenized. These samples were analysed for prodrug and active drug.

Pharmacokinetic parameters Under light ether anaesthesia, rats were provided with a catheter in the left femoral vein and artery as described (Smits & Struyker Boudier, 1985). After receovery (at least 24 h) the animals were injected with 10 mg kg^{-1} prodrug. At $t = 0$, 15, 30, 60, 90, 120, 180 and 300 min (agPP), $t = 0$, 5, 10, 15, 20, 30, 45 and 60 min (agPNA) or $t = 0$, 10, 20, 30, 45, 60, 90 and 120 min (agAFA), blood samples were taken via the femoral artery catheter and plasma was prepared. Plasma (100 µl) was treated with 100 µl acetonitrile, mixed and centrifuged; 50 µl was injected on high performance liquid chromatography (h.p.l.c.) system described below. Total clearance (Cl) was calculated by $Cl = D/AUC$, were D = dose of prodrug and AUC is area under the plasma concentration-time curve. AUC was obtained by the trapezoidal method. V_d was estimated by extrapolation to $t = 0$ of the concentration-time curve: $V_d = D/C_0$. Renal clearance (Cl_R) was calculated by $Cl_R = Cl.fe$, were fe is fraction of dose excreted unchanged in the urine.

Cumulative urinary excretion (0–20 h) was determined by use of metabolic cages after i.v. injection of 10 mg kg^{-1} prodrug.

Materials

The N-acetyl-L-γ-glutamyl prodrugs of propranolol (PP) and metoprolol (MP) were a gift of Ciba-Geigy (Basel, Switzerland). Aminophenylacetic acid (AFA) and 4'-aminoantipyrine (AAP) were supplied by Janssen Chimica (Beerse, Belgium). Sulphamethoxazole (SM), sulphadimethoxine (SDM), *p*-nitroanaline (PNA), γ-glutamyl-*p*-nitroanaline (gPNA), probenecid and buthionine sulphoximine (BuSO) were supplied by Sigma Chemical Co (St Louis, MO, U.S.A.). The N-acetyl-L-γ-glutamyl prodrugs of SM, SDM, PNA, AFA and AAP were prepared according to the general method of King & Kidd (1949), as modified by Orlowski (1980); see also Drieman (1989).

Analysis

All N-acetyl-L-γ-glutamyl prodrugs and other anionic drugs were analysed by the following system. Column: Rosil NH_2 90-5S, 150 × 4.6 mm (Biorad, Nazareth, Belgium); solvent: 20% acetonitrile/80% NaH_2PO_4-buffer (5–40 mM), flow rate 1.5 m min^{-1}; detection: u.v.; optimal wavelengths were determined with a diode array detector (ABI 1000 S, Applied Biosystems).

For each series of samples, a calibration curve was made. These curves were linear and reproducible in the concentration-range used. PNA and AAP were assayed on a C-18 column (Lichrosorb 5 RP18, 150 × 4.6 mm, Chrompack, The Netherlands); solvent: 60% acetonitrile, 40% methanol, 0.1% H_3PO_4 (0.5 M) and 0.05% perchloric acid (0.1 M), pH of solvent: 6.0. SDM was analysed on the same system at SM (see Drieman *et al.*, 1990b). Propranolol and metoprolol were analysed as described by Mooij *et al.* (1987).

Results

Experiments in vitro

Kidney slice uptake The uptake of PAH by rat kidney slices was used to assess the integrity of the anion transporters of the slices. The slice to medium ratio (S/M) of PAH uptake was 7.4 ± 0.3. The results of the slice uptake and conversion of the prodrugs by rat kidney homogenates are presented in Table 1.

The S/M of the prodrug concentrations is a result of the following processes: (i) transport across membranes (passive diffusion and carrier-mediated transport); (ii) metabolism (conversion) of the prodrug and (iii) tissue binding. This implies that a high (> 1) S/M does not necessarily mean carrier-mediated transport; tissue binding could also be involved. On the other hand, a relatively low (< 1) S/M does not exclude the involvement of carrier-mediated transport. Therefore, the influence of the transport inhibitors probenecid and buthionine sulphoximine (BuSO; Griffith *et al.*, 1979; Anderson & Meister, 1983) was also investigated. Table 1 shows that: (i) agPNA, agAFA and agSM were accumulated in the slices via a probenecid- and BuSO-sensitive process. These prodrugs were also converted to the parent compound by kidney homogenate. (ii) The transport of agPP and agMP is probenecid- but not BuSO-sensitive. Both compounds were converted by kidney homogenate, but agMP only to a limited extent. (iii) agAAP was not accumulated in the kidney slices via a probenid- or BuSO-sensitive carrier. Furthermore, this compound was not converted to the parent compound by rat kidney homogenate in detectable amounts. The accumulation of agSDM in kidney slices is probenecid-sensitive; the BuSO-sensitivity of the renal uptake of this prodrug could not be evaluated due to analytical interference of BuSO with agSDM.

The differences in sensitivity of the transport of the prodrugs to BuSO and probenecid indicates that at least two transporters are involved in the accumulation of the prodrugs, one of which is BuSO-sensitive and one that is not.

Experiments in vivo

The *tissue distribution* of agPP, agPNA and agAFA are depicted in Figure 1. agPP accumulated in the liver and to a lesser extent in the kidney: the tissue to plasma ratio was 11.2 and 2.1 for liver and kidney, respectively. The parent compound, PP, was only found in the liver. agAFA and agPNA accumulated in the kidney and not in the liver (kidney to plasma ratios were 3.2 and 22.2, respectively). The parent compound AFA was only found in the kidney, whereas PNA is found both in kidney and in plasma.

The deacetylated prodrug gPNA was also administered *in vivo*. No plasma or tissue concentrations of this compound could be measured at 20 min after its i.v. administration, but the parent compound PNA (0.3 µg ml^{-1} in plasma) and the acetylated metabolite agPNA (12.5 and 1.1 µg g^{-1} or ml^{-1} in kidney and plasma, respectively) were found.

The *urinary excretion* of the prodrugs (Table 2) shows that the kidney-selective prodrugs are for the most part excreted

Table 1 Uptake of prodrugs by rat kidney slices[a]

Prodrug[b]	*Control* ($n = 12$)	*+ Probenecid*[c] ($n = 6$)	*+ BuSO*[c] ($n = 6$)	*Conversion* (ng 20 min^{-1} mg^{-1})
agPNA	2.4 ± 0.2	0.4 ± 0.1* (83)[d]	1.6 ± 0.2* (33)	720 ± 100
agAAP	2.7 ± 0.2	2.6 ± 0.2 (4)	2.8 ± 0.2 (0)	< 10
agAFA	1.0 ± 0.1	0.30 ± 0.05* (70)	0.68 ± 0.05* (32)	400 ± 50
agSM	2.5 ± 0.2	0.45 ± 0.02* (82)	1.0 ± 0.1* (60)	350 ± 50
agSDM	2.8 ± 0.4	0.70 ± 0.04* (75)	not determined	270 ± 30
agPP	2.2 ± 0.3	1.2 ± 0.2* (46)	2.0 ± 0.2 (9)	120 ± 20
agMP	2.8 ± 0.3	0.4 ± 0.1* (86)	2.6 ± 0.3 (7)	40 ± 10

[a]Uptake is expressed as S/M ratio of prodrug, average ± s.e.mean.
[b]Substrate concentration was 100 μM (slice uptake) or 1 mM (conversion by homogenates). ag stands for N-acetyl-γ-glutamyl prodrug; PNA: *p*-nitroaniline, AAP: 4'-aminoantipyrine, AFA: aminophenylacetic acid, SM: sulphamethoxazole, SDM: sulphadimethoxine, PP: propranolol, MP: metoprolol.
[c]Inhibitor concentration was 1 mM.
[d]Percentage inhibition compared to control.
*Significantly different from control uptake ($P < 0.05$; Student's *t* test).

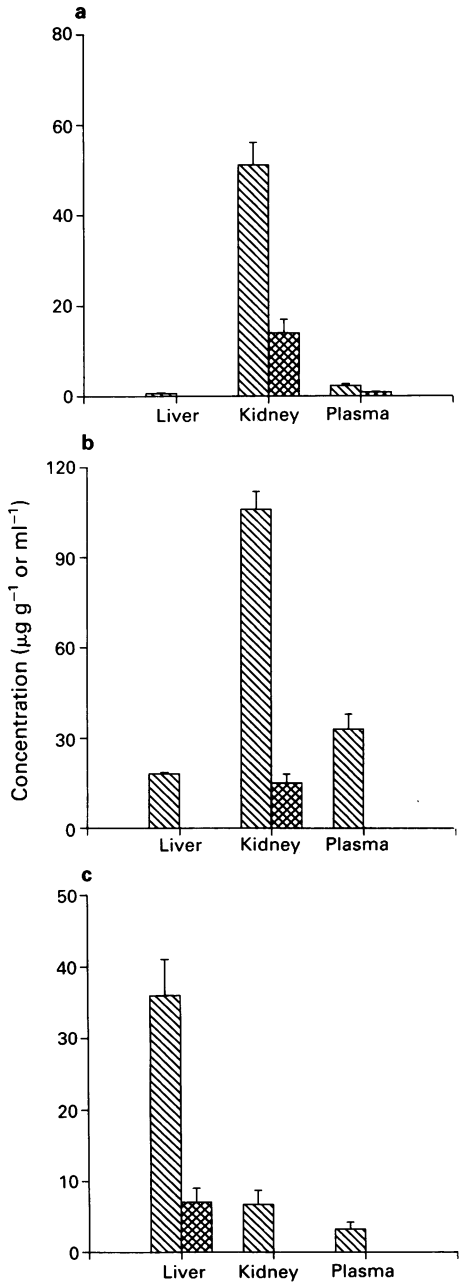


Figure 1 Tissue distribution (average ± s.e.mean; n = 4) of N-acetyl-L-γ-glutamyl prodrugs (hatched columns) and parent compounds (cross-hatched columns), 20 min after prodrug administration: (a) prodrug and *p*-nitroaniline; (b) prodrug and aminophenylacetic acid; (c) prodrug and propranolol.

unchanged and only to a limited extent as the parent compound; this is in contrast with the non-selective prodrug agPP (cf. 4'-aminowarfarin: 4% as prodrug and 2% as parent compound; Drieman & Thijssen, 1991). Remarkably, when gPNA was administered, 20% was excreted as agPNA; no gPNA and only 1% PNA was found in urine.

Pharmacokinetic parameters: The results are given in Table 2. The slope of the log plasma concentration-time curve of the three prodrugs was found to be linear. The volume of distribution of agPP was markedly higher than that of the other prodrugs, which may be explained by the high accumulation in the liver of this prodrug. The fraction of the dose excreted unchanged and the renal clearance (Cl_R) showed large differences between the prodrugs. A factor which could explain these differences is the renal transport (carrier mediated) of these prodrugs: the prodrugs with a high fe_u (agAFA and agPNA) accumulate in the kidney (see Figure 1). This corresponds with earlier results (Drieman *et al.*, 1990b; Drieman & Thijssen, 1991): the prodrug of sulphame-thoxazole ($fe_u = 0.72$) accumulated in the kidney, unlike the prodrug of 4'-aminowarfarin ($fe_u = 0.04$).

Discussion

In the last 15 years, several γ-glutamyl- and N-acetyl-L-γ-glutamyl prodrugs have been designed and tested for their renal selectivity, for instance: gludopa (Wilk *et al.*, 1978; Lee, 1987; Barthelmebs *et al.*, 1990; Cummings *et al.*, 1990), prodrugs of sulphamethoxazole (Orlowski *et al.*, 1980; Drieman *et al.*, 1990b), adamantine (Nagasawa *et al.*, 1980; Magnan *et al.*, 1982), CGP 22979 (Smits & Struyker-Boudier, 1985; Drieman *et al.*, 1990a), xanthine amine congener (XAC, Barone *et al.*, 1989) and SC 47792 (a dopamine β-hydroxylase inhibitor, Koepke *et al.*, 1991). Gludopa, sulpha-

Table 2 Pharmacokinetic parameters of N-acetyl-L-γ-glutamyl prodrugs[a]

	agAFA	*agPP*	*agPNA*	*gPNA*
Cl (ml min^{-1} kg^{-1})	9.9 ± 1.6	17.4 ± 2.5	39 ± 3	–
Vd (ml kg^{-1})	270 ± 50	670 ± 60	350 ± 50	–
$t_{1/2}$ (min)	18.9 ± 3	26 ± 3	6.3 ± 1.0	–
fe_u[b] prodrug	70 ± 10	14 ± 3	40 ± 5	0[c]
fe_u drug	3 ± 0.2	1.5 ± 0.1	2 ± 0.1	1 ± 0.1
Cl_R (ml min^{-1} kg^{-1})	6.9 ± 1.4	2.4 ± 0.5	16 ± 3	–

[a]Data are expressed as mean ± s.e.mean; $n = 4$; for abbreviations see Table 1.
[b]Fraction excreted in urine; percentage of dose of prodrug administered.
[c]20% of gPNA (γ-glutamyl PNA) was excreted as agPNA.

methoxazole, CGP 22979 and SC 47792 proved to be kidney-selective, while adamantine, XAC and 4'-aminowarfarin (Drieman & Thijssen, 1991) were not. In the present study a number of model prodrugs were investigated *in vitro* with slices and homogenates of rat kidney to determine their potential selectivity; three of these prodrugs were tested *in vivo*.

The aim of these experiments was to investigate how universally applicable the N-acetyl-L-γ-glutamyl prodrug approach is for drug targeting to the kidney and to investigate the usefulness of the *in vitro* screening studies for predicting *in vivo* selectivity.

The slice uptake studies show that not all N-acetyl-L-γ-glutamyl prodrugs are transported via a carrier-mediated system. The differences in transport inhibition by probenecid and BuSO indicate the involvement of at least two carriers in the transport of the prodrugs; this complicates structure-transport relationships. One major discrepancy between *in vitro* and *in vivo* accumulation is the behaviour of agPP. This prodrug does accumulate *in vitro*, an accumulation that can be inhibited by probenecid but not by buthionine sulphoximine. *In vivo*, agPP accumulates in the liver but not in the kidney. Possibly buthionine sulphoximine inhibition *in vitro* is a better predictor of kidney selectivity *in vivo*, indicating a major role for the buthionine sulphoximine-sensitive carrier compared to the probenecid-sensitive carrier *in vivo*. In general, the rat kidney slices seem to be a practicable *in vitro* system for screening prodrugs for possible kidney selectivity.

As carrier-mediated transport is a prerequisite for renal selectivity in our model (Drieman *et al.*, 1990b), only the prodrugs that were accumulated by the slices were investigated *in vivo*. Other investigators have also indicated the involvement of transport and intracellular conversion in the renal selectivity of both prodrugs (Hwang & Elfarra, 1989; 1991) and toxic compounds (Kramer *et al.*, 1987; Monks & Lau, 1987; Bennet, 1989; Zhang & Stevens, 1989).

The *in vitro* studies together with the *in vivo* tissue distribution of the prodrugs show that the N-acetyl-L-γ-glutamyl prodrugs of AFA and PNA are kidney-selective; agPP, agMP and agAAP are not selective. In combination with results obtained earlier (Drieman *et al.*, 1990a,b; Drieman & Thijssen, 1991) and with results described by others (Orlowski, 1980; Koepke *et al.*, 1991), this indicates that the concept of N-acetyl-L-γ-glutamyl prodrugs can be successfully applied to a number of structurally different drugs but also that this concept is not universally applicable.

The lack of selectivity of agAAP, and possibly XAC-prodrug (Barone *et al.*, 1989) is probably due to lack of affinity for the transporter or the converting enzyme. Steric hindrance at the nitrogen atom in the amide bond of agAAP may explain this lack of affinity.

agPP, however, and also N-acetyl-L-γ-glutamyl-4'-aminowarfarin (agAW; Drieman *et al.*, 1991) are transported and converted *in vitro*, but are not kidney-selective *in vivo* . An explanation for their lack of selectivity could be the following. *In vitro*, the accumulation is determined at equilibrium; the rate of uptake is not determined. This rate of uptake by the kidney of agPP is clearly insufficient compared to other metabolic routes, such as uptake and conversion by the liver. The renal clearance of the prodrugs, compared to total clearance, reflects the differences in transport rates and seems to be a more important parameter and a better predictor of renal selective uptake than the kidney slice accumulation.

Important in the pharmacokinetic behaviour of the prodrugs is the balance between the rate of uptake by the kidney and by other organs (e.g. the liver), and the balance between the intracellular conversion rate and the transport rate in- and out of the tubular cells.

An illustration of this last point is the urinary excretion of the prodrugs that are renally selective. These prodrugs are excreted for a large part unchanged into the urine, and only a minor proportion is converted to the active drug. This is in accordance with the results obtained with agSM (Drieman *et al.*, 1990b). A change in residence time in the proximal tubular cell or a change in the rate of conversion will have a profound effect on the renal concentrations of the prodrug and active drug. A difference in residence time and conversion rate could in part explain the differences in selectivity between agAFA and agPNA. This is supported by the *in vitro* kidney slice uptake studies (Table 1), which show that agPNA is accumulated to a larger extent, and the *in vivo* kinetic parameters (Table 2), which show that the plasma half-life time of agPNA is much shorter. These observations can explain the high kidney to plasma ratio of agPNA compared to agAFA.

In general, there is a correlation between the renal clearance and kidney concentrations of the prodrugs *in vivo*. This is a strong indication for trans-tubular transport by carrier-mediated uptake from the blood across the basolateral membrane and excretion in the tubular lumen. Tubular reabsorption can result in high kidney concentrations but not in a high renal clearance. This supports the model described earlier (Drieman *et al.*, 1990b).

One of the conversion steps is deacetylation of the γ-glutamyl moiety. The balance between acetylation and deacetylation has been shown to be important for the renal toxicity of cysteine conjugates (Commandeur *et al.*, 1991). The same may apply to the renal selectivity of the N-acetyl-L-γ-glutamyl prodrugs: relatively large amounts of acetylated prodrug were found in the urine following administration of γ-glutamyl PNA, in contrast to γ-glutamyl SM, where no acetylated prodrug was found in the urine (Drieman *et al.*, 1990b).

One important point which we did not investigate, but which is known from literature, is the ability of the tubular cells to retain the active drug (Stella & Himmelstein, 1980). If the parent compound has a high lipophilicity, it is probable that it will redistribute throughout the whole body.

In summary, it may be concluded that the rat kidney slice *in vitro* system is useful in screening drugs for possible kidney selectivity, but accumulation in the slices is not a guarantee for *in vivo* selectivity. Furthermore, this study shows that some, but not all N-acetyl-L-γ-glutamyl prodrugs are kidney selective. This can be explained by the strong influence of the active drug on the pharmacokinetic behaviour of the prodrug, in contrast to the influence of the active drug on marcomolecular carriers (though some macromolecular targeting devices have been described to be strongly influenced by the drug attached, van der Sluijs *et al.*, 1986). More prodrugs will have to be evaluated to gain a thorough insight in the structure-transport and structure-conversion relationships of these prodrugs, considering the multiple systems involved (at least two carriers and two intracellular converting enzymes). Meanwhile, in view of the success of some of the N-acetyl-L-γ-glutamyl prodrugs (see Smits & Struyker Boudier, 1985; Koepke *et al.*, 1991), it is in our view a good strategy to investigate prodrugs of pharmacologically active compounds that might be more beneficial when delivered intrarenally only, such as angiotensin converting enzyme (ACE) inhibitors, calcium antagonists, immunosuppressants or anti-inflammatory drugs.

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Differential sensitivity of antinociceptive assays to the bradykinin antagonist Hoe 140

Christopher G. Heapy, [1]John S. Shaw & Susannah C. Farmer

Bioscience 1, ICI Pharmaceuticals, Alderley Park, Macclesfield, Cheshire SK10 4TG

1 The antinociceptive activity of the bradykinin (BK) BK_2 receptor antagonist D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]BK (Hoe 140) was determined in a range of mouse abdominal constriction assays.

2 Hoe 140 potently inhibited the response induced by i.p. injection of 10 μg BK/mouse, and 1 μg BK/mouse in mice pre-sensitized by i.p injection of prostaglandin E_2 (PGE_2). The ED_{50} values in these assays were 1.9 and 3.7 μg kg^{-1} respectively. This confirms that Hoe 140 is a potent antagonist of BK *in vivo*.

3 Hoe 140 produced potent, but incomplete inhibition of the responses evoked by i.p injection of kaolin or 0.25% acetic acid. ED_{25} values in these assays were 2.7 and 16.1 μg kg^{-1}, and the maximum inhibition produced was 60% and 70% respectively.

4 At doses up to 1 mg kg^{-1}, Hoe 140 was completely ineffective against the abdominal constriction response induced by zymosan. In contrast, morphine, ibuprofen and indomethacin had similar potencies against zymosan, kaolin and acetic acid-induced abdominal constriction.

5 Although zymosan, acetic acid and kaolin all produce qualitatively similar responses, it is appears that they achieve this by different mechanisms. The extent to which BK is involved as a mediator differs between the various types of abdominal constriction assay.

Keywords: Analgesia; bradykinin antagonist; BK_2 receptor; abdominal constriction; indomethacin; ibuprofen

Introduction

Injection of bradykinin (BK) or its application to a blister base causes pain in human volunteers (Whalley *et al.*, 1987). Moreover, significant levels of BK are found at sites of inflammation (Melmon *et al.*, 1967). This has led to speculation that BK antagonists may be useful for the relief of inflammatory pain. However, the extent to which BK contributes to the pain associated with pathological conditions remains to be determined. Although there are significant amounts of BK in inflammatory exudates, other mediators such as prostaglandin E_2 (PGE_2) and PGI_2 are also present. The relative contribution of these mediators to pain and hyperalgesia remains largely unknown.

In animal models it had been established that BK is involved in the pain and hyperalgesia caused by a variety of irritant substances. The hyperalgesia which follows the injection of sodium urate crystals into the rat paw is attenuated by the BK_2 receptor antagonists D-Arg-[Hyp^3,D-Phe^7]BK (NPC 567) and D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]BK (NPC 349). These same compounds have also been reported to reduce the abdominal constriction response induced by intraperitoneal injection of 0.6% acetic acid (Steranka *et al.*, 1987). However, the low potency of these antagonists, and their susceptibility to peptidases has severely limited further investigation of their antinociceptive efficacy.

Recently a much more potent and stable BK_2 receptor antagonist has become available. In radiogland binding studies D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]BK (Hoe 140) was reported to displace BK with a K_i value of 8×10^{-10} M (Hock *et al.*, 1991). In isolated tissue assays, Hoe 140 antagonized the action of BK at the BK_2-receptor with pA_2 values ranging from 9.3 in the rat uterus to 8.1 in the guinea-pig ileum (Lembeck *et al.*, 1991; Hock *et al.*, 1991). The compound was reported to be devoid of activity at the BK_1 receptor (Hock *et al.*, 1991). When administered to rats, Hoe 140 inhibited the hypotensive response to intravenous BK for up to 5 h (Wirth *et al.*, 1991). This compound therefore presents the first opportunity to evaluate in detail the involvement of BK in animal models of antinociceptive activity.

The aim of this study was to determine the antinociceptive activity of Hoe 140 in the abdominal constriction model. A range of stimuli were employed. The BK-induced response was used to determine the dose of Hoe 140 required to antagonize BK receptors *in vivo*. Other studies employed kaolin, which is reported to activate the kallikrein-kinin system (Fujiyoshi *et al.*, 1989b,c), and zymosan which activates the complement cascade and induces PGI_2 synthesis (Rampart *et al.*, 1981; Doherty *et al.*, 1987). The precise mechanism by which dilute acetic acid induces the abdominal constriction response has yet to be elucidated. However, in common with zymosan and kaolin, intraperitoneal injection of acetic acid induces the synthesis of cyclo-oxygenase products, and the resulting abdominal constriction response is attenuated by cyclo-oxygenase inhibitors (Doherty *et al.*, 1987; Fujiyoshi *et al.*, 1989a; Deraedt *et al.*, 1980). A preliminary account of this work has already been published (Heapy *et al.*, 1991).

Methods

All studies were conducted on groups of 10 female mice of the Alderley Park strain weighing between 18 and 25 g. Each experiment was repeated on at least 3 separate occasions. Drugs were dissolved in saline, to which a small amount of $NaHCO_3$ was added in the case of indomethacin and ibuprofen (final pH 7.0–7.4). All compounds were administered subcutaneously in a volume of 10 ml kg^{-1} 30 min before challenge with the constriction-inducing agent.

The abdominal constriction response was induced by injecting 0.4 ml of either acetic acid (0.25% v/v), kaolin (7.5 mg ml^{-1}), zymosan (2.4 mg ml^{-1}) or bradykinin (25 μg ml^{-1}). When BK was used in combination with PGE_2, mice were injected with 0.2 ml PGE_2 (0.5 μg ml^{-1}) 15 min prior to

[1] Author for correspondence.

a 0.2 ml challenge with BK (5 μg ml^{-1}).

With the exception of groups challenged with BK, the responses were counted over a 15 min period by a trained observer who pressed the appropriate key on a microcomputer each time a response occurred. The computer recorded the time at which each response was logged, allowing subsequent analysis of the time course of the response. ED_{50} values (dose to reduce the number of responses to 50% of control) were calculated by linear regression from the data accumulated over the full 15 min observation period.

Because the response to BK was transient, the BK-treated groups were observed for 30 s following challenge, and the number of mice responding during this period was recorded. ED_{50} values (dose which protected 50% of animals) were calculated by the method of Litchfield & Wilcoxon (1949).

Materials

D-Arg-[Hyp^3,Thi^5,D-Tic^{7}Oic^8]BK (Hoe 140) was synthesized by Dr R. Cotton (ICI Pharmaceuticals). Other materials were obtained from commercial sources: morphine from Macfarlan-Smith, Edinburgh; zymosan, PGE_2, bradykinin, indomethacin and ibuprofen from Sigma, Poole, England.

Results

Antagonism of bradykinin by Hoe 140

To determine the doses of Hoe 140 required to antagonize the actions of BK *in vivo*, the ability of the compound to inhibit BK-induced abdominal constriction responses was determined. Two protocols were employed. In the first, responses were induced by the intraperitoneal injection of 10 μg BK. In the second, mice were pretreated with PGE_2 (100 ng, i.p.) 15 min prior to BK injection. The effect of the PGE_2 was to sensitize the mice, thus allowing the dose of BK to be reduced ten fold to 1 μg.

Animals given PGE_2 (100 ng, i.p.) alone did not exhibit the abdominal constriction response (data not shown). In both assays Hoe 140 produced a dose-related inhibition and completely abolished the response at a dose of 30 μg kg^{-1} (Figure 1a, Table 1).

Antinociceptive tests

Antinociceptive activity was measured in three abdominal constriction models employing acetic acid, zymosan and kaolin as stimuli. Preliminary experiments were conducted to select concentrations of the three algesic agents which produced similar levels of response. At the chosen concentrations, the mean number of responses per 15 min observation period was 15.6 ± 0.8 for zymosan, 18.5 ± 0.9 for acetic acid and 18.3 ± 0.7 for kaolin. Further confirmation that the three agents produce stimuli of similar intensity is provided by the finding that morphine had similar potency in each assay (Figure 1).

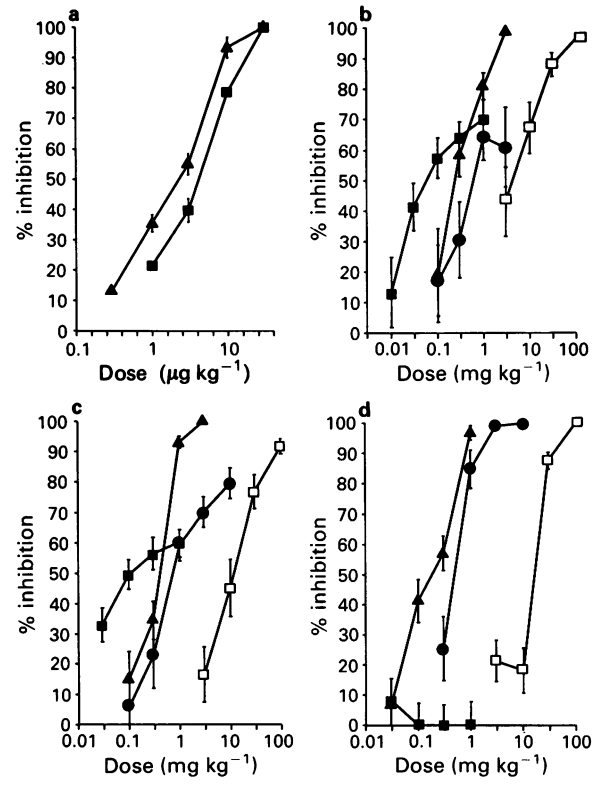


Figure 1 Dose-response curves: (a) Inhibition by Hoe 140 of the percentage of mice exhibiting an abdominal constriction response after 10 μg bradykinin (BK)/mouse (▲), or 1 μg BK/mouse in mice pretreated 15 min earlier with 100 ng PGE_2 (■). (b, c, d) Inhibition by Hoe 140 (■), indomethacin (●), ibuprofen (□) and morphine (▲) of the number of abdominal constriction responses induced by (b) 0.25% acetic acid. (c) kaolin and (d) zymosan. All compounds were injected subcutaneously 30 min before challenge. Data points represent the mean of 3 experiments, each of which employed groups of 10 mice; vertical bars show s.e.mean.

Antinociceptive activity of Hoe 140

Hoe 140 produced potent inhibition of the acetic acid induced-abdominal constriction response (Figure 1b). However, the bradykinin antagonist was unable to inhibit the response completely. The highest dose tested (1 mg kg^{-1}) produced a maximum inhibition of 60–70%. In contrast, morphine was able to abolish the response. An unexpected finding was the difference between the two cyclo-oxygenase

Table 1 Potencies of Hoe 140 and standard antinociceptive agents in abdominal constriction models

	ED_{50} (mg kg^{-1}, s.c.) *and 95% confidence interval*			
	Hoe 140	*Morphine*	*Indomethacin*	*Ibuprofen*
Zymosan	NA	0.17 (0.12–0.25)	0.52 (0.31–0.78)	13.4 (6.3–22.4)
Acetic acid	0.016[a] (0.008–0.024)	0.33 (0.21–0.53)	0.47 (0.18–2.51)	3.8 (1.1–7.9)
Kaolin	0.0027[a] (0.002–0.004)	0.27 (0.20–0.35)	0.86 (0.49–1.66)	12.3 (6.9–20.8)
Bradykinin	0.0019 (0.001–0.004)	–	–	–
Bradykinin + PGE_2	0.0037 (0.003–0.005)	–	–	–

[a]ED_{25} values quoted.

inhibitors. Whilst ibuprofen was able to abolish the response, indomethacin appeared capable of inhibiting it by only 70%.

Similar findings were obtained when kaolin was used as the stimulus (Figure 1c). Only a portion of the response (60%) was sensitive to Hoe 140, whilst both ibuprofen and morphine produced complete inhibition. As in the acetic acid assay, a small portion of the response appeared to be insensitive to indomethacin.

In marked contrast to the other assays, Hoe 140 was completely without effect against zymosan-induced abdominal constriction (Figure 1d). Morphine, indomethacin and ibuprofen all abolished the response at doses similar to those which were effective in the other assays.

Time course studies

In addition to measuring the total number of responses during the 15 min observation period, the time at which each response occurred was also recorded. The time courses following injection of acetic acid (Figure 2), kaolin (Figure 3) and zymosan (not shown) were broadly similar. The response to each agent peaked 4 to 5 min after injection, and slowly declined thereafter. The rate at which the acetic acid response decayed was somewhat slower than with the other two agents, and the response appeared to be biphasic, with an early peak occurring 4 min after injection followed by a less intense but more sustained response between 8 and 13 min.

Hoe 140 reduced both the early and late parts of the acetic acid response, but even at the highest dose the compound was unable to suppress completely either component (Figure 2a). In contrast, when kaolin was used to induce the response

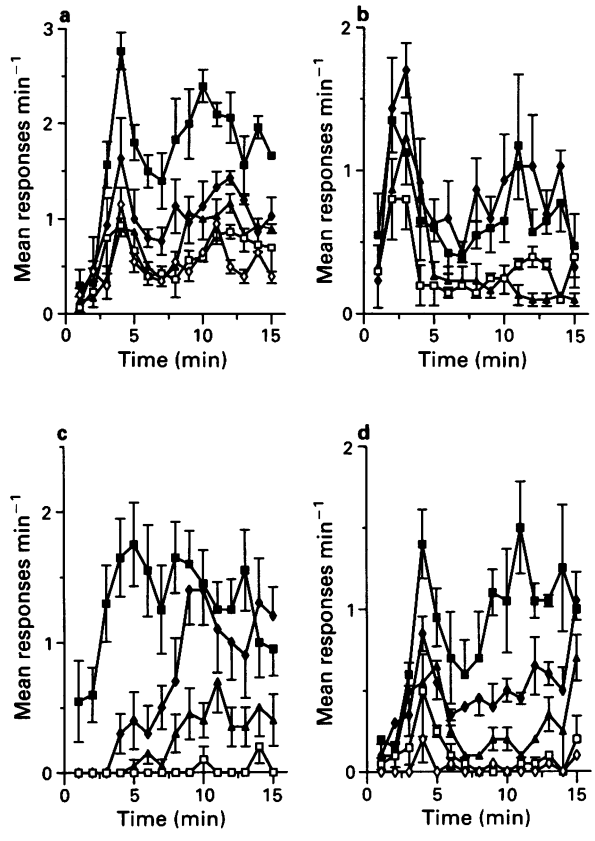


Figure 2 Time course for the inhibition of acetic acid-induced abdominal constriction response by (a) Hoe 140, (b) indomethacin, (c) morphine and (d) ibuprofen. (a) (■) Vehicle; (◆) Hoe 140 0.03 mg kg^{-1}, (▲) 0.1 mg kg^{-1}, (□) 0.3 mg kg^{-1} and (◇) 1 mg kg^{-1}. (b) (■) Vehicle; (◆) indomethacin 0.1 mg kg^{-1}, (▲) 1 mg kg^{-1} and (□) 3 mg kg^{-1}. (c) (■) Vehicle; (◆) morphine 0.3 mg kg^{-1}, (▲) 1 mg kg^{-1} and (□) 3 mg kg^{-1}. (d) (■) Vehicle; (◆) ibuprofen 3 mg kg^{-1}, (▲) 10 mg kg^{-1}, (□) 30 mg kg^{-1}, (◇) 100 mg kg^{-1}.

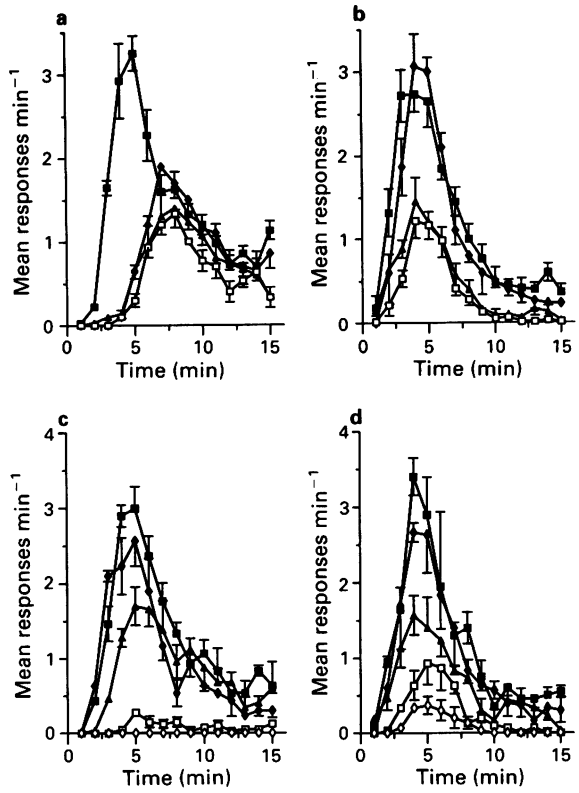


Figure 3 Time course for the inhibition of kaolin-induced abdominal constriction response by (a) Hoe 140, (b) indomethacin, (c) morphine and (d) ibuprofen. (a) Vehicle (■); (◆) Hoe 140 0.01 mg kg^{-1}, (▲) 0.1 mg kg^{-1}, (□) 1 mg kg^{-1}. (b) (■) Vehicle; (◆) indomethacin 0.1 mg kg^{-1}, (▲) 1 mg kg^{-1} and (□) 3 mg kg^{-1}. (c) (■) Vehicle; (◆) morphine 0.1 mg kg^{-1}, (▲) 0.3 mg kg^{-1}, (□) 1 mg kg^{-1}. (d) (■) Vehicle; (◆) ibuprofen 3 mg kg^{-1}, (▲) 10 mg kg^{-1}, (□) 30 mg kg^{-1}, and (◇) 100 mg kg^{-1}.

(Figure 3a), Hoe 140 abolished the effect at early time points, but was without any significant effect from 7 min onwards.

The difference in efficacy between indomethacin and ibuprofen is also apparent in the time course data. In both the acetic acid and the kaolin assays the cyclo-oxygenase inhibitors were less effective against the early part of the response than against the later component. However, whilst the early response was inhibited by higher doses of ibuprofen, a significant proportion of the response appeared to be completely resistant to indomethacin.

Discussion

The first part of this study clearly demonstrates that Hoe 140 is a potent antagonist of bradykinin following subcutaneous administration to the mouse. This confirms and extends earlier findings in the dog, guinea-pig and rat (Wirth *et al.*, 1991; Lembeck *et al.*, 1991, Bao *et al.*, 1991). Hoe 140 therefore represents a valuable tool with which to investigate the role of bradykinin in other *in vivo* assays.

Earlier studies using BK antagonists such as D-Arg-[Hyp^3,D-Phe^7]BK and D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]BK have revealed that BK antagonists display antinociceptive activity against BK-induced and urate-induced hyperalgesia of the rat paw (Steranka *et al.*, 1988) and in an acetic acid-induced abdominal constriction assay (Steranka *et al.*, 1987). In the latter study, these compounds failed to inhibit totally the response to acetic acid. Given the relatively low potency and short duration of action of these early antagonists, it was impossible to determine whether this remaining response was due to mediators other than BK, or due to the poor phar-

macokinetics of the antagonists used. The present study clarifies this point. At doses thirty times greater than were required to abolish BK-induced responses, Hoe 140 produced only 70% inhibition of the acetic acid-induced response. This clearly demonstrates that BK is a major component, but not the only mediator of the acetic acid response. However, there is little evidence from the time course study of any temporal separation between the components of the acetic acid response.

The effects seen against kaolin were qualitatively similar to those obtained with acetic acid. The proportion of the response that was sensitive to Hoe 140 was similar to both assays. It has previously been reported that the kaolin-induced abdominal constriction response is abolished by soyabean trypsin inhibitor, a non-specific kallikrein inhibitor (Fujiyoshi *et al.*, 1989c). This would imply that the response is almost entirely due to BK. The present finding that a significant proportion of the response is insensitive to a potent antagonist at the BK_2 receptor could be interpreted as suggesting the action of BK involves both BK_1 and BK_2 receptors. Hoe 140 is not an antagonist at the BK_1 site (Hock *et al.*, 1991). Alternatively, additional mediators could contribute to the response. Data from the time course study, which show that Hoe 140 abolished the kaolin response only at early time points (<6 min), confirm that this response consists of more than one component.

Of particular interest is the finding that Hoe 140 had no effect against the zymosan-induced response. Nevertheless, in all three assays the response was inhibited by cyclo-oxygenase inhibitors. The most obvious interpretation is that a cyclo-oxygenase product is the final common mediator produced by all stimuli, but in the kaolin and acetic acid models, prostanoid synthesis is, at least in part, secondary to kinin generation. The prostanoid synthesis induced by zymosan is independent of the kinin system. This hypothesis is consistent with earlier observations that acetic acid, kaolin and zymosan all induce the release of prostanoids into the peritoneal cavity (Deraedt *et al.*, 1980; Doherty *et al.*, 1987; Fujiyoshi *et al.*, 1989a). It has also been shown that kinin levels in the peritoneal cavity increase following injection of kaolin (Fujiyoshi *et al.*, 1990), but do not increase in response to zymosan (Damas *et al.*, 1990). Furthermore, Ferreira *et al.* (1973) have demonstrated that BK is a potent stimulus for prostaglandin release.

An interesting finding was the apparent difference in antinociceptive efficacy between two cyclo-oxygenase inhibitors, ibuprofen and indomethacin. This finding is in accord with an earlier study by Luttinger (1985) in which ibuprofen was shown to increase the reaction time of mice in response to dipping their tails in water at 45°C, whereas indomethacin was without effect. Since both indomethacin and ibuprofen are assumed to share a common mode of action, there is no simple explanation for these findings.

The results of this study demonstrate that kinins play a major role in the abdominal constriction response induced by acetic acid and kaolin, but are minimally involved in the response to zymosan. Since cyclo-oxygenase inhibitors were effective in all three assays, one interpretation of these findings is that BK antagonists will prove to be less effective in the treatment of pain than the non-steroidal anti-inflammatory agents. On the other hand, it is known that kinins are present in synovial fluid from patients with rheumatoid arthritis. Moreover, the concentrations of BK found in patients are comparable to those which have been shown to activate nociceptors (Dunn & Rang, 1990). On this basis it could reasonably be argued that kinins must at least contribute to arthritic pain. Thus a BK_2 receptor antagonist may prove to be an effective treatment for inflammatory pain, and the predictive value of the zymosan abdominal constriction assay would be called into question. The data available at present do not allow either of these possibilities to be dismissed.

Given the wide range of mediators produced at inflammatory sites, it is difficult to know whether removing the action of just one of these, BK, will provide a significant therapeutic benefit. It is likely that this question will only be answered when a BK antagonist is evaluated in man.

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Charybdotoxin-sensitive K^+ channels regulate the myogenic tone in the resting state of arteries from spontaneously hypertensive rats

[1]Masahisa Asano, Kaoru Masuzawa-Ito & Tomohiro Matsuda

Department of Pharmacology, Nagoya City University Medical School, Mizuho-ku, Nagoya 467, Japan

1 To determine the possible role of Ca^{2+}-activated K^+ (K_{Ca}) channels in the regulation of resting tone of arteries from spontaneously hypertensive rats (SHR), the effects of agents which interact with these channels on tension and ^{86}Rb efflux were compared in endothelium-denuded strips of carotid, femoral and mesenteric arteries from SHR and normotensive Wistar-Kyoto rats (WKY).

2 Strips of carotid, femoral and mesenteric arteries from SHR exhibited a myogenic tone; that is, the resting tone decreased when either the Krebs solution was changed to a $0\text{-}Ca^{2+}$ solution or 10^{-7} M nifedipine was added.

3 The addition of charybdotoxin (ChTX, 10^{-9}–10^{-7} M), a blocker of large conductance K_{Ca} channels, to the resting strips of these arteries produced a concentration-dependent contraction, which was significantly greater in SHR than in WKY. Relatively low concentrations of tetraethylammonium (0.05–5 mM) produced a concentration-dependent contraction which was similar to the ChTX-induced contraction in these strips.

4 The ChTX-induced contractions in SHR were greatly attenuated by 10^{-7} M nifedipine and by 3×10^{-6} M cromakalim, a K^+ channel opener. Cromakalim alone abolished the myogenic tone in SHR.

5 The addition of apamin (a blocker of small conductance K_{Ca} channels, up to 10^{-6} M), or of glibenclamide (a blocker of ATP-sensitive K^+ channels, up to 5×10^{-6} M), to the resting strips failed to produce a contraction.

6 In resting strips of carotid, femoral and mesenteric arteries preloaded with ^{86}Rb, the basal ^{86}Rb efflux rate constants were significantly greater in SHR than in WKY. The addition of 10^{-7} M nifedipine to the resting strips decreased the basal ^{86}Rb efflux rate constants only in SHR.

7 The cellular Ca^{2+} uptake in the resting state of carotid and femoral arteries from SHR was significantly increased when compared to WKY, and this increase in SHR was significantly reduced by 10^{-7} M nifedipine.

8 These results suggest that the ChTX-sensitive K_{Ca} channels were highly activated to regulate the myogenic tone in the resting state of carotid, femoral and mesenteric arteries from SHR. The increased K_{Ca} channel functions in SHR arteries appeared to be secondary to the increased Ca^{2+} influx via L-type voltage-dependent Ca^{2+} channels in the resting state of these arteries.

Keywords: Ca^{2+}-activated K^+ channels; charybdotoxin; ^{86}Rb efflux; voltage-dependent Ca^{2+} channels; ^{45}Ca influx; Ca^{2+} uptake; spontaneously hypertensive rats (SHR); arterial smooth muscle

Introduction

The total peripheral resistance is increased in patients with hypertension and in various models of experimental hypertension. The increased resistance may be caused by increased arterial contractility. It has been demonstrated that the cellular Ca^{2+} uptake and/or content are increased in various tissues and cells isolated from patients and experimental animals with essential hypertension. Particularly, an abnormality in the Ca^{2+} handling of arterial smooth muscle has been demonstrated in spontaneously hypertensive rats (SHR) (for review, see Postnov & Orlov, 1985; Lau & Eby, 1985; Bohr & Webb, 1988). Since the myofilaments in arterial smooth muscle are activated by a rise in the cellular concentration of free Ca^{2+}, the alteration of the cellular Ca^{2+} uptake and/or content may conceivably provide a logical interpretation of some of the abnormal functions associated with hypertension, including an increased incidence of myogenic tone, hyperreactivity to vasoconstrictors and decreased relaxation to vasodilators.

Arteries isolated from SHR exhibit a spontaneous active tone (Noon *et al.*, 1978; Fitzpatrick & Szentivanyi, 1980; Winquist & Bohr, 1983; Asano *et al.*, 1986). The spontaneous active tone is independent of regional innervation and circulating hormones and has been termed myogenic. The myogenic tone is abolished by the removal of external Ca^{2+} (Noon *et al.*, 1978; Fitzpatrick & Szentivanyi, 1980; Winquist & Bohr, 1983) and by calcium channel blockers (Asano *et al.*, 1986), suggesting that the myogenic tone depends on Ca^{2+} influx via voltage-dependent Ca^{2+} channels. The ability of arteries to produce myogenic tone and to maintain it in the resting state also suggests the ability to produce membrane depolarization and subsequent Ca^{2+} influx. To counteract this ability, a negative feedback mechanism may exist that is activated by membrane depolarization and/or cellular Ca^{2+}. Ca^{2+}-activated K^+ (K_{Ca}) channels are an ideal candidate for such a mechanism because they are activated by both cellular Ca^{2+} and membrane depolarization (for review, see Cook, 1988; Castle *et al.*, 1989). K_{Ca} channels are subdivided into at least three types; large, intermediate and small conductance K_{Ca} channels, according to the difference in single channel conductance. Differences in the mechanisms of activation of these channels may reflect their functional roles, with some channels maintaining resting tone and others terminating or limiting contraction induced by vasoconstrictors. Pharmacologically, charybdotoxin (ChTX) and apamin are

[1] Author for correspondence.

relatively selective and potent blockers of large and small conductance K_{Ca} channels, respectively (Cook, 1988; Castle *et al.*, 1989). ChTX-sensitive large conductance K_{Ca} channels have been identified in single cells from vascular smooth muscles (Sugg *et al.*, 1990; Pavenstadt *et al.*, 1991; Brayden & Nelson, 1992). Although small conductance K_{Ca} channels have been found in vascular smooth muscles, it has not been determined whether the channels are sensitive to apamin (Inoue *et al.*, 1985; Benham *et al.*, 1986). In the present study, we examined the possible role of K_{Ca} channels in the regulation of myogenic tone in the resting state of arterial smooth muscle from SHR, by using standard tension-recording methods and ^{86}Rb efflux (used to monitor K^+ permeability) experiments.

Methods

Male SHR, 13 weeks of age, and age-matched male normotensive Wistar-Kyoto rats (WKY) were bred in our laboratory. Systolic blood pressures at this age, measured by the tail-cuff plethysmography, were significantly ($P<0.001$) higher in SHR (199 ± 3 mmHg, $n = 20$) than in WKY (138 ± 2 mmHg, $n = 20$). Body weights were not significantly different between SHR (250 ± 7 g, $n = 20$) and WKY (268 ± 7 g, $n = 20$).

Preparation of arterial strips and measurements of mechanical activity

The rats were stunned by a blow to the head and then exsanguinated. The common carotid artery (1.0–1.2 mm outside diameter), the femoral artery (0.7–0.9 mm o.d.) and the main branch of the superior mesenteric artery (0.7–0.9 mm o.d.) were excised and placed in Krebs solution of the following composition (in mM): NaCl 115.0, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and dextrose 10.0. Helical strips (0.8 mm × 8 mm) of these arteries were prepared as described previously (Asano *et al.*, 1988). The endothelium of the strip was removed by gently rubbing the endothelial surface with cotton pellets.

Strips were mounted vertically in water-jacketed muscle baths containing 10 ml Krebs solution (37°C). The Krebs solutions were aerated with 95% O_2 and 5% CO_2. The isometric tension was recorded with a force-displacement transducer (TB-612T, Nihon Kohden Kogyo Co., Tokyo, Japan). Strips were stretched passively to optimal length by imposing resting tension (carotid, 0.4 g; femoral, 0.6 g; mesenteric, 0.5 g) and a 90 min equilibration period preceded each experiment. The optimal resting tension was determined by a length-passive tension study (Asano *et al.*, 1988). All experiments were conducted in phenoxybenzamine-treated strips to eliminate possible α-adrenoceptor responses to noradrenaline that may be released from adrenergic nerve endings by depolarizing stimuli (Asano *et al.*, 1988).

After determination of the contractile responses of the strips to Krebs solution containing 65.9 mM KCl (K^+) (K^+ substitution for Na^+), the contractile responses to ChTX, apamin, tetraethylammonium (TEA; a relatively nonselective blocker of K^+ channels) or glibenclamide (a blocker of ATP-sensitive K^+ channels) were determined.

In some experiments, the effect of $0\text{-}Ca^{2+}$ solution, nifedipine (a blocker of L-type voltage-dependent Ca^{2+} channels) or cromakalim (a K^+ channel opener) on the resting tone was examined. The $0\text{-}Ca^{2+}$ solution was prepared by omission of Ca^{2+} from the Krebs solution and by addition of 0.1 mM EGTA.

Measurement of ^{86}Rb efflux from arterial strips

^{86}Rb efflux was measured simultaneously with tension changes as described previously (Masuzawa *et al.*, 1990; 1991). Briefly, after a 90 min equilibration, strips were incubated for an additional 3 h in the Krebs solution to which 14–20 μCi ml^{-1} ^{86}Rb had been added. Each strip was then dipped (15 s) into non-radioactive Krebs solution and transferred to a superfusion chamber. A resting tension of 1.2 g (carotid), 1.8 g (femoral) or 1.5 g (mesenteric) was applied because larger strips were used. The superfusate was sampled by use of a collection period of 2 min and counted for radioactivity in an Aloka autowell gamma counter. On completion of the efflux, each strip was solubilized. The rate constant of ^{86}Rb efflux was then calculated as described previously (Masuzawa *et al.*, 1990; 1991).

Measurement of cellular Ca^{2+} uptake

Cellular Ca^{2+} uptake was measured from the basal ^{45}Ca influx as described previously (Asano & Hidaka, 1985). Briefly, isolated arteries were equilibrated in a Tris-buffered solution of the following composition (in mM): NaCl 154.0, KCl 5.4, $CaCl_2$ 2.5, dextrose 11.0 and Tris 6.0 (pH 7.4). The Tris-buffered solutions were aerated with 100% O_2. Arteries were then transferred to the Tris-buffered solution to which 1 μCi ml^{-1} ^{45}Ca had been added. After a 5 min incubation time, the arteries were placed in test tubes containing the 80.8 mM La^{3+}-substituted solution at 0.5°C for 60 min. Each artery was then placed in a glass scintillation vial containing 0.2 ml NCS tissue solubilizer (Amersham International, Buckinghamshire). Solubilized tissues were mixed with 5 ml Amersham ACS II scintillant and counted for radioactivity in an Aloka liquid scintillation counter. Values for cellular Ca^{2+} uptake (expressed as nmol g^{-1} tissue wet weight) were then calculated as described previously (Asano & Hidaka, 1985).

Statistical analysis

Unless specified, results shown in the text, tables and figures are expressed as means ± s.e.mean (n = number of preparations). Student's t test for paired or unpaired data, or variance analysis was used to determine the significance of differences between means, and a P value of <0.05 was taken as significant.

Drugs and isotopes

The drugs used were ChTX (Peptide Institute Inc., Minoh, Japan), TEA chloride (Sigma Chemical Co., St. Louis, MO, U.S.A.), apamin (Sigma), glibenclamide (Sigma), nifedipine (Bayer Yakuhin Ltd., Osaka, Japan), papaverine hydrochloride (Wako Pure Chemical Industries, Osaka, Japan), cromakalim (Beecham Pharmaceuticals Research Division, Harlow, Essex, UK) and phenoxybenzamine hydrochloride (Nakarai Chemicals, Kyoto, Japan). $^{86}RbCl$ (specific activity initially 1.5–2.9 mCi mg^{-1}) and $^{45}CaCl_2$ (specific activity initially 16.7–28.5 mCi mg^{-1}) were obtained from Amersham International (Buckinghamshire).

Nifedipine (1 mM) and phenoxybenzamine (1 mM) were dissolved in 50% ethanol with further dilution in distilled water before use. Cromakalim (10 mM) was dissolved in 60% ethanol with further dilution in distilled water before use. Glibenclamide (1 mM) was dissolved in 50% ethanol with further dilution in the same solvent before use. Aqueous stock solutions were prepared for other drugs. Concentrations of drugs are expressed as final molar concentrations.

Results

Myogenic tone in the resting state of spontaneously hypertensive rat arteries

Carotid arteries isolated from SHR exhibited a myogenic tone; typically small magnitude oscillations superimposed on a tonic contraction (Figures 1 and 2). Such oscillation was

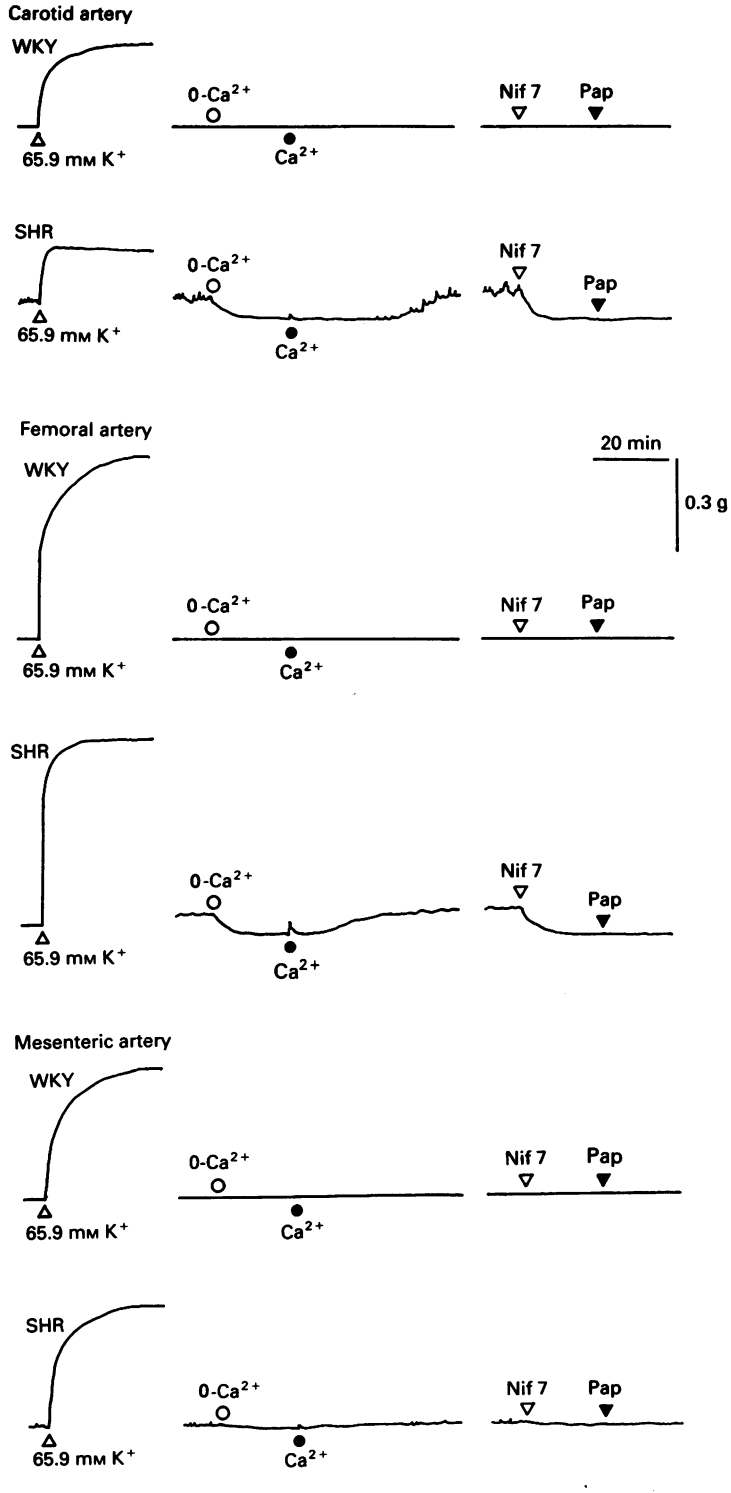


Figure 1 Typical recordings of the effects of $0-Ca^{2+}$ solution ($0-Ca^{2+}$) and nifedipine (Nif) on resting strips of carotid, femoral and mesenteric arteries from 13 week old WKY and SHR. After the 90 min equilibration, strips were maximally activated by repeated application of 65.9 mM K^+ until the responses were reproducible. Following washout with a Krebs solution, the solution was replaced with a $0-Ca^{2+}$ solution for 20 min, and then 2.5 mM Ca^{2+} (Ca^{2+}) was added to the $0-Ca^{2+}$ solution. The strips were then washed with a Krebs solution for 40 min, and were exposed to 10^{-7} M Nif. At the end of each experiment, 10^{-4} M papaverine (Pap) was added to identify the position of the maximum relaxation. Note the existence of an oscillatory contraction in carotid and mesenteric arteries from SHR and of a tonic contraction in femoral arteries from SHR in the resting state.

occasionally observed in mesenteric arteries from SHR (13 out of 35 preparations), but was rare in femoral arteries from SHR (9 out of 51 preparations) in which a tonic contraction was usually observed (Figures 1 and 2). These arteries relaxed from the resting tone when placed in a $0-Ca^{2+}$ solution (Figure 1). The relaxation in these arteries was $28.9 \pm 3.1\%$ (carotid, $n = 14$), $9.8 \pm 1.8\%$ (femoral, $n = 22$) and $2.7 \pm 0.6\%$ (mesenteric, $n = 12$), respectively, of the 65.9 mM K^+-induced maximum contraction. After the 20 min exposure to the $0-Ca^{2+}$ solution, the addition of 2.5 mM Ca^{2+} restored the myogenic tone completely. The addition of 10^{-7} M nifedipine to the resting strips of these arteries placed in a Krebs solution also caused a relaxation in SHR which was comparable to the relaxation induced by Ca^{2+} removal

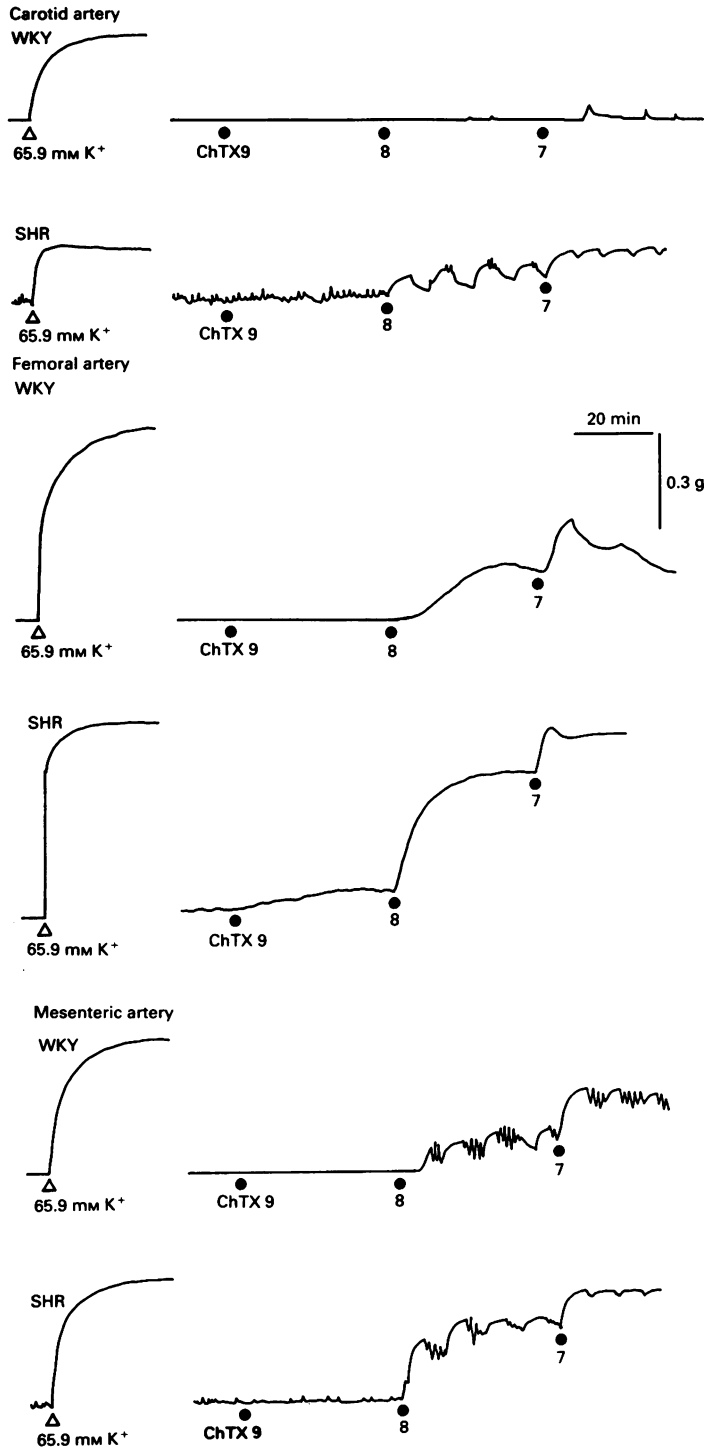


Figure 2 Typical recordings of the contractile effect of charybdotoxin (ChTX) on resting strips of carotid, femoral and mesenteric arteries from 13 week old WKY and SHR. After the application of 65.9 mM K^+, and washout with a Krebs solution as in Figure 1, the concentration-response curve for ChTX was constructed in a cumulative fashion. Concentrations of ChTX are expressed as the negative log of the molar concentration.

(Figure 1). No further relaxation was obtained when 10^{-4} M papaverine was added to these arteries.

Carotid, femoral and mesenteric arteries isolated from WKY did not respond to Ca^{2+} removal or nifedipine (Figure 1).

Charybdotoxin and tetraethylammonium-induced contraction

The addition of ChTX to the resting strips of SHR arteries caused a concentration-dependent oscillatory contraction in carotid and mesenteric arteries and a tonic contraction in femoral arteries (Figure 2). On the other hand, the ChTX-induced contractions in WKY arteries were relatively weak and transient (Figure 2). Concentration-response curves for the contractile effect of ChTX are compared in arteries from SHR and WKY in Figure 3. In the three arteries used, the ChTX-induced contractions were significantly greater in SHR than in WKY. The difference in the ChTX-induced contraction between SHR and WKY was in order of carotid> femoral> mesenteric. The peak contractions induced by 10^{-7} M ChTX in arteries from SHR were comparable to the

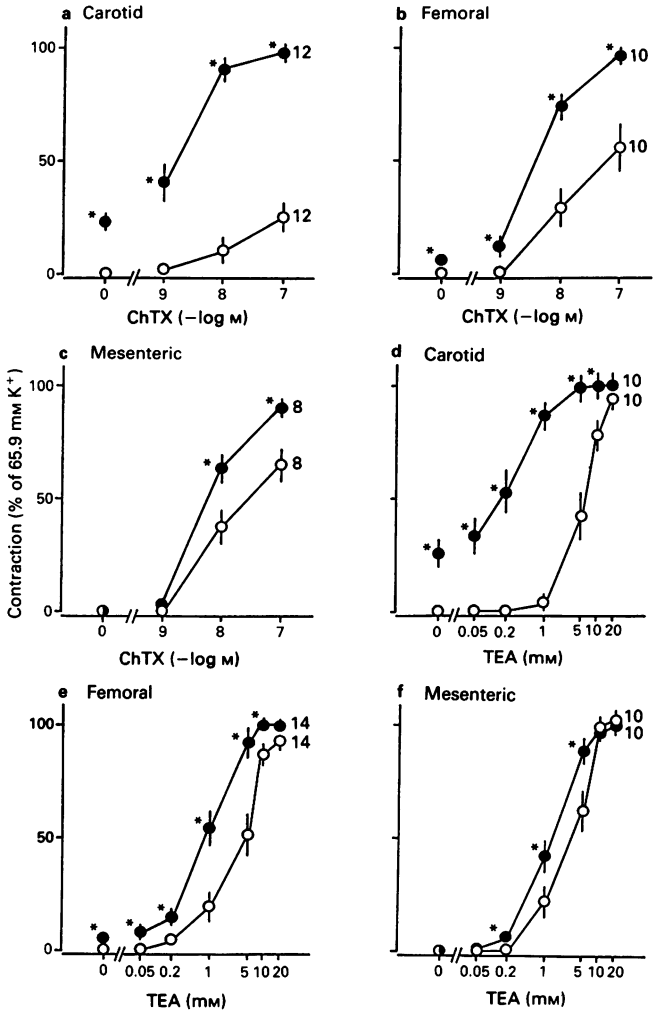


Figure 3 Concentration-response curves for the contractile effects of charybdotoxin (ChTX; a,b,c) and tetraethylammonium (TEA; d,e,f) on resting strips of carotid (a,d), femoral (b,e) and mesenteric (c,f) arteries from WKY (○) and SHR (●). Experimental conditions were the same as in Figure 2. The peak contractions induced by each concentration of ChTX and TEA are expressed as % of the maximum contraction induced by 65.9 mM K^+. At zero on the abscissa scale, the oscillatory contraction or the tonic contraction before the addition of ChTX or TEA is expressed as % of the maximum contraction induced by 65.9 mM K^+. Data points are means of the number of preparations indicated by each curve, and s.e.mean is shown by vertical bars. *Significantly different from WKY ($P<0.05$).

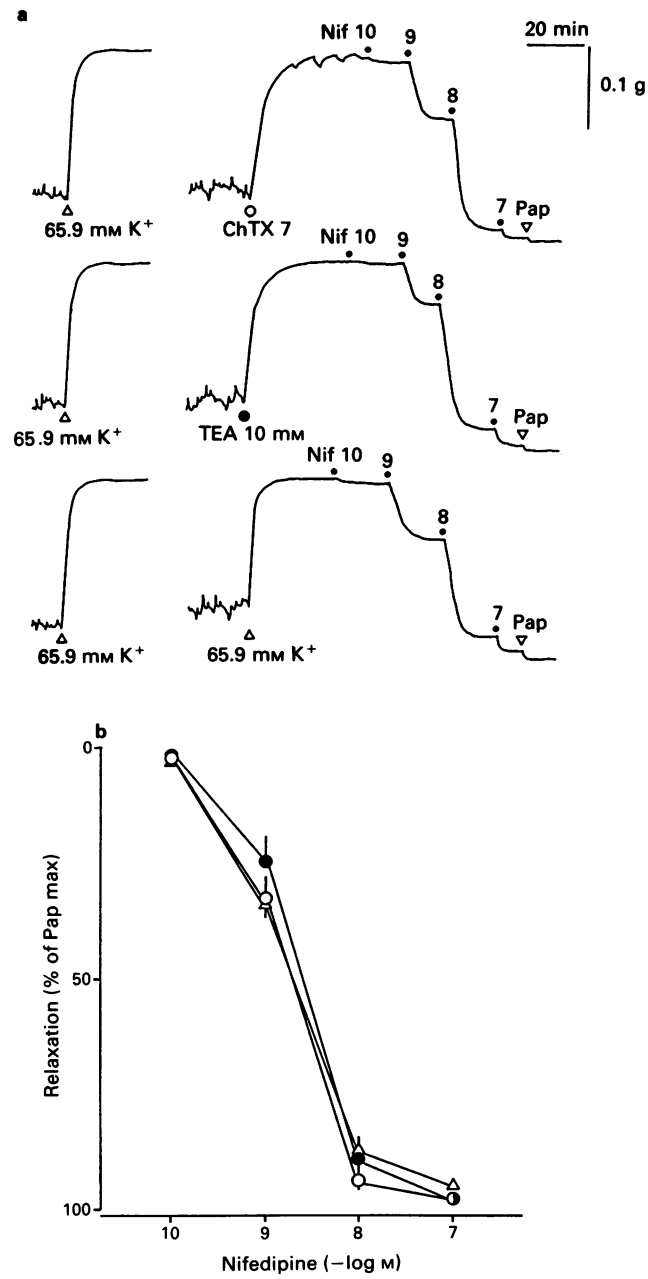


Figure 4 The relaxant effect of nifedipine (Nif) on (13 weeks old) SHR carotid arteries contracted with charybdotoxin (ChTX), tetraethylammonium (TEA) and 65.9 mM K^+. (a) Typical recordings. After the application of 65.9 mM K^+, and washout with a Krebs solution as in Figure 1, the strips were contracted with 10^{-7} M ChTX, 10 mM TEA or 65.9 mM K^+. Nif was added in a cumulative fashion after the contraction had reached a plateau. At the end of each experiment, 10^{-4} M papaverine (Pap) was added to identify the position of the maximum relaxation. Concentrations of Nif are expressed as the negative log of the molar concentration. (b) Concentration-response curves for the relaxant effect of Nif on 10^{-7} M ChTX (○), 10 mM TEA (●) and 65.9 mM K^+ (△)-contracted strips. Relaxations induced by each concentration of Nif are expressed as % of the maximum relaxation induced by Pap. Data points are means of the number of preparations (ChTX, TEA and K^+, $n=5$) and s.e.mean is shown by vertical bars.

maximum contractions induced by 65.9 mM K^+.

The addition of TEA to the resting strips caused a concentration-dependent contraction in carotid, femoral and mesenteric arteries from SHR and WKY (Figure 3). Although the maximum contraction induced by 20 mM TEA in each artery was not significantly different between SHR and WKY, the threshold concentration for initiating contraction and the EC_{50} value for TEA in each artery were significantly lower in SHR than in WKY. It is noteworthy that the contractions induced by relatively low concentrations (below 5 mM) of TEA in these arteries were similar to the ChTX-induced contractions. The difference in the TEA-induced contraction between SHR and WKY was in the order of carotid > femoral > mesenteric.

The addition of apamin (up to 10^{-6} M, $n=3-5$), or of glibenclamide (up to 5×10^{-6} M, $n=3-5$), to the resting strips of the three arteries failed to cause a contraction in either SHR or WKY (data not shown).

Mechanism of charybdotoxin-induced contraction

After the contraction of SHR carotid arteries induced by 10^{-7} M ChTX had reached a plateau, the addition of nifedipine produced a concentration-dependent relaxation (Figure 4a). Nifedipine also relaxed the arteries contracted by

either 10 mM TEA or 65.9 mM K^+ (Figure 4a). Concentration-response curves for the relaxant effect of nifedipine were similar among the ChTX-, TEA- and K^+-contracted arteries (Figure 4b). The contractile responses of SHR carotid arteries to ChTX were greatly attenuated by 10^{-7} M nifedipine or by 3×10^{-6} M cromakalim (Figure 5). Nifedipine or cromakalim alone abolished the myogenic tone in SHR carotid arteries (Figure 5a). In the presence of either drug, ChTX produced only a transient contraction which was similar to that of WKY carotid arteries shown in Figures 2 and 3.

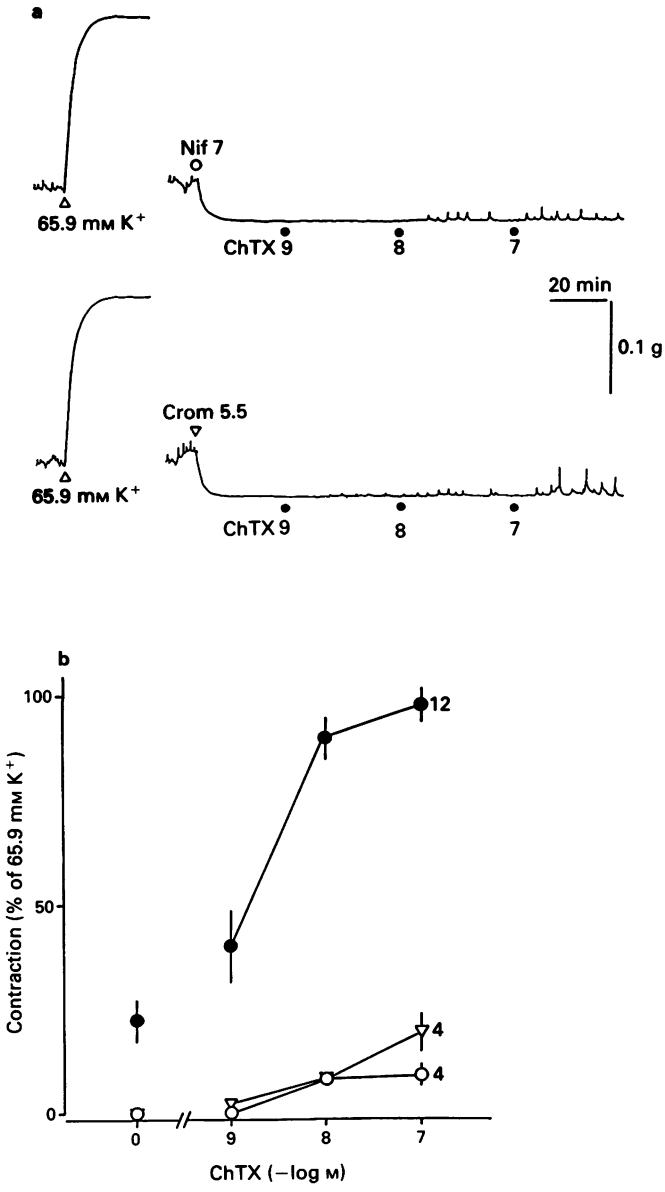


Figure 5 Effects of nifedipine (Nif) and cromakalim (Crom) on the charybdotoxin (ChTX)-induced contraction in (13 weeks old) SHR carotid arteries. (a) Typical recordings. Nif (10^{-7} M) or Crom (3×10^{-6} M) was added 30 min before the determination of the ChTX-induced contraction. The ChTX-induced contraction was determined as in Figure 2. Concentrations of Nif, Crom and ChTX are expressed as the negative log of the molar concentration. (b) Concentration-response curves for the ChTX-induced contraction in the absence (control, ●) and presence of either 10^{-7} M Nif (○) or 3×10^{-6} M Crom (▽). The peak contractions induced by each concentration of ChTX are expressed as % of the maximum contraction induced by 65.9 mM K^+. At zero on the abscissa scale, the oscillatory contraction before the addition of ChTX is expressed as % of the maximum contraction induced by 65.9 mM K^+. Data points are means of the number of preparations indicated by each curve, and s.e.mean is shown by vertical bars.

^{86}Rb efflux from resting strips

The basal ^{86}Rb efflux rate constants in resting strips of carotid, femoral or mesenteric arteries were significantly greater in SHR than in WKY (Figure 6, Table 1). The addition of 10^{-7} M nifedipine to the resting strips of carotid, femoral and mesenteric arteries from SHR significantly decreased the basal ^{86}Rb efflux rate constants (Figure 6, Table 1). Nifedipine also produced a relaxation in carotid and femoral arteries from SHR (Figure 6). However, 10^{-7} M nifedipine did not have an effect on ^{86}Rb efflux and tension in the three arteries from WKY (Figure 6, Table 1). After exposure to 10^{-7} M nifedipine, the ^{86}Rb efflux rate constants in carotid, femoral or mesenteric arteries from SHR were still significantly greater than those from WKY (Table 1).

Cellular Ca^{2+} uptake

The cellular Ca^{2+} uptake in the resting state of carotid and femoral arteries from SHR was significantly increased when compared to WKY (Table 2). In carotid arteries, 10^{-7} M nifedipine abolished the increase in the cellular Ca^{2+} uptake in SHR, but had no effect on WKY. The cellular Ca^{2+} uptake in the presence of nifedipine became similar in the carotid arteries from SHR and WKY (Table 2). In the femoral arteries, although nifedipine reduced the cellular Ca^{2+} uptake only in SHR, the cellular Ca^{2+} uptake in the presence of nifedipine was still significantly increased in SHR when compared to WKY (Table 2).

Discussion

The present study clearly demonstrated that the ChTX-sensitive K_{Ca} channels were highly activated in the resting state of carotid, femoral and mesenteric arteries from SHR when compared to WKY. In SHR arteries, the primary defect responsible for the increased activation of ChTX-sensitive K_{Ca} channels could be an increase in the cellular Ca^{2+} uptake. Such K_{Ca} channel activation may act as a negative feedback mechanism to regulate the level of myogenic tone in the resting state of SHR arteries. This conclusion arises from the following observations: (1) the addition of ChTX to the resting strips from SHR produced a potent contraction which was comparable to the maximum contraction induced by high K^+-depolarization; (2) a similar contraction was obtained in SHR when relatively low concentrations (below 5 mM) of TEA were added to the resting strips; (3) arterial contractions induced by ChTX and TEA were weak in WKY; (4) the ^{86}Rb efflux from resting strips was significantly increased in SHR; (5) the cellular Ca^{2+} uptake in resting arteries was significantly increased in SHR; and (6) nifedipine reduced the ^{86}Rb efflux and the cellular Ca^{2+} uptake only in SHR.

We initially compared the effect of ChTX on carotid, femoral and mesenteric arteries from SHR and WKY, because SHR arteries have been demonstrated to exhibit a myogenic tone in the resting state (Noon *et al.*, 1978; Fitzpatrick & Szentivanyi, 1980; Winquist & Bohr, 1983; Asano *et al.*, 1986). ChTX was used to determine if K_{Ca} channels play a role in regulating resting tone in isolated arteries. The assumption was that if ChTX-sensitive K_{Ca} channels regulate the resting tone, the blockade of the K_{Ca} channels will contract arterial smooth muscle by causing membrane depolarization and the subsequent Ca^{2+} influx via voltage-dependent Ca^{2+} channels (Brayden & Nelson, 1992). If the depolarization and Ca^{2+} influx activate K_{Ca} channels, then a hyperpolarization of the membrane or inhibition of Ca^{2+} influx should diminish the arterial contraction induced by K_{Ca} channel blockers. This is the probable mechanism of ChTX-induced contractions demonstrated in the present study, because either the hyperpolarization of the membrane through the activation of K^+ channels by cromakalim or the

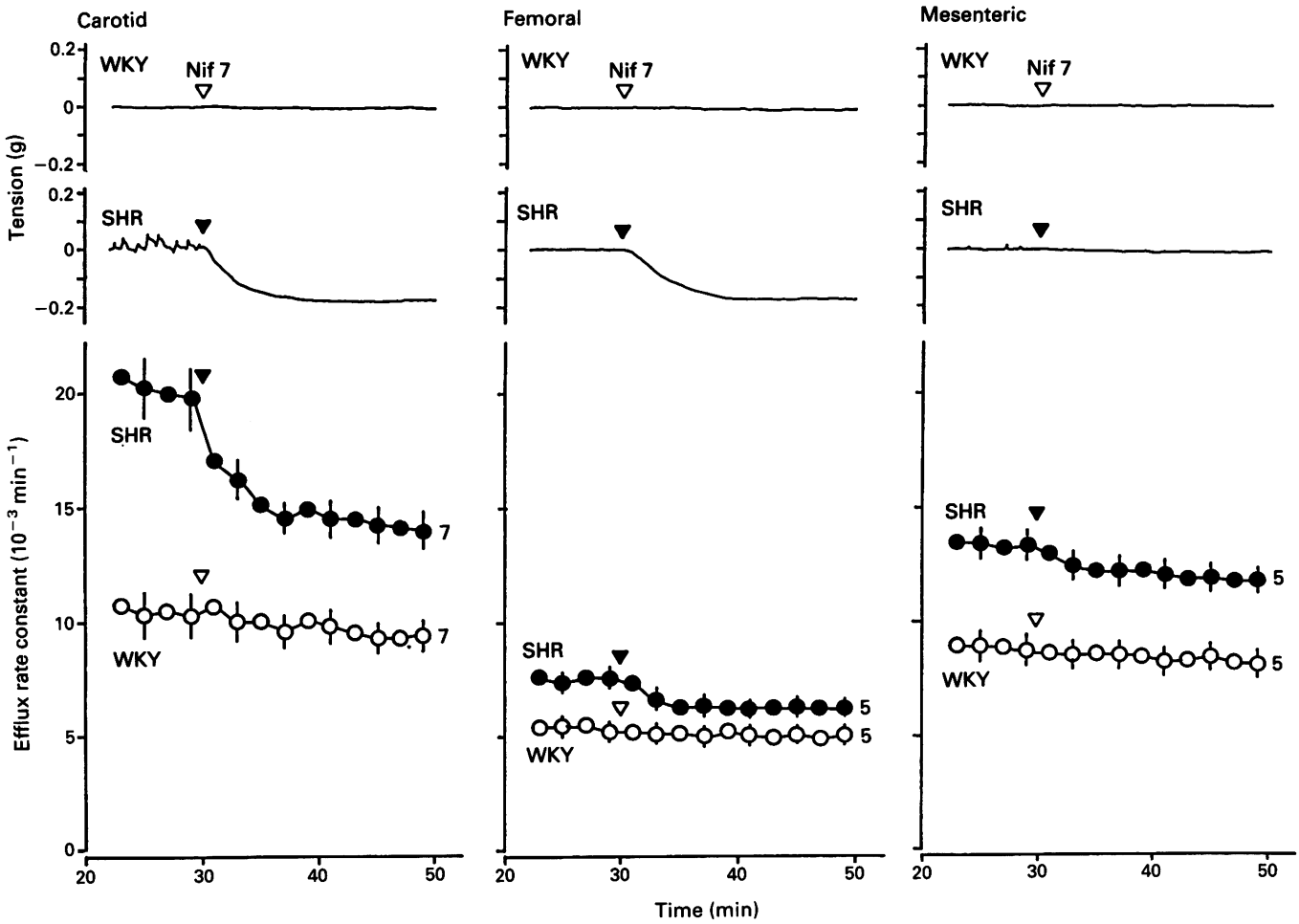


Figure 6 The effect of nifedipine (Nif) on tension (two top panels) and ^{86}Rb efflux (bottom panels) in resting strips of carotid, femoral and mesenteric arteries from WKY and SHR. The strips were incubated with ^{86}Rb for 3 h in Krebs solution and then were washed out for the first 30 min with Krebs solution, followed by a 20 min washout with Krebs solution containing 10^{-7} M Nif. Ordinate scale: change in tension expressed as g or ^{86}Rb efflux rate constant expressed as 10^{-3} min^{-1}. Abscissa scale: time (min) after start of the efflux period. The rate constants are means of the number of preparations indicated by each curve, and s.e.mean (representative values) is shown by vertical bars.

Table 1 The effect of 10^{-7} M nifedipine on ^{86}Rb efflux from resting strips of carotid, femoral and mesenteric arteries from WKY and SHR

			^{86}Rb efflux rate constant (10^{-3} min^{-1})[a]	
Artery	*Rat*	n	*Before Nif*[b]	*After Nif*[c]
Carotid	WKY	7	10.2 ± 1.0	9.9 ± 0.7
	SHR	7	19.7 ± 1.4*	14.5 ± 0.8*†
Femoral	WKY	5	5.2 ± 0.4	5.1 ± 0.3
	SHR	5	7.2 ± 0.4*	6.2 ± 0.4*†
Mesenteric	WKY	5	8.8 ± 0.7	8.5 ± 0.6
	SHR	5	13.4 ± 0.7*	12.0 ± 0.6*†

[a] The experimental conditions were the same as in Figure 6.
[b] ^{86}Rb efflux rate constants before the exposure to 10^{-7} M nifedipine (Nif) were measured between the 26th and 30th min of efflux.
[c] ^{86}Rb efflux rate constants after the exposure to 10^{-7} M nifedipine (Nif) were measured between the 40th and 44th min of efflux.
Data are expressed as means ± s.e.mean, and *n* indicates the number of preparations used.
*Significantly different from WKY ($P<0.05$).
†Significantly different from 'Before Nif' ($P<0.05$).

inhibition of Ca^{2+} influx by nifedipine almost abolished the ChTX-induced contraction. The present study clearly demonstrates the increased function of ChTX-sensitive K_{Ca} channels in SHR arteries. The ChTX-induced contractions can be explained pharmacologically by a blocking action of ChTX on large conductance K_{Ca} channels. A similar explanation can be given for the TEA-induced contractions. In the present study, TEA, at concentrations below 5 mM, caused a contraction which was similar to the ChTX-induced contraction in each artery. At low concentrations, TEA preferentially blocks large conductance K_{Ca} channels, although at high concentrations it blocks other types of K^+ channels (Cook, 1988; Nelson *et al.*, 1990; Brayden & Nelson, 1992). Furthermore, the large conductance K_{Ca} channel is the only known K^+ channel in arterial smooth muscle that is blocked by either ChTX or low concentrations of TEA (Nelson *et al.*, 1990).

The ^{86}Rb efflux experiments also demonstrated the increased K^+ permeability in carotid, femoral and mesenteric arteries from SHR as compared to WKY. An increased K^+ permeability in SHR arteries was initially reported by Jones (1973; 1974a,b). The finding that nifedipine reduced the basal ^{86}Rb efflux rate constant only in SHR arteries clearly indicates that extracellular Ca^{2+} enters the cell via L-type voltage-dependent Ca^{2+} channels in the resting state of SHR arteries, activates K_{Ca} channels, and thus contributes to the increased K^+ permeability. Similar results were reported for the resting state of the aorta from aldosterone-salt hypertensive rats in which the effects of calcium channel blockers on basal tension and the elevated ^{42}K efflux were evaluated (Smith & Jones, 1990). Judging from the effect of nifedipine

Table 2 The effect of 10^{-7} M nifedipine on the cellular Ca^{2+} uptake in the resting state of carotid and femoral arteries from WKY and SHR

Condition	*Rat*	*Cellular Ca^{2+} uptake* (nmol g^{-1} wet tissue)[a] *Carotid*	*Femoral*
Control	WKY	60.1 ± 1.6 (18)	82.1 ± 2.4 (15)
	SHR	71.2 ± 1.7* (18)	103.1 ± 3.4* (15)
Nifedipine 10^{-7} M[b]	WKY	58.6 ± 1.3 (18)	84.3 ± 2.8 (15)
	SHR	61.4 ± 2.0† (18)	94.1 ± 2.0*† (15)

[a]Isolated carotid and femoral arteries were incubated with ^{45}Ca for 5 min in Tris-buffered solution (5.4 mM K^+) before a 60 min washout in 80.8 mM La^{3+}-substituted solution.
[b]Nifedipine was added 60 min before the application of and also during the ^{45}Ca incubation period.
Data are expressed as means ± s.e.mean, and numbers in parentheses indicate the number of preparations used.
*Significantly different from WKY ($P<0.05$).
†Significantly different from 'Control' ($P<0.05$).

on ^{86}Rb efflux shown in Figure 6, the contribution of the Ca^{2+} influx to the increased K^+ permeability was greater in carotid and femoral arteries than in mesenteric arteries.

High activation of K_{Ca} channels in the resting state of SHR arteries may reflect the increased cellular Ca^{2+} uptake in these arteries, because the K_{Ca} channels are activated by both cellular Ca^{2+} and membrane depolarization (Cook, 1988; Castle *et al.*, 1989). The increased cellular Ca^{2+} uptake or content in the resting state has been demonstrated in aortae from SHR (van Breemen *et al.*, 1986; Jelicks & Gupta, 1990; Sada *et al.*, 1990). The present study also demonstrated the increased cellular Ca^{2+} uptake in the resting state of carotid and femoral arteries from SHR. The mechanisms responsible for the increased cellular Ca^{2+} uptake in SHR was different between carotid and femoral arteries. In SHR carotid arteries, the increase in cellular Ca^{2+} uptake was abolished by nifedipine, suggesting that the increased Ca^{2+} influx through the opening of L-type voltage-dependent Ca^{2+} channels contributed to the increased cellular Ca^{2+} uptake. However, additional factors are also contributing to the increased cellular Ca^{2+} uptake in SHR femoral arteries, because the increase in the cellular Ca^{2+} uptake was not completely abolished by nifedipine. The opening of voltage-dependent Ca^{2+} channels in arterial smooth muscle normally requires membrane depolarization (Bean *et al.*, 1986). Therefore, a likely explanation for the increased Ca^{2+} influx in resting strips of SHR appears to be that SHR arteries are more depolarized in the resting state than WKY arteries (Asano *et al.*, 1986). Although no measurements of the resting membrane potential are available in the present study, membrane depolarization in SHR arteries has been demonstrated in the mesenteric artery (Fujii *et al.*, 1992), the tail artery (Cheung, 1984), and the aorta (Tomobe *et al.*, 1991). The level of the resting membrane potential in arterial smooth muscle largely determines the resting tone and the sensitivity to vasoconstrictors, since the distribution and conductance of ions across the plasma membrane are influenced by the resting membrane potential. However, we do not exclude the possibility that the voltage-dependent Ca^{2+} channels in resting strips of SHR exhibited a different dependence on membrane potential when compared to WKY. Verification that the SHR arteries are depolarized or that the voltage-dependent Ca^{2+} channels in these arteries behave differently at a normal resting membrane potential will require detailed electrophysiological studies on each artery.

The increased cellular Ca^{2+} uptake produces the myogenic tone. The assumption was that if the membrane depolarization and the subsequent Ca^{2+} influx produce the myogenic tone, then a hyperpolarization of the membrane or inhibition of Ca^{2+} influx should reduce the myogenic tone. This is the probable mechanism of the appearance of myogenic tone, because either the hyperpolarization of the membrane through the activation of K^+ channels by cromakalim or the inhibition of Ca^{2+} influx by nifedipine almost abolished the myogenic tone. Therefore, the resting tone of SHR arteries is determined by the net balance of two opposing phenomena; a contraction due to the increased cellular Ca^{2+} uptake (probably through the activation of myosin light chain kinase), and a relaxation due to the activation of K_{Ca} channels. The net balance of the two phenomena resulted in the oscillatory and/or tonic contraction that we observe in resting strips of SHR. The finding of myogenic tone in resting strips of SHR clearly indicates that the contraction due to the increased cellular Ca^{2+} uptake exceeded the relaxation due to the activation of K_{Ca} channels. Our results were consistent with a previous report of enhanced TEA-induced contractions in carotid arteries of stroke-prone SHR compared with WKY (Thompson *et al.*, 1987). These authors concluded that an alteration of voltage-dependent Ca^{2+} channels in these arteries was a likely explanation for the differential effect of TEA.

In the resting state of WKY arteries, K_{Ca} channels must be very infrequently open, because the function of K_{Ca} channels in arterial smooth muscle is to open when cellular Ca^{2+} rises and so exert a repolarizing action on the membrane potential. This may be the case in carotid arteries. However, the K_{Ca} channels were moderately activated in the resting state of femoral and mesenteric arteries from WKY, because ChTX produced moderate contractions in these arteries. The K_{Ca} channels in these two arteries probably were involved in the repolarization of the membrane which resulted in the disappearance of the myogenic tone in these arteries. The moderate activation of K_{Ca} channels in femoral and mesenteric arteries suggests that the basal Ca^{2+} contents in these arteries are higher than the carotid arteries. The difference in the basal Ca^{2+} content among the WKY arteries may result in the variation of the K_{Ca} channel function in these arteries, and hence the regional difference in the ChTX- or TEA-induced contraction between SHR and WKY. The difference in the basal Ca^{2+} content among the WKY arteries did not depend on the Ca^{2+} influx via L-type voltage-dependent Ca^{2+} channels. These observations suggest a direct link between the increased Ca^{2+} influx via L-type voltage-dependent Ca^{2+} channels and the activation of K_{Ca} channels in SHR arteries.

Small conductance K_{Ca} channels might be activated in the resting state of SHR arteries by the increased cellular Ca^{2+} uptake. However, this is unlikely, because apamin had no effect on the myogenic tone. Although small conductance K_{Ca} channels have been found in some vascular smooth muscles, it has not been determined whether the channels are sensitive to apamin (Inoue *et al.*, 1985; Benham *et al.*, 1986). Thus, it is likely that apamin-sensitive K_{Ca} channels do not exist in the arteries used in the present study. The myogenic tone was not affected by glibenclamide but was abolished by cromakalim, suggesting that the cromakalim-activated K^+ channels are present but do not appear to be activated in association with the myogenic tone in SHR arteries.

In conclusion, the present study clearly demonstrated that the ChTX-sensitive K_{Ca} channels were highly activated in the

resting state of SHR arteries. We propose that the activation of ChTX-sensitive K_{Ca} channels, caused by the increase in the cellular Ca^{2+} uptake, is an important negative feedback mechanism that regulates the resting tone in SHR arteries. This regulatory mechanism is likely to influence the resting tone in many arteries, including carotid, femoral and mesenteric arteries. In these arteries, the primary defect responsible for the increased K^+ permeability could be an increase in the transmembrane Ca^{2+} influx via L-type voltage-dependent Ca^{2+} channels.

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Non-peptide antagonists, CP-96,345 and RP 67580, distinguish species variants in tachykinin NK_1 receptors

[1]A.J. Barr & S.P. Watson

University Department of Pharmacology, Mansfield Road, Oxford OX1 3QT

1 The potency of the non-peptide antagonists CP-96,345 and RP 67580 on NK_1 receptor-stimulated [^{3}H]-inositol phosphate accumulation in cell lines or tissue from three different species has been examined.

2 We have used: UC11 cells, derived from a human astrocytoma, and rat LRM55 glial cells, both of which express large numbers of functional NK_1 receptors, and the well characterized guinea-pig ileum which expresses both NK_1 and NK_3 receptors.

3 RP 67580 has an ~25 fold lower affinity for NK_1 receptors in human UC11 cells ($Kd = 194$ nM) than in rat LRM55 cells ($Kd = 7.9$ nM), in contrast CP-96,345 has an ~200 fold lower affinity in rat LRM55 cells ($Kd = 210$ nM) relative to human UC11 cells ($Kd = 0.99$ nM). The pharmacological profile of CP-96,345 and RP 67580 in guinea-pig ileum was similar to that observed in human UC11 cells.

4 In conclusion, we have demonstrated that previously reported species differences in binding affinities for the non-peptide NK_1 antagonists, CP-96,345 and RP 67580, are also observed in inhibition of NK_1 receptor stimulated hydrolysis of inositol phospholipids.

Keywords: Tachykinin NK_1 receptors; 'species variants'; substance P; CP-96,345; RP 67580; non-peptide antagonists; inositol phospholipid hydrolysis

Introduction

Receptors for mammalian tachykinins, substances P (SP), neurokinin A and neurokinin B have been classified on pharmacological criteria into three types termed NK_1, NK_2 and NK_3 (see review by Guard & Watson, 1991). This classification has been confirmed by the cloning of each receptor (Nakanishi, 1991; Gerard *et al.*, 1990; 1991; Takeda *et al.*, 1991; Buell *et al.*, 1992). All three tachykinin receptor types stimulate phosphoinositide metabolism. NK_1-stimulation of this pathway has been reported in guinea-pig ileum longitudinal smooth muscle (Watson & Downes, 1983); NK_2-stimulation in the hamster urinary bladder (Bristow *et al.*, 1987) and NK_3 stimulation in myenteric plexus of guinea-pig ileum (Guard *et al.*, 1988). Recently, Nakajima *et al.* (1992) demonstrated that individual tachykinin receptors stably transfected in Chinese hamster ovary cells couple to phosphoinositide hydrolysis.

Recently, novel potent non-peptide antagonists of the NK_1 receptor have been described. CP-96,345 was reported to bind to bovine caudate membranes with an affinity in the low nanomolar range (Snider *et al.*, 1991) and RP 67580 was reported to inhibit [^{3}H]-SP binding to rat brain membranes with a similar affinity (Garret *et al.*, 1991). The affinity of both non-peptide antagonists, however is species-dependent. For example, in binding studies CP-96,345 has approximately two orders of magnitude higher affinity for NK_1 receptors in human and guinea-pig relative to rat and mouse (Gitter *et al.*, 1991; Snider *et al.*, 1991, Watling *et al.*, 1991), whilst the converse is true for RP 67580 (Garret *et al.*, 1991, Fardin *et al.*, 1992). These species-differences in binding affinity are also apparent at functional NK_1 receptors in isolated preparations (Bereford *et al.*, 1991). A number of the above studies, however, have used the racemic mixture (±)-CP-96,345 rather than the (2S,3S) active isomer, and a number of non-selective actions of the (2R,3R) isomer have been reported (Snider *et al.*, 1991; Boyle *et al.*, 1991; Guard & Watling, 1992).

In the present study we have measured the ability of CP-96,345 and RP 67580 to inhibit NK_1 receptor-stimulated [^{3}H]-inositol phosphate accumulation by substance P methyl ester (SPOMe), a selective NK_1 agonist (Watson *et al.*, 1983), in cell lines or tissue from three different species: man, rat and guinea-pig. We have used: UC11 cells, derived from a human astrocytoma, on which a high density of functional NK_1 receptors have been characterized by radioligland binding and inositol phosphate formation (Johnson & Johnson, 1992); the rat LRM55 glial cell line which contains large numbers of functional NK_1 receptors (Perrone *et al.*, 1986) and the well-characterized guinea-pig ileum which expresses both NK_1 and NK_3 receptors (Guard *et al.*, 1988). These cell lines and the tissue have been used to demonstrate that the potency of both non-peptide antagonists, in antagonizing a biochemical response, is species-dependent.

Methods

Agonist-induced accumulation of [^{3}H]-inositol phosphates in guinea-pig ileum longitudinal muscle

Hartley strain guinea-pigs of either sex (300–500 g) were killed by cervical dislocation. The entire length of the small intestine, apart from the duodenum, was removed into pre-gassed (95% O_2:5% CO_2) Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10 (pH 7.4) at room temperature. Longitudinal muscle strips of guinea-pig ileal smooth muscle (prepared essentially as described by Rang, 1964) were cross-chopped (350 × 350 μM) with a McIlwain tissue chopper. The slices were washed twice with 10 ml Krebs solution and incubated in 5 ml Krebs solution containing 30 μ Ci [^{3}H]-*myo*-inositol under an atmosphere of 95% O_2:5% CO_2 for 1 h in a shaking water bath at 37°C. The prelabelled slices were subsequently washed with 10 ml Krebs solution at 37°C every 15 min for 1 h.

Ileal slices were suspended in 2 ml of Krebs solution and 30 μl aliquots were transferred to plastic tubes containing 200 μl of pre-gassed Krebs solution and LiCl (final conc.

[1] Author for correspondence.

10 mM). Agonist was added in 10 µl volumes, the tubes gassed, capped and incubated in a shaking water bath at 37°C for 30 min. In experiments where tachykinin antagonists were used, tissue was incubated with these drugs 5 min before exposure to agonists.

Cell culture

The UC11 human astrocytoma cells were originally isolated at the University of Cincinnati (Liwnicz *et al.*, 1986) and were kindly provided by Dr C.L. Johnson. The cells were grown in RPMI 1640 medium (supplemented with 2 mM glutamine, 25 mM HEPES and antibiotics) plus 10% calf serum.

The LRM55 rat glial cell line (Martin & Shain, 1979) was grown in DMEM nutrient mix F12 (1:1) medium (supplemented with 2 mM glutamine and antibiotics) plus 10% calf serum. All cell culture reagents, media and calf serum were from GIBCO Ltd., Paisley, Scotland.

Agonist-induced accumulation of [^{3}H]-inositol phosphates in cell lines

Cells grown in 75 cm^2 flasks were labelled for ~24 h in medium containing 1 µCi ml^{-1} [^{3}H]-inositol. The cells were washed in phosphate-buffered saline, trypsinised and resuspended in Hanks solution, of the following composition (mM): NaCl 143, KCl 5.6, $CaCl_2$ 1.3, $MgCl_2$ 2, glucose 10 and HEPES 10 (pH 7.4 with NaOH at 37°C), gassed with 100% O_2, at a cell density of 5×10^5 cells ml^{-1}.

Aliquots (200 µl) of the cell suspension were transferred to plastic tubes containing LiCl (final conc. 10 mM) and 30 µl of Hanks solution. Agonist was added in 10 µl volumes, the tubes gassed, capped and incubated in a shaking water bath at 37°C for 30 min. In experiments where tachykinin antagonists were used, cells were incubated with these drugs 5 min before exposure to agonists.

Extraction of water-soluble [^{3}H]-inositol phosphates

Incubations were terminated by addition of 0.94 ml of chloroform:methanol:HCl (100:200:1, v/v/v). Total [^{3}H]-inositol phosphates were extracted essentially as described by Berridge *et al.* (1982). Chloroform (0.31 ml) and water (0.31 ml) were added, the samples vortexed and the phases separated by centrifugation at 1000 *g* for 5 min. A portion of the upper aqueous phase (0.7 ml) was applied to a glass column containing 1 ml of an approximately 1:1 (w/v) slurry of Dowex AGI-X8 resin (100–200 mesh, formate form) and distilled water. [^{3}H]-inositol and [^{3}H]-glycerophosphoinositol were removed with 5 mM disodium tetraborate/60 mM ammonium formate (2 × 8 ml). Total [^{3}H]-inositol phosphates were eluted with 6 ml of 800 mM ammonium formate/0.1 M formic acid. This eluant was collected in scintillation vials and counted in the gel phase for radioactivity following the addition of 10 ml Liquiscint.

Incorporation of [^{3}H]-inositol into phospholipids was measured by transferring 200 µl of the lower organic phase to a scintillation vial, evaporating and counting for radioactivity following the addition of 5 ml Liquiscint.

Analysis of results

[^{3}H]-inositol phosphate results in the guinea-pig ileum, were normalised to phosphoinositides as described previously (Watson & Downes, 1983). Normalisation was not necessary for the cell lines.

Agonist concentration-response curves and antagonist inhibition curves, from three separate experiments performed in triplicate, were fitted for a four parameter logistic equation by the non-linear regression programme GraphPad (ISI) to obtain EC_{50}, IC_{50} and Hill coefficient values. Antagonist dissociation constants (*K*d) were estimated by a modification of the null method described by Lazareno & Roberts, (1987). Briefly, a concentration-response curve to substance P methyl ester (SPOMe) was generated and a concentration (C = 300 nM) of SPOMe was chosen which gave a response greater than 50% of the maximum agonist response. The concentration of antagonist (IC_{50}) required to reduce the response of this concentration (C) of SPOMe by 50% was then determined. From the SPOMe concentration-response curve a concentration of SPOMe (C′) was identified which yielded a response equivalent to 50% of that produced by concentration C (in the absence of antagonist). The apparent *K*d was then determined from the relationship:

$$C/C' = IC_{50}/Kd + 1$$

Chemicals

[^{3}H]-*myo*-inositol (17.9 Ci mmol^{-1}) was from Amersham International plc. Substance P methyl ester (SPOMe) was from Bachem Biochemicals, Basel, Switzerland. SPOMe was dissolved in 0.1% acetic acid and stored in aliquots at −20°C. CP-96,345 [(2**S**,3**S**)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octam-3-amine], was a gift from Dr J.G. Stam, Pfizer Inc., Groton, CT, U.S.A. RP 67580, 2-(1-imino-2-(2-methoxy-phenyl)-ethyl)-7,7-diphenyl-4 perhydroisoindolone (3a**R**, 7a**R**) was a gift from Dr C. Garret, Rhône-Poulenc Rorer, Vitry-sur-Seine, France. Neurokinin A (NKA), [βAla^8]NKA(4–10) and senktide (succinyl-[Asp^6,N-MePhe^8]-SP_{6-11}) were obtained from Peninsula Laboratories, CA, U.S.A. Histamine and carbachol were from Sigma, Dorset.

Results

Characterization of tachykinin receptor types on cell lines

It is well established that the guinea-pig ileum expresses both NK_1 and NK_3 receptors, with the latter found on nerves of the myenteric plexus. UC11 and LRM55 cells have been shown to express NK_1 receptors (Johnson & Johnson, 1992; Perrone *et al.*, 1986). In order to determine whether other tachykinin receptors are also present on LRM55 and UC11 cells we measured the ability of senktide, a selective NK_3 agonist (Wormser *et al.*, 1986), and [βAla^8]NKA(4–10), a selective NK_2 agonist (Rovero *et al.*, 1989), to stimulate [^{3}H]-inositol phosphate accumulation, as both of these receptor types have been shown to stimulate inositol phospholipid hydrolysis (Bristow *et al.*, 1987; Guard *et al.*, 1988). [βAla^8]NKA(4–10) (300 nM) and senktide (1 µM), had no significant effect on accumulation of [^{3}H]-inositol phosphates in UC11 cells. Results expressed as a percentage of basal (± s.e.mean) were as follows: basal 100 ± 2.5%; senktide (1 µM) 96 ± 4.1% and [βAla^8]NKA(4–10) (300 nM) 98 ± 5.0%. similar results were obtained in the LRM55 cell line (basal 100 ± 3.0%; senktide (1 µM) 94 ± 7.3% and [βAla^8]NKA(4–10) (300 nM) 101 ± 5.0%). These results indicate that NK_2 and NK_3 receptors are absent from both cell lines.

Substance P methyl ester-stimulated accumulation of total [^{3}H]-inositol phosphates

SPOMe stimulated a concentration-dependent increase in total [^{3}H]-inositol phosphates giving rise to a mean ± s.d. fold maximal increase above basal in UC11 cells of 8.3 ± 2.6 (n = 4). Using the method described by Johnson & Johnson (1992), in which experiments are performed on monolayers of cells in multi-well plates we observed a maximal 41 ± 3 fold increase in total [^{3}H]-inositol phosphates with SPOMe. The

essential difference between the two methods would seem to be whether experiments are performed in monolayers or in suspension. The maximal fold increase in total [^{3}H]-inositol phosphates in LRM55 cells was 4.3 ± 2.1 ($n = 3$) and in ileum longitudinal muscle was 2.4 ± 0.5 ($n = 4$), during a 30 min incubation.

Dose-response curves (Figures 1a, b and c) and a comparison of EC_{50} values and Hill coefficients for SPOMe are shown (Table 1). All EC_{50} values were similar, falling within the range 50–70 nM, and Hill slopes were close to unity, consistent with a single population of receptors.

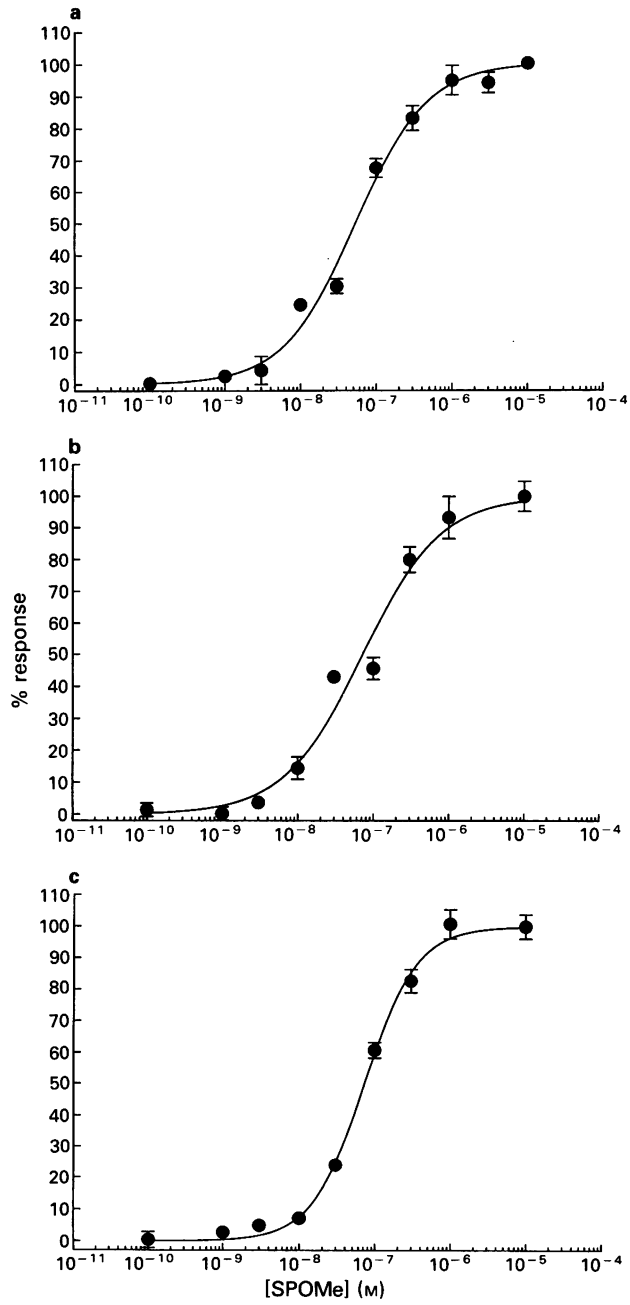


Figure 1 Substance P methyl ester (SPOMe) stimulated total [^{3}H]-inositol phosphate accumulation in: (a) UC11 human astrocytoma cells; (b) LRM55 rat glial cells and (c) guinea-pig ileum longitudinal smooth muscle. Results are expressed as a percentage of the maximal SPOMe response. Each point represents the mean of three or four separate experiments, performed in triplicate, with s.e.mean shown by vertical lines.

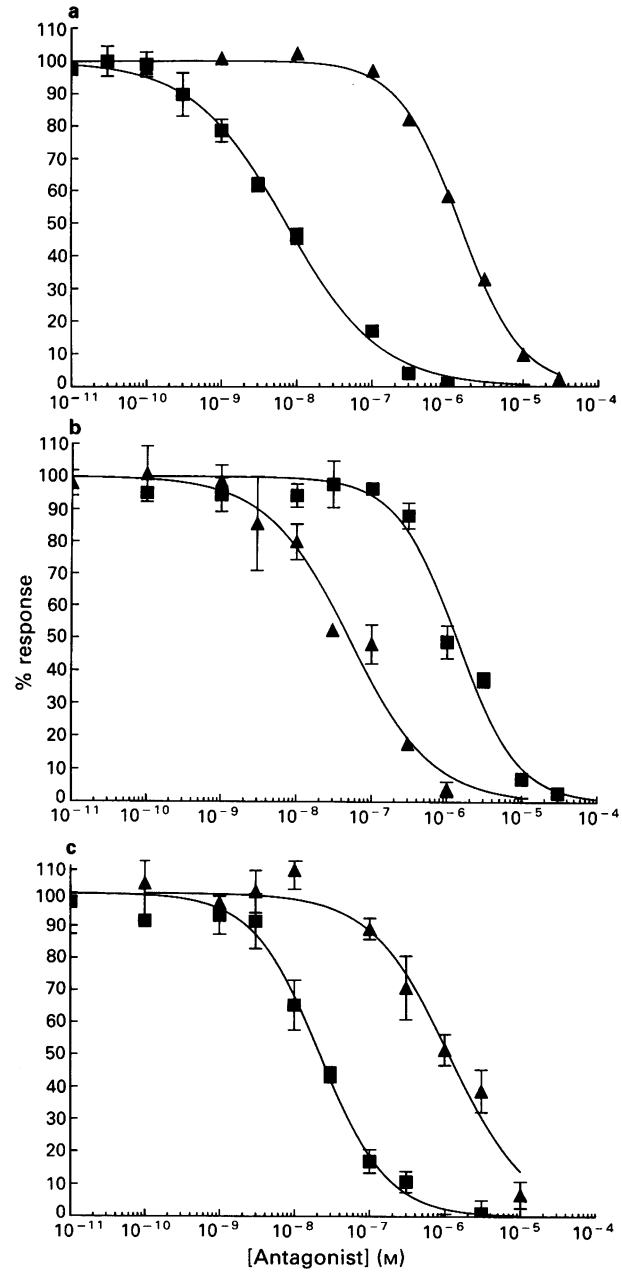


Figure 2 Effect of CP-96,345 (■) and RP 67580 (▲) on (300 nM) substance P methyl ester (SPOMe) stimulated total [^{3}H]-inositol phosphate accumulation in: (a) human astrocytoma cells; (b) LRM55 rat glial cells and (c) guinea-pig ileum longitudinal muscle. Results are expressed as a percentage of the maximal SPOMe response. Each point represents the mean of three separate experiments, performed in triplicate, with s.e.mean shown by vertical lines. Mean pIC_{50} values were calculated from three experiments. CP-96,345 is the single active (2S,3S) isomer.

Effects of CP-96,345 and RP 67580 on substance P methyl ester-stimulated accumulation of total [^{3}H]-inositol phosphates

CP-96,345 inhibited SPOMe (300 nM)-stimulated accumulation of total [^{3}H]-inositol phosphates in the human UC11 cell line and in the guinea-pig ileum with nanomolar affinity (Table 1; Figure 2a and c). In contrast, CP-96,345 was ~200 fold less potent in the rat cell line, LRM55 (Table 1; Figure 2b).

On the other hand RP 67580 inhibited SPOMe (300 nM)-stimulated accumulation of [^{3}H]-inositol phosphates in the

Table 1 Comparison of: EC_{50} values (nM) for substance P methyl ester (SPOMe) stimulated total [^{3}H]-inositol phosphate production; pIC_{50} (nM) and apparent *Kd* (nM) for CP 96,345 and RP 67580 in human UC11 cells, rat LRM55 cells and guinea-pig ileum

	SPOMe		*CP 96,345*			*RP 67580*		
	EC_{50} (nM) (−,+s.e.mean)	nH	pIC_{50} (nM)	nH	Apparent *Kd* (nM)	pIC_{50} (nM)	nH	Apparent *Kd* (nM)
(Human) UC11	53.4 (−4.4,+5.5)	0.92 ± 0.08	8.15 ± 0.04	0.69 ± 0.04	0.99	5.86 ± 0.02	1.06 ± 0.05	194
(Rat) LRM55	72.3 (−12.0,+14.4)	0.84 ± 0.12	5.88 ± 0.06	1.07 ± 0.14	210	7.30 ± 0.08	0.79 ± 0.10	7.90
(Guinea-pig) Ileum	73.3 (−4.3,+4.5)	1.24 ± 0.08	7.66 ± 0.05	0.97 ± 0.10	5.20	5.95 ± 0.07	0.84 ± 0.12	270

Mean EC_{50} values (concentration required to induce 50% of maximal response, as defined by 10 μM substance P methyl ester). EC_{50} values represent geometric means, from three separate experiments and are expressed as antilog of mean (−, + s.e.mean). Mean pIC_{50} values (negative log of the concentration of antagonist required to reduce the maximal SPOMe (300 nM) response by 50%) were determined from three separate experiments. pIC_{50} values are expressed as mean ± s.e.mean. The apparent *Kd* was determined by the null method described by Lazareno & Roberts (1987). CP-96,345 is the single (2S,3S) enantiomer. 'nH' denotes Hill slope.

rat LRM55 cell line with nanomolar affinity (Figure 2b), but was ~25 fold less potent in human UC11 cells and guinea-pig ileum (Table 1; Figure 2a and c). Hill slopes were close to unity for all antagonist inhibition curves except for CP-96,345 on human UC11 cells where the Hill slope was 0.69 ± 0.04, possibly suggesting the presence of more than one class of receptor site.

Effects of CP-96,345 and RP 67580 on carbachol-stimulated accumulation of total [^{3}H]-inositol phosphates

The effects of CP-96,345 and RP 67580 on the response to carbachol in the guinea-pig ileum were examined. Carbachol (1 mM) stimulated an ~18 fold increase in [^{3}H]-inositol phosphates in the ileum which was unaffected by CP-96,345 (10 μM, 94 ± 1.1%) or RP 67580 (10 μM, 96 ± 1.5%). Results are expressed as a percentage of the carbachol response (mean ± s.d.) from three separate experiments performed in triplicate.

Discussion

Previous studies using radioligand binding have reported that the non-peptide NK_1 antagonist (±)-CP-96,345 has a 30–120 fold lower affinity for the rat or mouse receptor than for the human, guinea-pig or bovine receptor (Gitter *et al.*, 1991; Snider *et al.*, 1991; Beresford *et al.*, 1991; Watling *et al.*, 1991). In contrast the non-peptide NK_1 antagonist RP 67580 shows the reverse species variation, although this is less marked than with (±)-CP-96,345 (Fardin *et al.*, 1992). Thus, affinities of (±)-CP-96,345 and RP 67580 for different species can be separated into two groups with human, guinea-pig and bovine having high affinity for (±)-CP-96,345 and low affinity for RP 67580; while rat and mouse have low affinity for (±)-CP-96,345 and high affinity for RP 67580.

Importantly, the species-differences observed in binding affinity are also present in functional studies (Beresford *et al.*, 1991). In the present study we show differences in the potency of CP-96,345 and RP 67580 in antagonizing biochemical responses in a human cell line when compared with a rat cell line. RP 67580 was ~25 fold less potent in human UC11 cells than in rat LRM55 cells while CP-96,345 was ~200 fold less potent in LRM55 cells relative to UC11 cells. The pharmacological profile of CP-96,345 and RP 67580 in guinea-pig ileum was similar to that observed in human UC11 cells. The observed Hill coefficient value of 0.69 ± 0.04 for CP-96,345 on the human UC11 cell line may indicate the presence of more than one class of binding site. This may be explained by the report that two isoforms of the human NK_1 receptor, a short and long form, exist (Fong *et al.*, 1992). These isoforms have been cloned and characterized in expression systems and both are expressed in human brain; however it is not known whether both isoforms are present in human UC11 cells.

Comparison of apparent *Kd* values for CP-96,345, on human UC11 cells (0.99 nM) and on guinea-pig ileum (5.2 nM), calculated by the method of Lazereno & Roberts (1987) are in reasonable agreement with previously reported values. Snider *et al.* (1991) report a pA_2 for CP-96,345 of 8.7 in the dog carotid artery; Beresford *et al.* (1991) report a pK_B of 8.9 in the guinea-pig ileum and Rouissi *et al.* (1991) report a pA_2 of 9.52 for (±)-CP-96,345 in rabbit cava vein. Also the calculated *Kd* of CP-96,345 in rat LRM55 cells, 210 nM, is in the same range as the pK_B of 7.13 in rat spinal cord obtained by Beresford *et al.* (1991).

Previous studies have shown non-specific effects with (±)-CP-96,345. For example Guard & Watling, (1992) reported interaction of the NK_1 receptor antagonist (±)-CP-96,345 with calcium channels in the rat cerebral cortex and Boyle *et al.* (1991) demonstrated a non-competitive block at NK_1 and NK_3 receptors in guinea-pig longitudinal muscle. The effects of CP-96,345 may be explained by the recent report that both enantiomers, (2S,3S) and (2R,3R) or CP-96,344 displace [^{3}H]-diltiazem binding and enhance [^{3}H]-nitrendipine binding in high nanomolar concentrations, suggesting an interaction with L-type calcium channels (Schmidt *et al.*, 1992).

Comparison of the amino acid sequences of human and rat NK_1 receptors reveals that only 22 of 407 residues are different (Takeda *et al.*, 1991). Of these, 6 residues, 2 of which are relatively conservative changes, are located in the putative transmembrane spanning domains. These domains have been identified as the ligand binding region for many G-protein coupled receptors (Strader *et al.*, 1989; Bockaert, 1991). Thus, it is likely that if the hydrophobic core of the NK_1 receptor is involved in ligand binding, some of these residues are important in determining antagonist binding affinity.

Johnson & Johnson, (1992) found a high density of NK_1 tachykinin receptors, using radioligand binding and inositol phosphate assays, on UC11 cells and Perrone *et al.* (1986) have identified and characterized NK_1 recptors on LRM55 rat glial cells. The lack of response to the selective NK_2 agonist [βAla^8]NKA(4–10) (1 μM) and selective NK_3 agonist senktide (1 μM) suggest that NK_2 and NK_3 tachykinin receptor types are absent from UC11 and LRM55 cell lines. Thus

the UC11 cell line is an excellent model system for studying the human NK_1 receptor and its mode of signal transduction.

In conclusion, we have demonstrated that the species differences in binding affinities reported with the non-peptide NK_1 antagonists CP-96,345 and RP 67580 are also observed in antagonist potency inhibiting a biochemical response. The availability of high affinity compounds such as CP-96,345, and a human cell line, UC11, should facilitate research into NK_1 tachykinin receptors.

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Regulation of NANC neural bronchoconstriction *in vivo* in the guinea-pig: involvement of nitric oxide, vasoactive intestinal peptide and soluble guanylyl cyclase

Yu-Hong Lei, Peter J. Barnes & [1]Duncan F. Rogers

Department of Thoracic Medicine, National Heart & Lung Institute, Dovehouse Street, London SW3 6LY

1 We investigated the effect of the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) and the peptidase α-chymotrypsin on non-adrenergic, non-cholinergic (NANC neural) bronchoconstriction induced by electrical stimulation of the vagus nerves and by capsaicin in anaesthetized guinea-pigs *in vivo* using pulmonary insufflation pressure (PIP) as an index of bronchial tone. We also investigated the contribution of soluble guanylyl cyclase (SGC) to NANC neural relaxant mechanisms.

2 In the presence of atropine and propranolol, electrical stimulation of the vagus nerves induced a frequency-dependent increase in PIP above baseline of 67% at 2.5 Hz, of 128% at 5 Hz and of 230% at 10 Hz. L-NAME (1–50 mg kg^{-1}, i.v.), at doses inducing increases in systemic blood pressure, dose-relatedly potentiated NANC bronchoconstriction. At 10 mg kg^{-1} i.v., L-NAME significantly ($P<0.05$) potentiated NANC bronchoconstriction by a further 106% at 2.5 Hz and a further 147% at 5 Hz but did not potentiate the increase in PIP at 10 Hz. L-NAME did not induce bronchocontriction in sham-stimulated control animals. D-NAME did not potentiate NANC bronchoconstriction. Raising systemic blood pressure with phenylephrine did not potentiate vagally-induced bronchoconstriction (2.5 Hz).

3 The NO precursor L-arginine, but not D-arginine, (100 mg kg^{-1}, i.v.) significantly reversed the potentiation by L-NAME of NANC bronchoconstriction. L-Arginine alone significantly inhibited neurogenic bronchoconstriction at 10 Hz (by 74%); the inhibition of 25% at 2.5 Hz was not significant.

4 L-NAME did not significantly affect the increases in PIP induced by intravenous substance P, neurokinin A (NKA) or capsaicin.

5 The inhibitor of SGC, methylene blue (10 mg kg^{-1}, i.v.) potentiated (by 110–140%) NANC neural bronchoconstriction induced by lower frequencies of nerve stimulation and reversed the reduction in PIP induced by the SGC activator, sodium nitroprusside (SNP, 1.05 mg kg^{-1}, i.v.). SNP significantly ($P<0.05$) reduced by 65% the bronchoconstriction induced by nerve stimulation at 10 Hz. Methylene blue did not effect baseline PIP in sham-stimulated controls. The airway effects of methylene blue and SNP were not associated with their cardiovascular effects.

6 α-Chymotrypsin (2 units kg^{-1}, i.v.) significantly potentiated vagally-induced bronchoconstriction by a further 63% at 2.5 Hz, by a further 95.6% at 5 Hz but did not potentiate the increase in PIP at 10 Hz. α-Chymotrypsin also potentiated (by 116%) capsaicin-induced bronchoconstriction. Vasoactive intestinal peptide (VIP, 10 μg kg^{-1} i.v. infused over 1 min) significantly reduced by 70% the increase in PIP induced by NKA (0.1 μmol kg^{-1} i.v., infused over 30 s).

7 The combination of α-chymotrypsin (2 units kg^{-1}, i.v.) and L-NAME (5 mg kg^{-1}, i.v.) significantly potentiated NANC bronchoconstriction by a further 304% at 2.5 Hz, an increase in PIP which was greater than that induced by either α-chymotrypsin or L-NAME alone ($P<0.05$).

8 We conclude that endogenous NO and a bronchodilator peptide, possibly VIP, released in association with nerve stimulation, as well as activation of soluble guanylyl cyclase, regulate the magnitude of NANC neurogenic bronchoconstriction in guinea-pigs *in vivo*.

Keywords: Nitric oxide; vasoactive intestinal peptide; α-chymotrypsin; bronchoconstriction; non-adrenergic, non-cholinergic nerves; vagus nerve stimulation; methylene blue; nitrite; sensory nerve

Introduction

In addition to adrenergic and cholinergic nerves, there are neurally-mediated responses in the airways of several animal species, including man and guinea-pig, which are not blocked by adrenoceptor or cholinoceptor antagonists and termed non-adrenergic, non-cholinergic (NANC) (Barnes *et al.*, 1991). Activation of excitatory NANC (e-NANC) nerves induces a number of airway responses including smooth muscle contraction, mucus secretion, vasodilatation and plasma exudation. The identity of e-NANC nerves is a population of C-fibre afferent (sensory) nerves which are sensitive to capsaicin, the pungent principle of hot *Capsicum* peppers (Holzer, 1991). The neurotransmitters of these nerves are peptides including the tachykinins substance P (SP) and neurokinin A (NKA), as well as calcitonin gene-related peptide (Barnes *et al.*, 1991). Inhibitory NANC (i-NANC) nerves mediate bronchodilatation *in vitro* and *in vivo* in animal and human airways (Coburn & Tomita, 1973; Coleman & Levy, 1974; Richardson & Béland, 1976; Middendorf & Russell, 1980) and induce bronchodilatation *in vivo* in preconstricted cat airways (Diamond & O'Donnell, 1980; Irvin *et al.*, 1980). In guinea-pig trachea *in vitro* vasoactive intestinal peptide (VIP), peptide histidine isoleucine (PHI), although to a much lesser extent than VIP, and nitric oxide (NO) have been proposed as the mediators of the relaxation (Li & Rand, 1991; Ellis & Farmer, 1989a,b; Tucker *et al.*, 1990). In

[1] Author for correspondence.

guinea-pig airways, NO contributes to a significant proportion of the relaxation (Li & Rand, 1991; Tucker *et al.*, 1990; Belvisi *et al.*, 1991; Brave *et al.*, 1991) whereas in human bronchi *in vitro* the response is entirely due to NO (Belvisi *et al.*, 1992). It is not yet known whether NANC bronchoconstriction *in vivo* is regulated by NO or VIP and it was the purpose of the present study to determine if endogenous NO or VIP regulate the magnitude of NANC bronchoconstriction in guinea-pigs *in vivo*. In a number of tissues including vascular smooth muscle and adrenal glands, NO stimulates soluble guanylyl cyclase (SGC) (Gruetter & Ignarro, 1979; Palacios *et al.*, 1989), but the contribution of this mechanism to airway relaxation is unknown. In the present study, we have examined the action of N^G-nitro-L-arginine methyl ester (L-NAME), a specific inhibitor of endogenous NO biosynthesis (Rees *et al.*, 1990), methylene blue, an inhibitor of SGC (Gruetter *et al.*, 1981; Kukovetz & Holzmann, 1983), sodium nitroprusside, an activitor of SGC (Ignarro *et al.*, 1981), or α-chymotrypsin, a peptidase capable of degrading VIP (Altiere & Diamond, 1984), on bronchoconstriction induced by electrical stimulation of the vagus nerves in guinea-pig airways *in vivo*.

Methods

Animal preparation

Male Dunkin-Hartley guinea-pigs (350–450 g body weight) were anaesthetized with 2 g kg^{-1} urethane (25% w/v in 0.9% w/v saline) and laid supine on a heated blanket which maintained body temperature (rectal) at 37°C. An incision was made in the trachea immediately below the larynx and a cannula was tied into the rostral end of the tracheal lumen through which the animals were ventilated mechanically at a tidal volume of 10 ml kg^{-1} and a frequency of 60 breaths min^{-1} (small animal ventilator, Harvard Apparatus, South Natick, Massachusettes). A side arm in the expiratory limb of the tubing was connected to a pressure transducer (Sensortechnics, Rugby, Warwks.) and changes in pulmonary insufflation pressure (PIP) displayed on a recorder (Multitrace 2, Lectromed Limited, St. Peter, Jersey). The cervical jugular veins were exposed and all drugs except VIP, NKA or phenylephrine were administered intravenously as bolus injections by passing the injection needle through the *pectoralis major* to prevent bleeding on withdrawal. In animals for infusion of VIP, NKA, or phenylephrine, cannulae filled with 2 units ml^{-1} heparin saline were tied into the jugular veins and the drugs administered via these cannulae. To monitor the physiological condition of the animal and to establish drug activity, blood pressure in the left carotid artery was recorded on a two-channel recorder (Devices, Ormed, Welwyn Garden City) via an indwelling Portex cannula filled with heparin saline (2 u ml^{-1}) linked to a pressure transducer (Bell and Howell, Basingstoke). Both cervical vagus nerves were exposed, sectioned below the nodose ganglia (to avoid stimulating the central nervous system during vagus nerve stimulation) and placed on bipolar platinum electrodes (Subminiature electrode, Harvard Apparatus Ltd., Edenbridge, Kent). A double channel square wave stimulator (Model S88, Grass Instruments, Quincy, U.S.A.) was used to stimulate electrically the nerves at various frequencies (2.5, 5 of 10 Hz) with other parameters held constant at 5 V, 5 ms for 3 min. The effects of dissection and nerve manipulation on PIP were determined by repeating the above protocol but without electrical stimulation (sham-stimulated control).

Protocols

All animals for nerve or sham-stimulation were pretreated with atropine and propranolol (each at 1 mg kg^{-1}, i.v.), given 30 min prior to stimulation, to eliminate respectively cholinergic and adrenergic influences. One mg kg^{-1} i.v. atropine has been shown previously to block vagally induced bronchoconstriction in capsaicin-treated guinea-pigs pretreated with propranolol (Stretton *et al.*, 1992). The effectiveness of the propranolol dose was determined in the present animals. Without propranolol, vagal stimulation at 10 Hz reduced blood pressure by $10.5 \pm 6.2\%$ ($n = 3$). With 1 mg kg^{-1} propranolol the reduction was increased to $48.7 \pm 3.5\%$ ($n = 3$) which indicates that the dose used was effective. L-NAME (1–50 mg kg^{-1}, i.v.) or H-D-Arg(NO_2)-oMe·HCl (D-NAME, 10 mg kg^{-1}, i.v.) or methylene blue (10 mg kg^{-1}, i.v.) or α-chymotrypsin (2 u kg^{-1}, i.v.) were administered 10 min (L-NAME and D-NAME) or 5 min (methylene blue) or 60 min (α-chymotrypsin, 30 min before atropine and propranolol) before nerve stimulation. In separate animals, L-arginine or D-arginine (100 mg kg^{-1}, i.v.) were administered 9 min after L-NAME (10 mg kg^{-1}, i.v.) (i.e. 1 min prior to nerve stimulation). Sodium nitroprusside (SNP, 1.05 mg kg^{-1}, i.v.) was administered 10 min prior to vagus nerve stimulation (2.5 or 10 Hz) in separate animals. In separate animals, phenylephrine (10 μg ml^{-1}, i.v. infused to a total volume of 5–6 ml per guinea pig) was injected for 10 min prior to and during nerve stimulation (2.5 Hz) to maintain a raised carotid artery pressure.

In separate animals, L-NAME (10 mg kg^{-1}, i.v.) was given 10 min prior to administration of SP (20 nmol kg^{-1}, i.v.), NKA (1 nmol kg^{-1}, i.v.) or capsaicin (15 μg kg^{-1}, i.v.) in the absence of atropine and propranolol. In separate animals, atropine and propranolol (1 mg kg^{-1}, i.v.) were administered 30 min after treatment with α-chymotrypsin (2 units kg^{-1}, i.v.) followed 20 min later by L-NAME (5 mg kg^{-1}, i.v.) and 10 min later by vagus nerve stimulation (2.5 Hz). In separate animals, VIP (10 μg kg^{-1}, i.v.) was infused for the 1 min preceding administration of NKA (0.1 μmol kg^{-1}, i.v. infused over 30 s).

Drugs

The following drugs were used: urethane, L-NAME, SP, NKA, capsaicin, L-arginine, D-arginine, methylene blue, SNP, VIP, α-chymotrypsin and L-phenylephrine hydrochloride (Sigma Chemical Company Ltd., Poole, Dorset); D-NAME (Bachem Feinchemikalen AG, Bubendorf, Switzerland); atropine sulphate (Pharma Hameln, G.m.b.H., W. Germany); propranolol hydrochloride (Imperial Chemical Industries PLC, Macclesfield, Cheshire); heparin sodium (CP Pharmaceuticals Ltd., Wrexham, North Wales). L-NAME, D-NAME, L-arginine, D-arginine and methylene blue were dissolved in distilled water on each day of experimentation. SP, NKA and α-chymotrypsin were dissolved in 0.9% saline at a concentration of 0.1 μmol ml^{-1} for SP and NKA and of 2 units ml^{-1} for α-chymotrypsin; capsaicin was dissolved in 100% ethanol (10 mg ml^{-1}); all solutions were stored at − 20°C. All stored solutions were diluted in saline to the final concentration before use. SNP and phenylephrine were dissolved in saline on each day of experimentation. All drugs were administered in a volume of 0.1 ml 100 g^{-1} body weight except for VIP which was infused in a volume of 0.2 ml 100 g^{-1} (stock solution of 5 μg ml^{-1} in 0.9% saline) and phenylephrine (see above).

Data analysis

Changes in PIP were converted to percentage increases above baseline and are expressed as mean ± s.e.mean. The Mann-Whitney U-test (two-tailed) was used to determine the significance of differences in change in PIP between groups. Mean arterial blood pressure was calculated from recorded traces as: diastolic pressure + 0.33(systolic pressure − diastolic pressure). Data for blood pressure (BP) were assessed

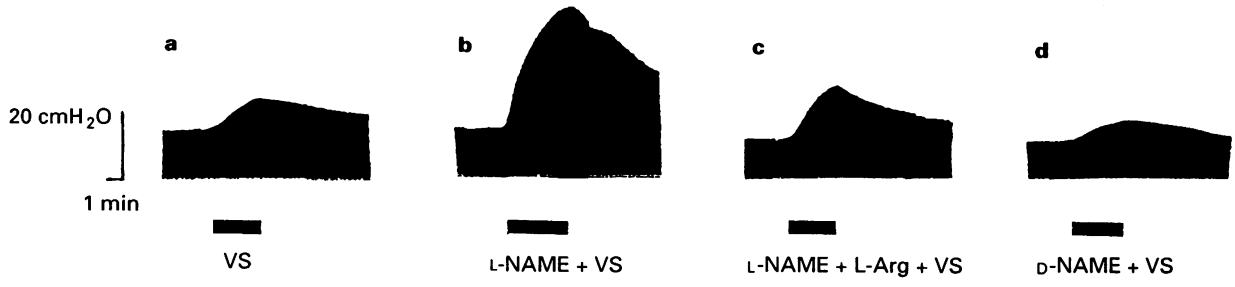


Figure 1 Examples of the effect of inhibition of NO synthase by N^G-nitro-L-arginine methyl ester (L-NAME) on bronchoconstriction induced by electrical stimulation of the vagus nerves (VS; 2.5 Hz, 5 V, 5 ms for 3 min) in the presence of atropine and propranolol (1 mg kg^{-1}, each) in anaesthetized guinea-pigs: (a) Increase in pulmonary insufflation pressure (PIP, cmH_20) in response to VS. (b) Effect of L-NAME (10 mg kg^{-1}, i.v.) on VS-induced inrease in PIP. (c) Effect of L-arginine (100 mg kg^{-1}, i.v.) on L-NAME (10 mg kg^{-1}, i.v.) potentiation of VS-induced increase in PIP. (d) Effect of D-NAME (10 mg kg^{-1}, i.v.) on VS-induced increase in PIP.

for group differences by one-way analysis of variance. The significance of differences between pre-selected groups was determined by Students t test for unpaired observations (two-tailed). The null hypothesis was rejected at $P<0.05$.

Variability in mean baseline BP within each of the 36 experimental groups was assessed by expressing the s.e.mean as a percentage of the mean. The mean of these percentage values was 7.7% ($\pm$ 0.6%) which indicates acceptable within-group variability. In order to assess between-group variability, we analysed the original baseline BP data for the 36 groups using one-way analysis of variance which showed that there were no significant differences to be found among the groups ($F = 1.38$, $P = 0.1$). Thus, all animal groups are likely to have come from the same population.

Results

Airway pressure

Mean baseline PIP (measured just prior to any treatment) was 14.3 cmH_2O ($\pm$ 0.2, $n = 170$). Electrical stimulation of the vagus nerves in the presence of atropine and propranolol increased PIP above baseline (Figures 1 and 2). The increase was frequency-dependent and produced increases in PIP of 67 $\pm$ 3% at 2.5 Hz, of 128 $\pm$ 21% at 5 Hz and of 230 $\pm$ 29% at 10 Hz ($n = 5-7$ per group, Figure 2).

Effect of NO synthase inhibitors and precursors on neurogenic bronchoconstriction

L-NAME (10 mg kg^{-1}, i.v.) significantly potentiated the increase in PIP above baseline at 2.5 and 5 Hz with further increases above those with stimulation alone of 106% and 147% respectively ($n = 4-7$ per group; $P<0.05$ compared with corresponding vagal stimulation group; Figures 1 and 2). L-NAME did not significantly potentiate the increase in PIP at 10 Hz ($n = 6$, Figure 2). Potentiation by L-NAME of the increase in PIP induced by 2.5 Hz was dose-dependent over the range 1–50 mg kg^{-1} ($n = 4-8$ per group, Figure 3). D-NAME (10 mg kg^{-1}, i.v.) did not significantly potentiate the increase in PIP at 2.5 Hz (increase of 39%, $n = 5$; Figure 1). The potentiation by L-NAME was significantly reversed by L-arginine, by 60% at 2.5 Hz and by 110% (i.e. below baseline) at 5 Hz ($n = 5$ per group; $P<0.05$ compared with increase in PIP without L-arginine; Figures 1 and 2) but not by D-arginine ($n = 5$ per group, Figure 2). Neither L-NAME nor L-arginine affected baseline PIP in sham controls (no change in PIP, $n = 5$). In contrast, at 2.5 Hz L-arginine alone showed a tendency to inhibit neurogenic bronchoconstriction

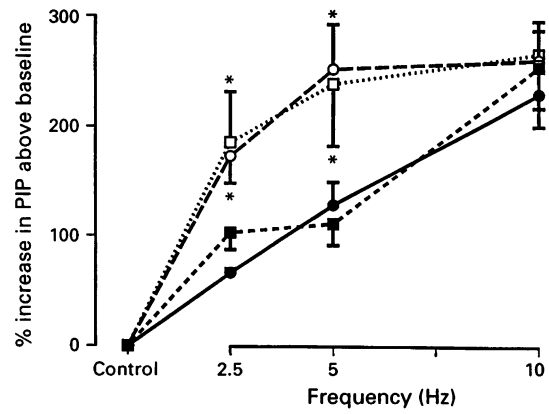


Figure 2 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on the increase in pulmonary insufflation pressure (PIP) above baseline induced by electrical stimulation of the vagus nerves (VS) in anaesthetized guinea-pigs (2.5, 5, or 10 Hz, 5 V, 5 ms for 3 min in the presence of atropine and propranolol). Increase in PIP above baseline induced by nerve stimulation (●); effect of L-NAME (10 mg kg^{-1}, i.v.) on VS-induced increase in PIP (○); effect of L-arginine (100 mg kg^{-1}, i.v.) on L-NAME potentiation of VS-induced increase in PIP (■); effect of D-arginine (100 mg kg^{-1}, i.v.) on L-NAME potentiation of VS-induced increase in PIP (□). Data are mean percentage increase in PIP above baseline; s.e.mean shown by vertical bars. $n = 4-8$ animals per point. $*P<0.05$ compared with corresponding value after vagal stimulation alone.

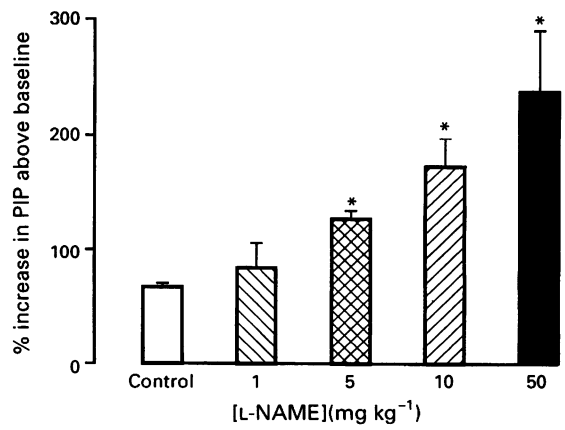


Figure 3 Dose-dependent potentiation by N^G-nitro-L-arginine methyl ester (L-NAME) of vagus nerve stimulation-induced increases in pulmonary insufflation pressure (PIP) (2.5 Hz, 5 V, 5 ms for 3 min in the presence of atropine and propranolol, open column) in anaesthetized guinea-pigs. Data are mean percentage increase in PIP above baseline ($n = 4-8$ per group), vertical bars show s.e.mean. $*P<0.05$ compared with corresponding value with vagal stimulation alone.

Table 1 Effect of inhibition of NO synthase by N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}, i.v.) on neuropeptide-induced bronchoconstriction in anaesthetized guinea-pigs

Administration	n	*Control*	*L-NAME*
Substance P (20 nmol kg^{-1}, i.v.)	5	147.9 ± 24.7	131.7 ± 24.2
Neurokinin A (1 nmol kg^{-1}, i.v.)	5	22.0 ± 5.5	25.0 ± 13.6
Capsaicin (15 μg kg^{-1}, i.v.)	5	123.0 ± 14	108.0 ± 26.0

Data are mean ± s.e.mean % increase in pulmonary insufflation pressure above baseline.

(reduction in PIP of 25.1 ± 11.0%, $n = 4$) albeit not significantly. However, at 10 Hz, L-arginine significantly reduced PIP by 74.3 ± 14% ($n = 6$).

L-NAME (10 mg kg^{-1}, i.v.) had no significant effect on increases in PIP induced by SP, NKA or capsaicin: changes in PIP between agonist alone and L-NAME plus agonist were an 11% reduction for SP, a 12% increase for NKA and a 16% reduction for capsaicin ($n = 5$ per group; Table 1).

Phenylephrine (10 μg ml^{-1}, i.v., infused in a volume of 5–6 ml per animal) increased mean BP by 106% but had no significant effect on vagal stimulation (2.5 Hz) induced increase in PIP (7% increased, $n = 4$).

Effect of alterations in soluble guanylyl cyclase metabolism on neurogenic bronchoconstriction

Methylene blue (10 mg kg^{-1}, i.v.) also significantly potentiated nerve stimulation-induced increases in PIP above baseline by a further 110% at 2.5 and by a further 141% at 5 Hz ($n = 4-5$ per group; $P<0.05$ compared with corresponding vagal stimulation group) but not at 10 Hz ($n = 5$; Figure 4). Methylene blue did not affect baseline PIP in sham controls (no change, $n = 4$). The potentiation induced by methylene blue at 2.5 Hz was reversed by SNP (1.05 mg kg^{-1}, i.v.) by 114 ± 3% (i.e. reduced below pre-methylene blue baseline) ($P<0.05$ compared with corresponding methylene blue group, $n = 4$). SNP did not significantly affect vagally induced increases in PIP above baseline at this frequency ($n = 4$). However, SNP significantly reduced the bronchoconstriction induced by vagal stimulation by 64.7 ± 10.8% at 10 Hz ($n = 4$; $P<0.05$ compared with vagal stimulation). Methylene blue (10 mg kg^{-1}, i.v.) caused a 65.1 ± 18.7% reversal of this reduction. ($n = 5$, $P<0.05$ compared with SNP group). SNP alone induced a non-significant increase in PIP of 10.7 ± 2.0% ($n = 16$).

Effect of α-chymotrypsin on neurogenic bronchoconstriction

α-Chymotrypsin (2 units kg^{-1}, i.v.) significantly potentiated the increase in PIP induced by vagus nerve stimulation by 63% at 2.5 and by 93.6% at 5 Hz ($P<0.05$ respectively, $n = 5$ each group) but not at 10 Hz (Figure 4). In addition, in animals pretreated with α-chymotrypsin (2 units kg^{-1}, i.v.), L-NAME (5 mg kg^{-1}, i.v.) caused a potentiation of the increase in PIP above baseline by a further 304% ($P<0.05$ compared with vagal stimulation or pretreatment with either α-chymotrypsin or L-NAME alone, $n = 5$, Figure 5). α-Chymotrypsin significantly ($P<0.05$) potentiated (by 116.2 ± 23.6%, $n = 4$) the increase in PIP induced by capsaicin (15 μg kg^{-1}, i.v.).

NKA (0.1 μmol kg^{-1}, i.v., infused over 30 s) induced an increase in PIP above baseline of 364 ± 49% ($n = 5$, Figure 5). VIP (10 μg kg^{-1} i.v., infused over the 1 min prior to NKA administration) significantly reduced this response by 70% ($n = 5$, $P<0.05$, Figure 5).

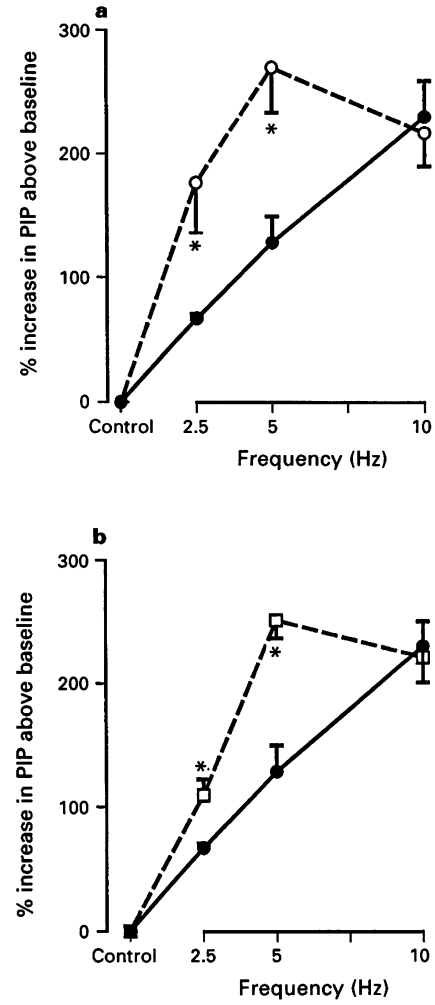


Figure 4 Potentiation by (a) methylene blue (10 mg kg^{-1}, i.v.) or (b) α-chymotrypsin (2 units kg^{-1}, i.v.) of the increase in pulmonary insufflation pressure (PIP) above baseline induced by vagus nerve stimulation (VS 2.5–10 Hz, 5 V, 5 ms for 3 min) in the presence of atropine and propranolol in anaesthetized guinea-pigs. Increase in PIP above baseline induced by VS (●); potentiation by methylene blue (a) (○) or α-chymotrypsin (b) (□) of VS-induced increase in PIP. Data are mean percentage increase in PIP above baseline ($n = 4-8$ animals per point), vertical lines show s.e.mean. *$P<0.05$ compared with corresponding value with vagal stimulation alone.

Blood pressure

Mean baseline carotid artery pressure in the 170 guinea-pigs used was 39.6 mmHg (± 0.6 mmHg). In the presence of atropine and propranolol, L-NAME increased BP above baseline by 15.0 mmHg (± 3.8, $n = 4$) (42%) at 1 mg kg^{-1} by 32.8 mmHg (± 2.0, $n = 4$) (69.8%) at 5 mg kg^{-1} by 28.3 mmHg (± 1.9, $n = 24$) (67.8%) at 10 mg kg^{-1} and by 26.8 mmHg (± 7.9, $n = 4$) (51.2%) at 50 mg kg^{-1}, i.v. In the absence of atropine and propranolol, L-NAME (10 mg kg^{-1}, i.v.) increased the BP above baseline by 50.8 mmHg (± 2.1, $n = 13$) (146.8%). In animals for administration of SP, NKA or capsaicin, L-NAME (10 mg kg^{-1}, i.v.) increased mean BP by 153%, 119% and 119% respectively. These increases were

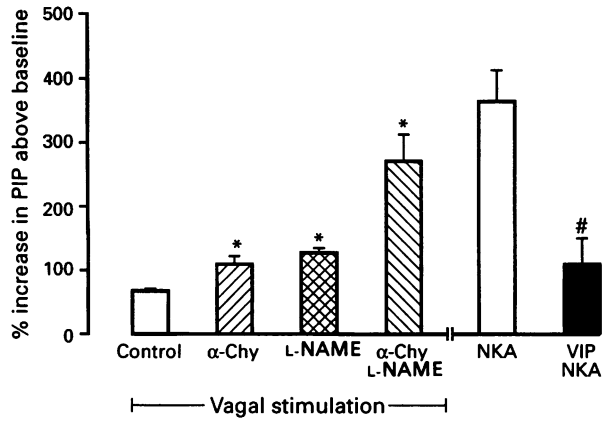


Figure 5 Potentiation and inhibition of bronchoconstriction in anaesthetized guinea-pigs. Left panel (four columns): effect of α-chymotrypsin (α-Chy, 2 units kg^{-1}, i.v.) and N^G-nitro-L-arginine methyl ester (L-NAME, 5 mg kg^{-1}, i.v.), either alone or in combination (hatched and cross-hatched columns) on the percentage increase in pulmonary insufflation pressure (PIP) induced by vagus nerve stimulation (open column: 2.5 Hz, 5 V, 5 ms for 3 min in the presence of atropine and propranolol). Right panel (two columns): effect of vasoactive intestinal peptide (VIP, 10 μg kg^{-1}, i.v.) on neurokinin A (NKA, 0.1 μmol kg^{-1}, i.v.)-induced increase in PIP. Data are mean percentage increases in PIP above baseline ($n = 5$ animals per group), vertical lines show s.e.mean. $*P < 0.05$ compared with control (vagal stimulation); $\#P < 0.05$ compared with NKA alone.

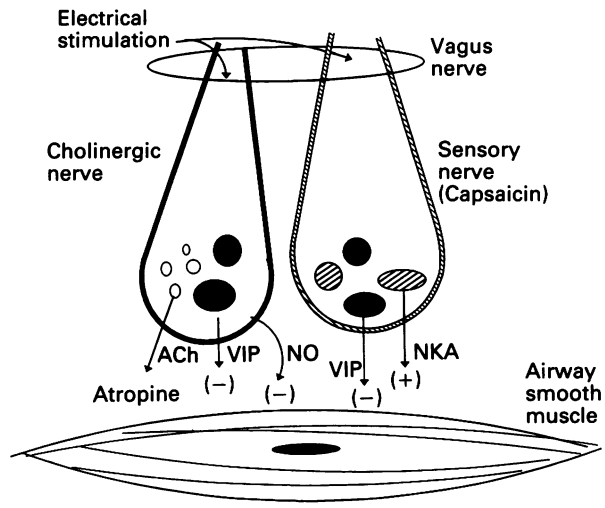


Figure 6 Model of possible excitatory and inhibitory neural mechanisms in guinea-pig airways. Electrical stimulation of the vagus nerves induces release of acetylcholine (ACh) from parasympathetic post-ganglionic cholinergic nerve terminals and antidromic release of sensory neuropeptides (including neurokinin A, NKA) from capsaicin-sensitive sensory nerves. In the presence of atropine, only bronchoconstrictor neuropeptides (e.g. NKA) will induce airway smooth muscle contraction (+). Nerve activation is also associated with release of endogenous bronchodilators (−): nitric oxide (NO) from cholinergic nerves and vasoactive intestinal peptide (VIP) from cholinergic nerves and sensory nerves. For details, see text.

reversed by 130% by SP (which represents a fall below pre-L-NAME baseline), by 81% by NKA and by 70% by capsaicin. Methylene blue induced a transient (no longer than 5 s) increase in mean BP above baseline of 34.6% ($n = 12$). SNP lowered the mean BP to 64.3% below the baseline BP ($n = 14$), an effect which had returned to baseline before vagus nerve stimulation.

Discussion

An airway inhibitory non-adrenergic, non-cholinergic (i-NANC) neural pathway has been demonstrated *in vitro* in a number of animal species including guinea-pig (Coburn & Tomita, 1973; Coleman & Levy, 1974; Richardson & Bouchard, 1975; Taylor *et al.*, 1984), baboon (Middendorf & Russell, 1980) and man (Richardson & Béland, 1976; Taylor *et al.*, 1984). The identities of the mediators of the relaxation have been determined *in vitro* for the guinea-pig trachea precontracted with carbachol or histamine. α-Chymotrypsin reduced i-NANC relaxations by 35–42% (Ellis & Farmer, 1989a; Li & Rand, 1991) and incubation with VIP antiserum reduced NANC relaxations by 23–70% (Ellis & Farmer, 1989b; Li & Rand, 1991; Matsuzaki *et al.*, 1980). Antisera to PHI only reduced relaxations by 20% (Ellis & Farmer, 1989b). These data indicate that VIP (and, to a lesser extent, PHI) mediates part of the relaxation. A significant proportion (38% to approximately 90%) of the remainder of the relaxation is abolished by NO-synthase inhibitors (Tucker *et al.*, 1990; Li & Rand, 1991) which indicates that NO also mediates a substantial part of the relaxation. In human trachea, NO appears to account completely for the relaxation induced at lower frequencies of stimulation (Belvisi *et al.*, 1992). The variability in the magnitude of inhibition of the relaxant response in the studies above may relate to the specificity of the α-chymotrypsin, the VIP antisera, and the NO-synthase inhibitor used, as well as to the frequency of stimulation and whether indomethacin was present in the bath. *In vivo* in the cat, NANC bronchodilatation can be demonstrated when the basal tone is raised with 5-hydroxytryptamine (Diamond & O'Donnel, 1980; Irvin *et al.*, 1980). *In vitro* data indicate that VIP and other peptides sensitive to α-chymotrypsin do not mediate relaxation in this species (Altiere & Diamond, 1985). Endogenous NO has also been shown *in vitro* to regulate the magnitude of cholinergic bronchoconstriction (Belvisi *et al.*, 1991). The guinea-pig trachea, isolated *in situ* and fluid-filled, has previously been shown to demonstrate an i-NANC relaxation (Chesrown *et al.*, 1980). In the present study, we wished to determine whether a neural inhibitory NANC mechanism regulated the magnitude of neural bronchoconstriction *in vivo* in the guinea-pig. We found that the NO synthase inhibitor L-NAME (Rees *et al.*, 1990), at doses inducing increases in carotid artery pressure, dose-relatedly potentiated neural bronchoconstriction in the guinea-pig *in vivo*. These data indicate that endogenous NO, or NO-releasing material, produced in association with stimulation of the vagus nerves, regulates the magnitude of the bronchoconstriction. The increase in blood pressure induced in the present study in guinea-pigs is consistent with removal of the vasorelaxant effects of NO by L-NAME inhibition of NO synthase (Rees *et al.*, 1990). The lack of a potentiating effect on neurogenic bronchoconstriction of phenylephrine, given in the present study at a dose inducing a marked increase in blood pressure, indicates that the potentiating effect of L-NAME was not a non-specific response to vasoconstriction. The specificity of the response was confirmed by the lack of potentiation by D-NAME, an inactive enantiomeric analogue of L-arginine, and the reversal of the effect of L-NAME by L-arginine (the NO precursor) but not D-arginine, its inactive enantiomer.

The precise source of the NO is unexplored in the present study although it is unlikely to be the epithelium (Brave *et al.*, 1991). A number of studies indicate a neural source. NO synthase protein has been localized immunocytochemically to central and peripheral nerves (Bredt *et al.*, 1990), which is consistent with the ability of brain synaptosomes to generate NO (Knowles *et al.*, 1989), and nerve stimulation has been

demonstrated to release NO (Bult *et al.*, 1990). The possible neural mechanisms involved in the present study are shown in the model of Figure 6. Electrical stimulation of the vagus nerves in guinea-pigs activates parasympathetic cholinergic nerves and capsaicin-sensitive sensory nerves. We chose to investigate neurogenic NANC bronchoconstriction rather than cholinergic bronchoconstriction because it is easier to eliminate adrenergic and cholinergic influences *in vivo* than it is to eliminate excitatory NANC influences. We used atropine to eliminate the constrictor influences of acetylcholine released from vagal cholinergic nerves and propranolol to prevent adrenergic influences, for example catecholamine release in response to vagally-induced hypotension, inducing airway relaxation. Thus, the bronchoconstriction seen in the present study is due primarily to the smooth muscle-contracting properties of substance P and neurokinin A released by vagus nerve stimulation from a population of sensory nerves sensitive to capsaicin, the pungent principle of hot *Capsicum* peppers (Holzer, 1991). L-NAME did not significantly affect bronchoconstriction induced by either SP or NKA which indicates that NO is released in association with nerve activation rather than being released by tachykinins or in response to bronchoconstriction. In addition, the released NO is not regulating bronchoconstriction by acting as a tachykinin receptor antagonist but rather is acting as a functional antagonist or is inhibiting some aspect of neurotransmission, for example inhibition of SP and NKA release. Brave *et al.* (1991) suggested that functional antagonism accounts for the inhibitory action of NO rather than inhibition of neurotransmitter release (Figure 6). Whether direct release of NO from NO-generating airway nerves accounts for the regulation of NANC bronchoconstriction remains to be determined.

The NO may be derived from the cholinergic or the sensory nerves. Capsaicin-induced bronchoconstriction was not affected by L-NAME which indicates that the cholinergic nerves were the source of the NO (Figure 6). However, certain differences in the mechanisms of nerve activation between electrical stimulation and capsaicin (Holzer, 1991) indicate further studies are required to clarify this suggestion.

The lack of potentiation by L-NAME of bronchoconstriction induced by capsaicin, as well as that induced by SP or NKA, may be due to a change in capillary transit time brought about by raised systemic blood pressure tending to increase clearance of the agonists. However, substance P, NKA and capsaicin decrease blood pressure and in the present study reversed the increase in blood pressure induced by L-NAME. These data indicate that the lack of potentiation by L-NAME was due primarily to factors other than changes in lung clearance of agonists.

In the present study we found that L-arginine alone partially inhibited neurogenic bronchoconstriction, particularly at the higher frequency of 10 Hz. This is in contrast to the vasculature where intracellular L-arginine is considered to be in excess (i.e. is not rate-limiting) and exogenous administration only produced relaxation when intracellular stores are depleted (Ignarro & Gold, 1990). These data suggest differences in NO production between vascular cells and nerve cells and merit further study.

In the present study we found that NO regulated NANC bronchoconstriction induced *in vivo* by stimulation of capsaicin-sensitive sensory nerves. A previous study *in vitro* in the guinea-pig found that L-NAME did not potentiate constrictor NANC responses to electrical field stimulation of the main or hilar bronchi (Belvisi *et al.*, 1991). The latter result is not surprising because guinea-pig bronchi fail to demonstrate an i-NANC relaxation (Grunstrom *et al.*, 1981), so that it is unlikely that sufficient NO is synthesized at this airway level for L-NAME to demonstrate any potentiating effect. The combined observations that L-NAME potentiates PIP *in vivo* but has no effect in main or hilar bronchi *in vitro* indicates that PIP is a measure of narrowing of airways other than extrapulmonary bronchi.

The actions of NO in a number of tissues are considered to be mediated via activation of soluble guanylyl cyclase (SGC), which leads to the production of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Moncada & Higgs, 1991). In the present study, we found that methylene blue, an inhibitor of activation of SGC by NO and other nitrovasodilators (Gruetter *et al.*, 1981; Martin *et al.*, 1985), potentiated the increase in PIP induced by vagal stimulation. The latter observation indicates that NO-activation of SGC and production of cyclic GMP mediates the endogenous regulation of neurogenic bronchoconstriction *in vivo*, a suggestion which is consistent with the demonstration *in vitro* that airway relaxation in response to NO and other nitrovasodilators is entirely cyclic GMP-dependent (Katsuki *et al.*, 1977). Our present data demonstrating a lack of potentiating effect for phenylephrine suggest that the potentiation was not a non-specific response to the transient increase in blood pressure induced by methylene blue. In the present study we also found that sodium nitroprusside, an activator of SGC leading to production of cyclic GMP (Schultz *et al.*, 1977; Ignarro *et al.*, 1981), inhibited neurogenic bronchoconstriction at 10 Hz, the latter effect being reversed by methylene blue, and completely inhibited methylene blue induced potentiation of neurogenic bronchoconstriction at 2.5 Hz. These observations indicate that guinea-pig airways express a functional SGC/cyclic GMP system which not only contributes to the endogenous regulation of NANC bronchoconstriction, via NO generation, but which can also be activated by exogenous administration of nitrovasodilator drugs.

VIP is another candidate i-NANC neurotransmitter, which has a potent relaxant effect in guinea-pig (Venugopalan *et al.*, 1986; Ellis & Farmer, 1989b) and feline (Altiere & Diamond, 1984) airway smooth muscle *in vitro*. Similarly, α-chymotrypsin, which degrades peptides including VIP, reduces the magnitude and duration of NANC neural relaxation *in vitro* (Ellis & Farmer, 1989; Li & Rand, 1991). In the present *in vivo* study, we found that VIP significantly reduced NKA-induced bronchoconstriction and that α-chymotrypsin potentiated bronchoconstriction induced by both capsaicin and electrical stimulation of the vagus nerves. These results are consistent with the involvement of VIP in the regulation of neurogenic bronchoconstriction. The source of VIP is most likely to be neural (see model in Figure 6). VIP is co-localized with acetylcholine in cholinergic nerves (Laitinen *et al.*, 1985) and would be released from parasympathetic postganglionic efferent fibres in response to vagus nerve stimulation (Figure 6). In addition, the potentiation by α-chymotrypsin of capsaicin-induced bronchoconstriction is consistent with the localization of VIP to some sensory nerves, including subepithelial nerves in the airways (Lundberg *et al.*, 1984; Luts & Sundler, 1989). Thus, antidromic electrical stimulation of vagal afferents may also induce VIP release (Figure 6). However, it should be noted that the relative lack of specificity of α-chymotrypsin for VIP means that potent and specific receptor antagonists are required before the precise role of this peptide as an inhibitory neurotransmitter can be deduced. In the present study, potentiation of NANC bronchoconstriction induced by α-chymotrypsin combined with L-NAME was greater than that induced by L-NAME or α-chymotrypsin alone. This suggests that NO and VIP are both involved in the regulation of NANC bronchoconstriction induced by vagus nerve stimulation in guinea-pig *in vivo* and act as i-NANC neurotransmitters (Figure 6).

The demonstration of NO-regulation of cholinergic bronchoconstriction in human airways *in vitro* (Belvisi *et al.*, 1992) indicates the possible relevance of this inhibitory pathway to the pathophysiology of asthma: a defect in endogenous regulation, possibly due to increased breakdown of NO in inflamed airways by the action of oxygen-derived free radicals such as superoxide anions released from activated inflammatory cells, may account in part for the exaggerated bronchoconstriction of asthmatic patients. Fur-

thermore, drugs designed to activate airway soluble guanylyl cyclase and increase cyclic GMP may have therapeutic potential in asthma.

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Potentiation of miniature endplate potential frequency by ATP in *Xenopus* tadpoles

[1]Wen-Mei Fu, Shiau-Hui Yang & Shoei-Yn Lin-Shiau

Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, Taiwan

1 Extracellular application of ATP (1 mM), a substance co-stored and co-released with acetylcholine in peripheral nervous systems, potentiated the spontaneous secretion of acetylcholine (ACh) but had no effect on the amplitude and decay time constant of miniature endplate potentials (m.e.p.ps) at neuromuscular synapses in *Xenopus* tadpoles.

2 α,β-Methylene ATP (0.3 mM) and GTP (1 mM) were also effective in increasing m.e.p.p. frequency. On the other hand, ADP, AMP and adenosine (all at 1 mM) decreased m.e.p.p. frequency.

3 Unlike the transient effect of ATP analogue and GTP on m.e.p.p. frequency, the phorbol ester TPA (2 μM) which is a protein kinase C activator, increased m.e.p.p. frequency consistently and the effects lasted as long as the presence of TPA.

4 Staurosporine (0.5 μM) and H-7 (10 μM), which are protein kinase C inhibitors, each decreased the basal level of m.e.p.p. frequency and markedly inhibited the effects of both ATP and TPA.

5 These results suggest that there is a basal activity of cytosolic protein kinases in the nerve terminals of *Xenopus* tadpoles and the effect of ATP is probably mediated by the binding of membrane surface purinoceptors which in turn activates cytosolic protein kinases and increases ACh release.

Keywords: ATP; protein kinase activation; ACh release

Introduction

Cell-cell interactions during the early phase of synaptogenesis are of crucial importance to the formation of neuronal connections. These interactions are responsible for cell-cell recognition and selective adhesion, and initiate the structural and functional differentiation in pre- and postsynaptic cells. The vertebrate neuromuscular junction forms as a result of intimate interactions between two specialized cells. Development of synaptic connections depends on electrical and trophic interactions between pre- and postsynaptic cells (Purves, 1986; Frank, 1987; Shatz, 1990). Release of trophic factors from the presynaptic nerve terminals may regulate the distribution and the ion-conducting properties of transmitter receptors in the postsynaptic membrane (Usdin & Fishbach, 1986; Nitkin *et al.*, 1987; Laufer & Changeux, 1987; Mulle *et al.*, 1988). Factors secreted by the postsynaptic cell may regulate the growth and survival of presynaptic neurones (Henderson *et al.*, 1981; Gurney, 1984; Oppenheim & Haverkamp, 1988). It is now widely recognized that many mature neurones contain more than one neurotransmitter (Hokfelt *et al.*, 1984), and in at least some instances, both substances have physiological effects on the postsynaptic cell (see, for example, Jan *et al.*, 1983). Adenosine-5'-triphosphate (ATP) is known to be released in association with acetylcholine (ACh) at synapses in the vertebrate peripheral nervous system (Silinsky & Hubbard, 1973; Silinsky, 1975; Zimmerman, 1978). Muscle cells are also known to secrete substantial amounts of ATP in response to electrical activity (Smith & Lindgren, 1986). Exogenous ATP and its derivative adenosine, at mature synapses, have inhibitory effects on the evoked and spontaneous ACh release (Ribeiro & Walker, 1975; Silinsky & Ginsborg, 1983), suggesting that ATP may serve as a negative feedback signal for regulating transmitter release. We were the first to report that ATP greatly potentiates the spontaneous ACh release at developing neuromuscular synapses in *Xenopus* cell culture (Fu & Poo, 1991). Since spontaneous ACh release is known to regulate the development of contractile properties of the postsynaptic muscle cell (Kidokoro & Saito, 1988), extracellular ATP may serve as a positive trophic factor at developing neuromuscular synapses. We report here that extracellular ATP also causes an increase in the frequency of miniature endplate potentials (m.e.p.ps) of *Xenopus* tadpoles, although the effects are much smaller than that of cultured cells. However, it is interesting to find that there are developmental changes in the modulation of ACh release by ATP.

Methods

The experiments were performed on the myotomal cells of tadpoles of *Xenopus laevis*. Stages of development of tadpoles of *Xenopus* were assessed by the criteria of Nieuwkoop & Faber (1967). We used tadpoles from stages 49 to 52 (about 2 to 3 weeks-old embryos). Tadpoles were placed in Ringer solution (composition mM: NaCl 115, $CaCl_2$ 2, KCl 2.5, HEPES 10, pH 7.3), where skin, head and viscera were gently removed, leaving the skinned tail. The tail then was transferred to a small sylgard-lined petri dish and pinned out on its side, and 1–3 μM tetrodotoxin and 10 mM $CaCl_2$ were added to the bathing solution to avoid muscle contraction and improve membrane resealing, respectively.

Synaptic miniature endplate potentials (m.e.p.ps) were measured by standard microelectrode techniques. The microelectrodes were filled with 3 M potassium acetate and had resistances ranging from 5–10 MΩ. All experiments were performed at room temperature (23–25°C). M.e.p.ps were processed on a digital waveform analyzer (Data 6100)-computer system. To avoid the large variance of the m.e.p.p. frequency between different endplates, we used the same endplate to monitor the effects of drugs. Spontaneous quantal release was estimated as the ratio of frequency after and before drug treatment. The results are expressed as the mean ± s.e.mean. The statistical significance was evaluated by Student's *t* test. However, ANOVA and Kruskal-Wallis one-way analysis were used to evaluate the statistical significance between the histograms in Figure 3.

[1] Author for correspondence.

The following chemicals were used: ATP, ADP, AMP, adenosine, GTP, α,β-methylene ATP, TPA (phorbol 12-myristate 13-acetate) (+)-tubocurarine and tetrodotoxin (Sigma); staurosporine (Boehringer) and H-7 (1- (5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride) (Seikagaku Kogyo Co., Japan).

Results

Effects of ATP on the miniature endplate potentials (m.e.p.ps)

In the tadpoles of stages 49 to 52 of *Xenopus*, the resting membrane potential was −80 to −100 mV. The mean frequency of m.e.p.p. was 118.6 ± 18.3 min^{-1} ($n = 42$). Many of these spontaneous ACh releases are capable of eliciting action potentials and contractions in the muscle cells. From observation under the microscope, application of 7 μM (+)-tubocurarine completely abolished the spontaneous contractions of the tadpoles. Therefore, m.e.p.ps were recorded from the same endplate in the presence of 1–3 μM tetrodotoxin to prevent muscle contractions. Figure 1a showed the effect of bath-applied 1 mM ATP on the frequency of m.e.p.ps. Each curve connects data collected from 1 synapse. The m.e.p.p. frequency increased transiently soon after ATP application, and the potentiation gradually subsided within 5 min. Figure 2 shows representative oscilloscopic traces of m.e.p.ps before (Figure 2a) and after (Figure 2b) ATP treatment. In addition, we examined the effects of α,β-methylene ATP, a slowly degraded derivative of ATP. We found that application of 0.3 mM α,β-methylene ATP also increased m.e.p.p. frequency transiently (Figure 1b). The increase of m.e.p.p. frequency by ATP is unlikely to result from the postsynaptic action of ATP because the m.e.p.p. amplitude histogram was unaffected by ATP (Figure 3). The average m.e.p.p. amplitude and

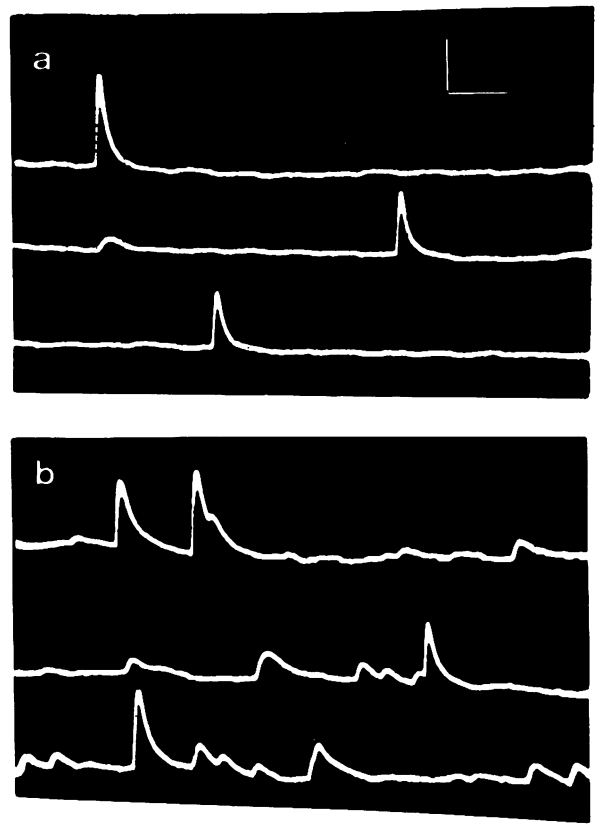


Figure 2 Potentiation of spontaneous acetylcholine (ACh) release by adenosine 5'-triphosphate (ATP). Samples of miniature endplate potentials (m.e.p.ps) before (a) and after (b) 1 mM ATP application are shown on the oscilloscope records. Scale bars are 2 mV and 20 ms, respectively.

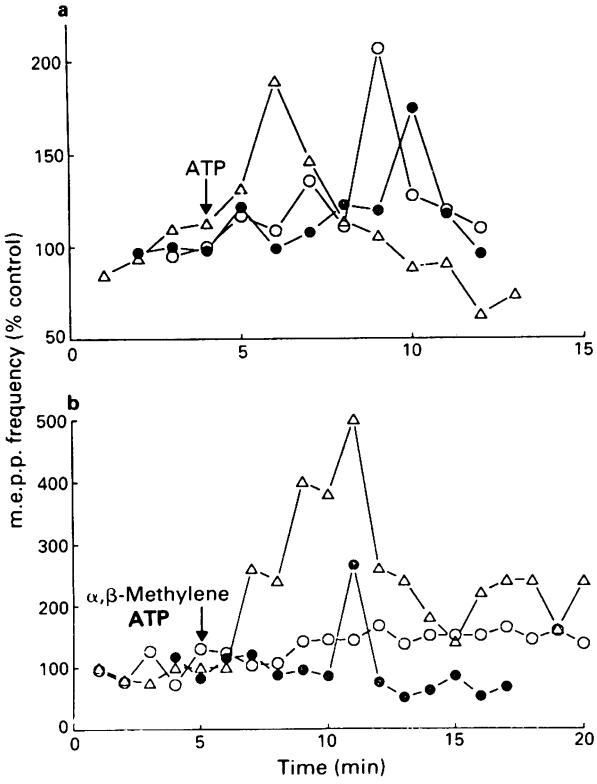


Figure 1 The effect of adenosine-5'-triphosphate (ATP) analogues on the frequency of miniature endplate potentials (m.e.p.ps). ATP at 1 mM (a) or α,β-methylene ATP at 0.3 mM (b) was bath applied at the time marked by the arrow. Each curve connects data collected from 1 synapse. Note that both drugs increased m.e.p.p. frequency transiently.

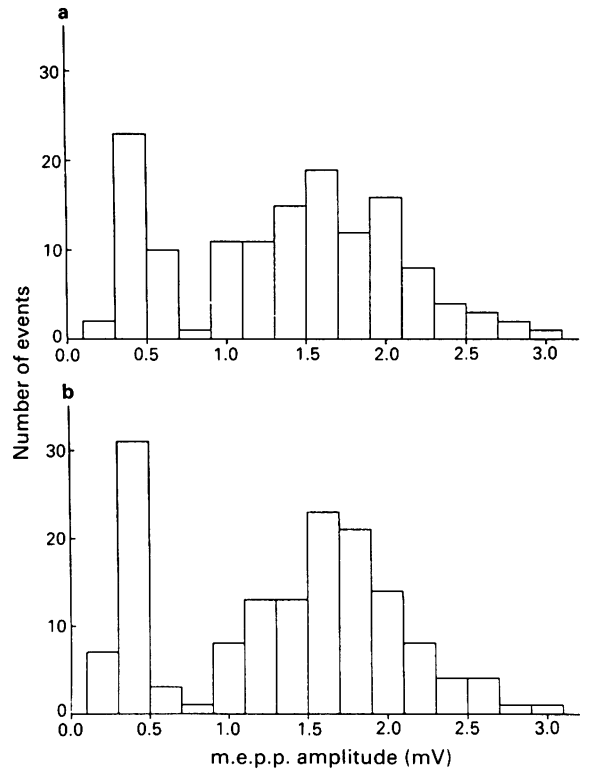


Figure 3 The effect of adenosine-5'-triphosphate (ATP) on the amplitude distribution of miniature endplate potentials (m.e.p.ps). Compared with control (a), 1 mM ATP had no significant effect on the m.e.p.p. amplitude (b). Amplitude histograms constructed from 140 m.e.p.ps.

decay time constant before and after ATP treatment was 1.56 ± 0.16 mV, 3.52 ± 0.13 ms and 1.34 ± 0.12 mV, 3.68 ± 0.13 ms, respectively ($n = 9$).

Potency of related nucleotides

Previous studies have indicated that P_2-purinoceptors mediate extracellular ATP effects in many cell types. We have examined the effects of various nucleotides on m.e.p.ps. We found that adenosine diphosphate (ADP), adenosine monophosphate (AMP) or adenosine (all at 1 mM) did not increase m.e.p.p. frequency but instead, decreased it (Figure 4). The ratios of the minimal m.e.p.p. frequency after treatment of various nucleotides to the control frequency were 61.6 ± 2.5% ($n = 6$), 48.2 ± 6.0% ($n = 9$) and 30.5 ± 7.1% ($n = 5$) for ADP, AMP and adenosine, respectively. These results indicated that the enhancing effect of ATP on ACh secretion was due to the action of ATP itself, not to any of its hydrolytic products. To our surprise, guanosine triphosphate (GTP) at 1 mM also increased m.e.p.p. frequency transiently as ATP did (Figure 5). The ratio of the maximal m.e.p.p. frequency after treatment of GTP to the control frequency was 295 ± 55% ($n = 5$). These results were summarized in Figure 6; ATP, α,β-methylene ATP and GTP increased but ADP, AMP and adenosine decreased m.e.p.p. frequency of tadpoles of *Xenopus*.

Involvement of protein kinases

It has been previously reported that ATP application to myotube cultures triggers the formation of inositol trisphosphate and diacylglycerol, which are known to activate protein kinase C (PKC). Enhancement of PKC activity by direct activation with phorbol esters is also known to increase transmitter release. To determine whether ATP potentiates the spontaneous ACh release by activating PKC, we have examined the effect of kinase inhibitors. Figure 7 showed that pretreatment with staurosporine (at 0.5 μM), a PKC inhibitor (Tamaoki *et al.*, 1986), completely inhibited the potentiating effect of ATP. As shown in Figure 7, pretreatment with staurosporine by itself caused a marked inhibition of the basal level of spontaneous ACh release. The ratio of minimal m.e.p.p. frequency after treatment with staurosporine to the control frequency was 36.3 ± 4.2% ($n = 7$). Another protein kinase C inhibitor, H-7 (10 μM) also had similar effects, inhibiting the basal level of m.e.p.p. and ATP effects by 40.0 ± 5.9% and 66.3 ± 13.5%, respectively ($n = 4$). For comparison, we also examined the effects of phorbol ester, TPA. TPA at 2 μM increased m.e.p.p. frequency for a long period as shown in Figure 8a. Pretreatment with staurosporine (0.5 μM) also significantly reduced the potentiation effect of TPA (Figure 8b). These are two similar replicates of these experiments. Taken together, these results are consistent with the notion that the ATP effect is probably mediated by PKC and that there is a tonic PKC activity in motor nerve terminals of tadpoles.

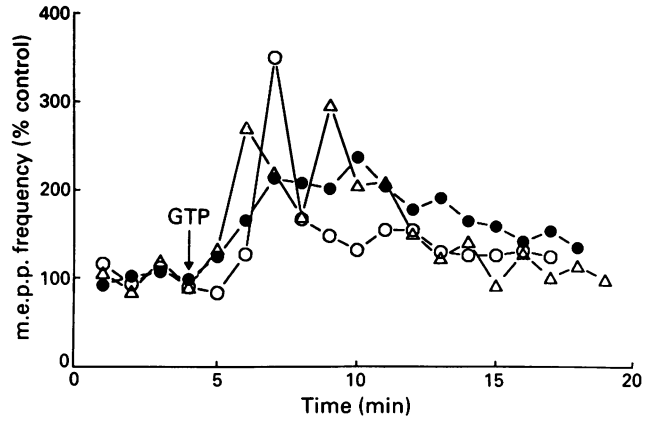


Figure 5 Effect of guanosine-5'-triphosphate (GTP) on the frequency of miniature endplate potentials (m.e.p.ps). Bath application of 1 mM GTP at the time marked by the arrow transiently increased the frequency of m.e.p.ps. Each curve represents the data collected from 1 synapse.

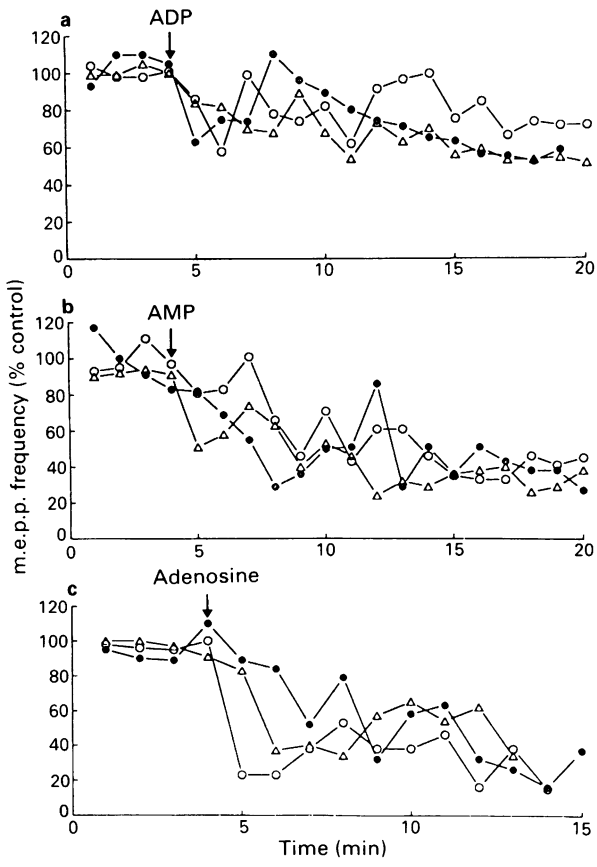


Figure 4 Effects of ADP, AMP and adenosine on the frequency of miniature endplate potentials (m.e.p.ps). Bath application of ADP (a), AMP (b) or adenosine (c) (at 1 mM each) at the time marked by the arrow decreased the frequency of m.e.p.ps. Each curve represents the data collected from 1 synapse.

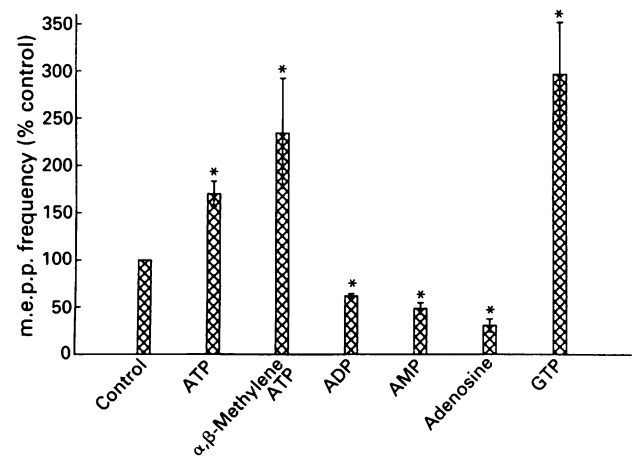


Figure 6 Effects of purine nucleotides on the frequency of miniature endplate potentials (m.e.p.ps). The maximum effects of purine nucleotides on m.e.p.p. frequency were determined. Note that ATP, α,β-methylene ATP and GTP increased but ADP, AMP and adenosine decreased m.e.p.p. frequency (all at 1 mM except α,β-methylene ATP which is 0.3 mM). Data are presented as mean ± s.e. (vertical bars) ($n = 4$–9). *$P < 0.05$ as compared with control.

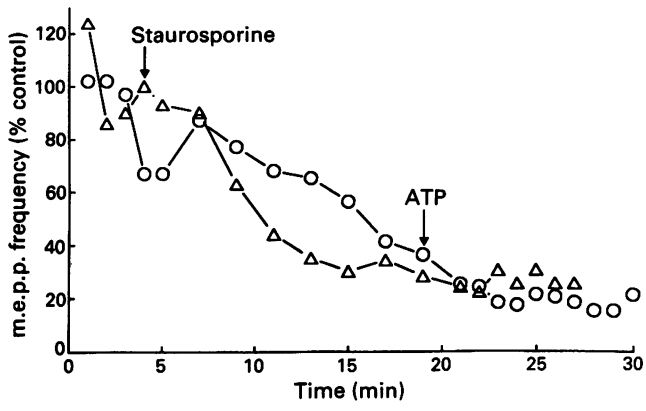


Figure 7 Antagonism by staurosporine of the potentiation effect of ATP on the frequency of miniature endplate potentials (m.e.p.ps). Pretreatment with staurosporine (0.5 μM) alone inhibited the m.e.p.p. frequency markedly. ATP was applied 15 min later and the potentiation effect was completely abolished by staurosporine. Each curve connects the data collected from 1 synapase.

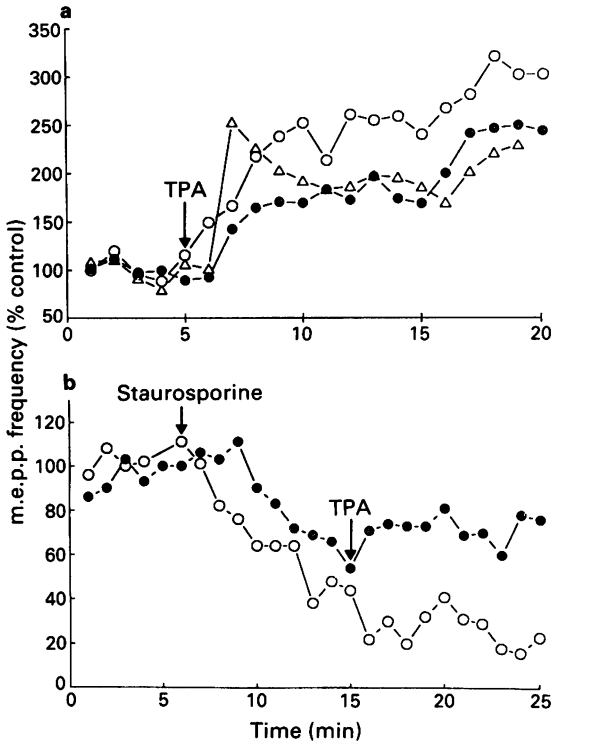


Figure 8 Effects of TPA (phorbol 12-myristate 13-acetate) on the frequency of miniature endplate potentials (m.e.p.ps). TPA (2 μM) was added at the time marked by the arrow and m.e.p.p. frequency was increased consistently and lasted as long as the presence of TPA (a). Pretreatment with staurosporine (0.5 μM) inhibited the potentiating action of TPA on m.e.p.p. frequency (b). Each curve connects the data obtained from 1 synapse.

Discussion

ATP is co-stored with ACh in the vesicle fraction of nerve endings (Dowdall *et al.*, 1974) and is a potential trophic substance at peripheral synapses. Physiological stimulation of a number of neuromuscular preparations has been shown to result in ATP release into the extracellular medium (Boyd & Forrester, 1968; Silinsky & Hubbard, 1973; Meunier *et al.*, 1975). However, ATP may also be released from stimulated postsynaptic cells. This has been observed in crayfish (Smith & Lindgren, 1986), frog (Abood *et al.*, 1962) and human muscle (Forrester, 1972) as well as in *Torpedo* electric organ (Israel *et al.*, 1976). Moreover, exogenous ATP has been shown to reduce transmitter release from presynaptic terminals of preganglionic sympathetic neurones (Silinsky & Ginsborg, 1983) and motoneurones (Ribeiro & Walker, 1975). We first found that ATP suppresses evoked ACh release, but greatly potentiates spontaneous ACh release in *Xenopus* cell culture (Fu & Poo, 1991). In the present study, we further demonstrated the positive regulation of ACh release by ATP in *Xenopus* tadpoles and suggest a possible positive trophic action of ATP during embryonic development.

That ATP had no effect on the m.e.p.p. amplitude distribution histogram further supported the idea that the increase in m.e.p.p. frequency resulted from the presynaptic increase of ACh release by ATP. The potentiation of ACh release by ATP could result directly from the action of ATP itself or of its hydrolytic products. Since we found that ADP, AMP and adenosine inhibited the frequency of m.e.p.ps, ATP must potentiate ACh release by itself, presumably through binding with cell surface ATP receptors. Although ADP is not as effective an agonist as ATP, GTP had a profound potentiating effect on the frequency of m.e.p.ps. GTP has been reported to act on P_2-purinoceptors in many cell systems (Keppens *et al.*, 1989; Okajima *et al.*, 1989). Taken together with the potentiating action of α,β-methylene ATP, ATP may act on cell surface P_2-purinoceptors. The action of GTP in tadpoles is quite different from its effects on *Xenopus* cell culture (plated from Day-1 embryos of stages 22-23) in which GTP produced no effect on spontaneous ACh release (Fu & Poo, 1991). On the other hand, adenosine produces no inhibition of ACh release in *Xenopus* cell culture (Fu & Poo, 1991) but inhibited ACh release in *Xenopus* tadpoles, which is very similar to the action at the mature nerve terminals and where it is supposed to act on adenosine A_1 receptors (Moody & Burnstock, 1982; Schubert *et al.*, 1982; Stone, 1983; Dunwiddie, 1984). The inhibitory action of ADP and AMP on m.e.p.p. frequency probably results from the hydrolytic product of adenosine as reported before (De May *et al.*, 1979; Silinsky, 1980). It is very interesting to find that compared with cell culture of earlier embryonic stages (stages 22-23), the potency of ATP is decreased (in *Xenopus* cell culture 0.3 mM ATP increased m.e.p.c. frequency a few hundred fold) and the effects of adenosine appeared in *Xenopus* tadpoles, which represent the later stages of embryos (stages 49 to 52). In recent years, increasing attention has been focused on age-related changes in receptor function. For instance, there are also developmental changes in the β-adrenoceptor modulation of L-type calcium current in enzymatically isolated adult and newborn rabbit ventricular cells (Osaka & Joyner, 1992). Thus, the potentiating action of spontaneous ACh release by ATP at earlier embryonic stage may be related to its positive trophic action.

Many hormones and neurotransmitters exert their calcium-mobilizing effects through G protein-mediated activation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol bisphosphate (PIP_2). This generates two second messengers, inositol 1,4,5-trisphosphate (IP_3), involved in the release of calcium from internal stores, and diacylglycerol (DAG) which activates protein kinase C (PKC) (Berridge, 1987). The phosphorylation product of IP_3, inositol 1,3,4,5-tetrakisphosphate (IP_4), has been proposed as an intracellular second messenger regulating Ca^{2+} influx from the extracellular to the intracellular space (Irvine & Moor, 1986; Morris *et al.*, 1987). The responses of several cell types to extracellular ATP involve phosphoinositide (PI) metabolism, ATP acting on P_2-purinoceptors coupled via G proteins to PLC (Haggblad & Heilbronn, 1987; Cockcroft & Stutchfield, 1989; Heilbronn & Eriksson, 1989; El-Moatassim *et al.*, 1992). Enhancement of PKC activity by direct activation with phorbol esters is also known to increase transmitter release (Shapira *et al.*,

1987). Since addition of staurosporine and H-7 inhibited the ATP effect, the action of ATP on the nerve terminals is probably mediated by PKC. Because of the lack of specificity of these inhibitors, involvement of other kinases cannot be excluded. In the absence of ATP, staurosporine as well as H-7 by itself inhibited significantly the basal level of spontaneous ACh release, suggesting that there is a tonic kinase activity in *Xenopus* tadpoles. Compared with the transient effect of ATP, TPA caused a prolonged increase in the frequency of m.e.p.ps, indicating that the cascades of transmitter release behind the purinoceptor activation behave normally. Thus, the decline in the ACh release frequency could be due to the desensitization of ATP purinoceptors. A similar desensitizing effect of ATP has been also observed in other cell systems (Jahar & Jessell, 1983; Krishtal *et al.*, 1983; Hume & Honig, 1986).

Spontaneous ACh release at the developing neuromuscular synapse may play a trophic function in the synapse maturation. In *Xenopus* nerve-muscle culture (Xie & Poo, 1986) as well as in *Xenopus* tadpoles, many of these spontaneous synaptic potentials are capable of eliciting action potentials and contractions in the muscle cells. Applications of (+)-tubocurarine abolished the spontaneous contractions of tadpoles. This frequent suprathreshold excitation produces a global influence on the development of the muscle cell, as shown by the acceleration of the appearance of myofibril striation (Kidokoro & Saito, 1988), the pattern of myosin isoforms and the level of oxidative and glycolytic enzymes (Salmons & Sreter, 1976). Because of developmental change of ATP action, exhibiting less effects at latter stages of embryonic development, ATP may thus contribute significantly to the synaptogenesis as well as the maturation of muscle cell during embryonic development.

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Actions and interactions of N^G-substituted analogues of L-arginine on NANC neurotransmission in the bovine retractor penis and rat anococcygeus muscles

[1]William Martin, John S. Gillespie & Ian F. Gibson

Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

1 The effects and interactions of a series of N^G-substituted analogues of L-arginine known to inhibit nitric oxide synthase were examined on non-adrenergic, non-cholinergic (NANC) neurotransmission in the bovine retractor penis (BRP) and rat anococcygeus muscles.

2 Treatment of BRP muscle strips with either N^G-nitro L-arginine (L-NOARG: 0.1–10 μM) or N^G-nitro L-arginine meythl ester (L-NAME; 0.1–100 μM) produced a concentration-dependent blockade of NANC relaxation: blockade was complete at the highest concentration of each.

3 Pretreatment with L-arginine (1–10 mM) had no effect on NANC relaxation by itself, but inhibited, in a concentration-dependent manner, the subsequent ability of both L-NOARG (0.1–300 μM) and L-NAME (0.1–1 mM) to produce blockade. L-Arginine (1–10 mM) reversed established submaximal blockade of NANC relaxation induced by L-NOARG (1 μM) or L-NAME (1 μM), but had little effect on maximal blockade induced by these agents.

4 In contrast to L-NOARG and L-NAME, N^G-monomethyl L-arginine (L-NMMA; 1 μM–1 mM) had no effect by itself on NANC relaxation of the BRP. L-NMMA (0.1–1 mM) did, however, like L-arginine, inhibit, in a concentration-dependent manner, the subsequent ability of both L-NOARG (0.1–1 mM) and L-NAME (0.1–3 mM) to produce blockade, but was more potent. As with L-arginine, L-NMMA (0.1–1 mM) reversed established submaximal blockade of NANC relaxation induced by L-NOARG (1 μM) or L-NAME (1 μM), but had little effect on maximal blockade induced by these agents.

5 In contrast to the effects on BRP, treatment of rat anococcygeus muscle with either L-NOARG (0.1–10 μM) or L-NMMA (1–100 μM) produced concentration-dependent inhibition of NANC relaxation: the maximal inhibition induced by L-NOARG and L-NMMA was 100% and 40.1 ± 5.9% ($n = 8$), respectively. L-Arginine (1–10 mM) reversed established submaximal inhibition of NANC relaxation induced by L-NOARG (1 μM), had little effect on maximal blockade by this agent, and reversed maximal blockade induced by L-NMMA (100 μM).

6 In the presence of partial blockade of NANC relaxation on rat anococcygeus by a maximal concentration of L-NMMA (100 μM), subsequent blockade by L-NOARG (0.1–100 μM) was inhibited. L-NMMA (100 μM) produced a partial reversal of established submaximal blockade of NANC relaxation induced by L-NOARG (1 μM), but had little effect on maximal blockade induced by this agent.

7 These findings suggest a complex series of interactions between L-arginine and certain of its N^G-substituted analogues that are commonly used to inhibit nitric oxide synthase. The most striking new finding is that L-NMMA does not block NANC relaxation in the BRP, but acts with greater potency than the endogenous substrate, L-arginine, to inhibit the blockade induced by L-NOARG or L-NAME. Even on rat anococcygeus where L-NMMA acts as a partial blocker of NANC relaxation, further blockade by L-NOARG is inhibited.

Keywords: Anococcygeus muscle; retractor penis muscle; NANC nerves; nitric oxide; L-arginine; N^G-monomethyl L-arginine; N^G-nitro L-arginine; N^G-nitro L-arginine methyl ester.

Introduction

There is increasing evidence that the L-arginine-nitric oxide pathway mediates neurotransmission by certain non-adrenergic, non-cholinergic (NANC) nerves in the peripheral autonomic nervous system (Gillespie *et al.*, 1989; Li & Rand, 1989), as well as neurotransmission or neuromodulation in the central nervous system (Garthwaite *et al.*, 1989; Bredt *et al.*, 1990). Early evidence of a link between the L-arginine-nitric oxide system and NANC nerves came from experiments on the rat and mouse anococcygeus and bovine retractor penis (BRP) muscles in which haemoglobin, methylene blue, free radical generators and hypoxia, which inhibit the formation or actions of endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980; Martin *et al.*, 1985; Moncada *et al.*, 1986), were shown to inhibit neurogenic relaxation (Bowman *et al.*, 1982; Bowman & McGrath, 1985; Bowman *et al.*, 1986; Gibson & Mirzazadeh, 1989; Gillespie & Sheng, 1990). Furthermore, NANC transmission, like relaxation mediated by EDRF (Rapoport & Murad, 1983; Griffith *et al.*, 1985; Martin *et al.*, 1986), was shown to proceed through elevation of cellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) content and was potentiated by inhibitors of cyclic GMP phosphodiesterase (Bowman & Drummond, 1984). The most compelling evidence linking nitric oxide to NANC transmission was obtained, however, when N^G-monomethyl L-arginine (L-NMMA), an inhibitor of nitric oxide synthase (Rees *et al.*, 1989), was shown to produce a partial inhibition of neurogenic relaxation in the anococcygeus of the rat and mouse (Gillespie *et al.*, 1989; Li & Rand, 1989; Gibson *et al.*, 1990). The inhibition was stereospecific and competitive in nature, i.e., the D-isomer was inactive, and blockade was reversed by L-, but not D-arginine; it therefore exhibited a profile similar to that for

[1] Author for correspondence.

inhibition of EDRF synthesis (Rees *et al.*, 1989). N^G-nitro L-arginine (L-NOARG) and its methyl ester (L-NAME) were subsequently shown to be more potent inhibitors of NANC relaxation than L-NMMA in the anococcygeus of the rat and mouse, and could abolish relaxation (Gibson *et al.*, 1990; Hobbs & Gibson, 1990; Liu *et al.*, 1991). Following these earlier studies, inhibitors of nitric oxide synthase were shown to inhibit certain forms of NANC inhibitory transmission in the gut (Bult *et al.*, 1990; Dalziel *et al.*, 1991; Desai *et al.*, 1991), respiratory tract (Tucker *et al.*, 1990; Li & Rand, 1991), reproductive system (Ignarro *et al.*, 1990; Liu *et al.*, 1991) and cerebral (Toda & Okamura, 1990) and penile arteries (Liu *et al.*, 1991). Nitric oxide synthase has recently been identified in homogenates of BRP and rat anococcygeus muscle (Mitchell *et al.*, 1991; Sheng *et al.*, 1991; 1992) and consists of two fractions; a soluble form similar to that isolated from brain (Bredt & Snyder, 1990; Schmidt *et al.*, 1991) and a particulate form, both of which are activated by calcium, and like NANC relaxation, are blocked by L-NOARG. Furthermore, a nitric oxide-like substance has been detected in the superfusion fluid following stimulation of NANC nerves in the isolated ileocolonic sphincter of the dog (Bult *et al.*, 1990).

Although NANC relaxation of the BRP and bovine penile artery is abolished by L-NOARG (Liu *et al.*, 1991), suggesting involvement of the L-arginine-nitric oxide pathway, we have surprisingly found that relaxation is completely unaffected by another inhibitor of nitric oxide synthase, L-NMMA. In this paper we show that L-NMMA, like the endogenous precursor, L-arginine, inhibits the ability of L-NOARG and L-NAME to block NANC relaxation, but is considerably more potent.

Methods

Preparation of tissues

BRP muscles were obtained from a local abattoir and transported to the laboratory. Some tissues were used that day, but others were stored at 4°C in Krebs solution for use the following day. BRP muscle strips 2–3 mm wide and 1 cm long were cut for tension recording. Rat anococcygeus muscles were dissected out and freed of connective tissue as previously described (Gillespie, 1972). BRP and anococcygeus muscle strips were mounted under 2 g and 1 g resting tension, respectively, within Ag-AgCl ring electrodes in 12 ml organ chambers and bathed at 37°C in Krebs solution of the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11 and gassed with 95% O_2 and 5% CO_2. Tension was measured with Grass FTO3C isometric transducers and displayed on a Grass Polygraph. In all experiments, adrenergic motor responses were blocked and the tone raised on BRP and anococcygeus muscle strips by use of guanethidine (30 μM). Electrical field stimulation was delivered at a pulse width of 0.5 ms and at supramaximal voltage.

Experimental protocols

Two separate protocols were adopted to examine the effects of L-arginine and L-NMMA on the ability of L-NOARG and L-NAME to block NANC relaxation. In the first, tissues were pretreated with L-arginine or L-NMMA to determine if this inhibited the subsequent blockade of NANC relaxation induced by L-NOARG or L-NAME. In the second, blockade of NANC relaxation was first established with L-NOARG or L-NAME and the subseqeunt ability of L-arginine or L-NMMA to reverse blockade was determined.

Drugs

L-Arginine hydrochloride, N^G-nitro L-arginine (L-NOARG) and N^G-nitro L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma. N^G-monomethyl L-arginine and N^G-monomethyl D-arginine acetate were generously provided by Wellcome Laboratories, Beckenham. Guanethidine sulphate was purchased from CIBA. All drugs were dissolved in saline (0.9%).

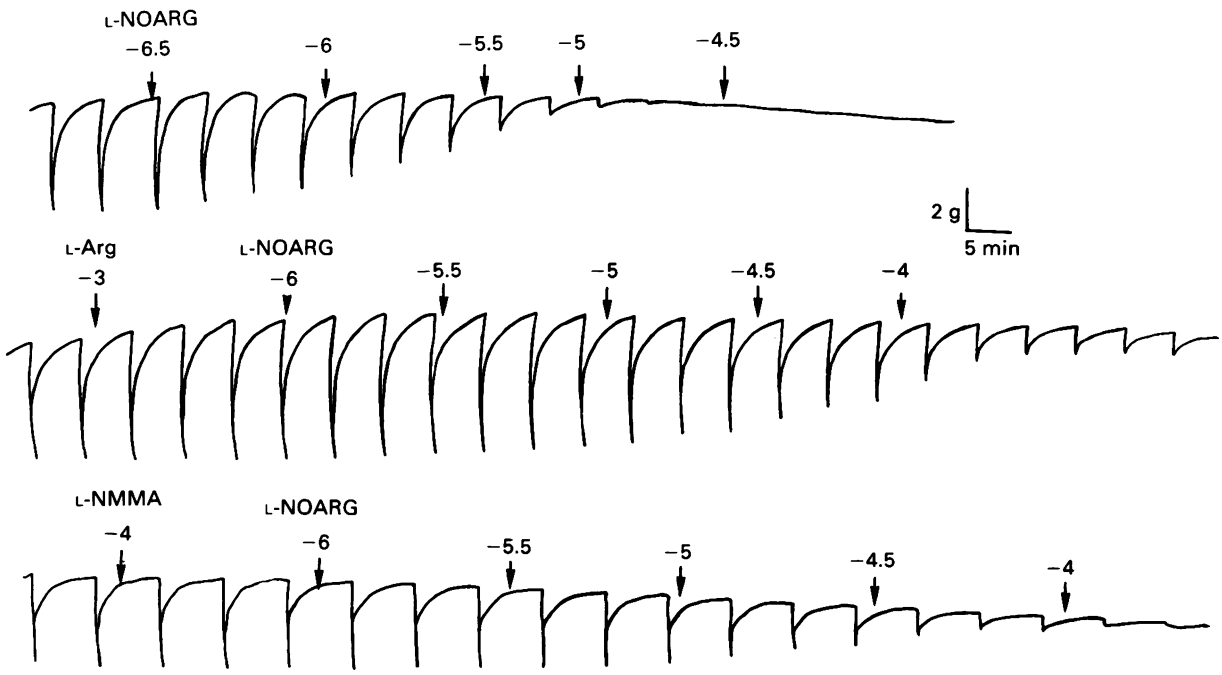


Figure 1 In the presence of guanethidine (30 μM)-induced tone, electrical field stimulation of strips of bovine retractor penis muscle at 10 Hz (100 pulses, 0.5 ms, supramaximal voltage) elicited a powerful non-adrenergic, non-cholinergic (NANC) relaxation. This relaxation was blocked in a concentration-dependent manner by N^G-nitro L-arginine (L-NOARG, 0.3–10 μM). Neither L-arginine (L-Arg, 1 mM) nor N^G-monomethyl L-arginine (L-NMMA, 100 μM) had any effect by itself on NANC relaxation, but both inhibited the ability of L-NOARG to block relaxation. All final molar concentrations are given in log units.

Results

Effects of L-NMMA, L-NOARG and L-NAME on the bovine retractor penis muscle

On strips of BRP muscle in the presence of guanethidine (30 μM)-induced tone, electrical field stimulation induced frequency (0.1–20 Hz)-dependent NANC relaxation. Treatment with L-NOARG (0.1–10 μM) or L-NAME (0.1–100 μM) produced a concentration-dependent blockade of NANC relaxation at all frequencies and complete abolition was obtained with the highest concentration of each. In contrast, L-NMMA (1 μM–1 mM) had no effect on NANC relaxation at any frequency. Figures 1–5 show the effects of these treatments on responses obtained following stimulation at 10 Hz (100 pulses).

Pretreatment with L-arginine (1–10 mM) had no effect by itself on NANC relaxation (Figure 1), but produced a concentration-dependent inhibition of the blockade induced by subsequent treatment with L-NOARG (0.1–300 μM; Figures 1 and 3a) or L-NAME (0.1–1 mM; data not shown). L-NMMA, but not D-NMMA, was also able to inhibit the blockade induced by subsequent treatment with L-NOARG (0.1–300 μM; Figures 1 and 3b) or L-NAME (0.1–3 mM; Figure 4), but was more potent than L-arginine: a concentration-dependent inhibition of the effects of L-NOARG was seen with L-NMMA over the range 100–300 μM, but concentrations of 1 mM or greater had no additional effect, whereas, the effects of L-NAME were blocked concentration-dependently by L-NMMA over the range 0.1–1 mM. In a second series of experiments, attempts were made to reverse L-NOARG- and L-NAME-induced blockade of NANC relaxation by subsequent addition of L-arginine or L-NMMA. Both L-arginine (1–10 mM) and L-NMMA (0.1–1 mM) reversed established submaximal inhibition of NANC relaxation induced by L-NOARG (1 μM) or L-NAME (1 μM), but had little effect on maximal inhibition by these agents (data not shown).

Effects of L-NMMA and L-NOARG on the rat anococcygeus muscle

In keeping with our previous findings (Liu *et al.*, 1991) on the rat anococcygeus muscle in the presence of guanethidine (30 μM)-induced tone, L-NOARG (0.1–10 μM) and L-NMMA (1–100 μM) each produced a concentration-dependent blockade of the NANC relaxation induced by electrical field stimulation: at 10 Hz (100 pulses) L-NOARG produced complete blockade (Figure 5), whereas the maximum blockade induced by L-NMMA was 40.1 ± 5.9% ($n = 8$). L-Arginine (1–10 mM) reversed established submaximal blockade of NANC relaxation induced by L-NOARG (1 μM), had little effect on maximal blockade by this agent, and reversed maximal blockade induced by L-NMMA (100 μM) (data not shown). Under conditions where the NANC response was partially blocked by a maximal concentration of L-NMMA (100 μM), protection was seen against the inhibitory actions of L-NOARG (0.1–100 μM; Figure 5). L-NMMA (100 μM) was able to produce a partial reversal of established submaximal blockade of NANC relaxation induced by L-NOARG (1 μM), but had little effect on maximal blockade induced by L-NOARG (10–30 μM) (data not shown).

Discussion

With the advent of compounds that inhibit the enzyme nitric oxide synthase (Rees *et al.*, 1989; 1990), the concept of the L-arginine-nitric oxide pathway mediating neurotransmission in the peripheral autonomic nervous system (Gillespie *et al.*, 1989; Li & Rand, 1989; Gibson *et al.*, 1990) as well as neurotransmission or neuromodulation in the central nervous system (Garthwaite *et al.*, 1988; 1989; Knowles *et al.*, 1989; Bredt *et al.*, 1990) has become widely accepted. In the autonomic nervous system L-NMMA, L-NOARG or L-NAME inhibit NANC relaxation of smooth muscle in a diverse array of tissues such as rat and mouse anococcygeus (Gillespie *et al.*, 1989; Li & Rand, 1989; Gibson *et al.*, 1990), dog and opossum lower oesophageal sphincter (De Man *et al.*, 1991; Tottrup *et al.*, 1991), guinea-pig and rat gastric fundus (Desai *et al.*, 1991; Boeckxstaens *et al.*, 1991; Lefebvre *et al.*, 1992), human ileum (Maggi *et al.*, 1991), dog ileocolonic sphincter (Bult *et al.*, 1990) and proximal colon (Dalziel *et al.*, 1991), guinea-pig trachea (Tucker *et al.*, 1990; Li & Rand, 1991), rabbit and human corpus cavernosum (Ignarro *et al.*, 1990; Pickard *et al.*, 1991), and dog cerebral arteries (Toda & Okamura, 1990).

The NANC relaxant responses of the BRP and bovine

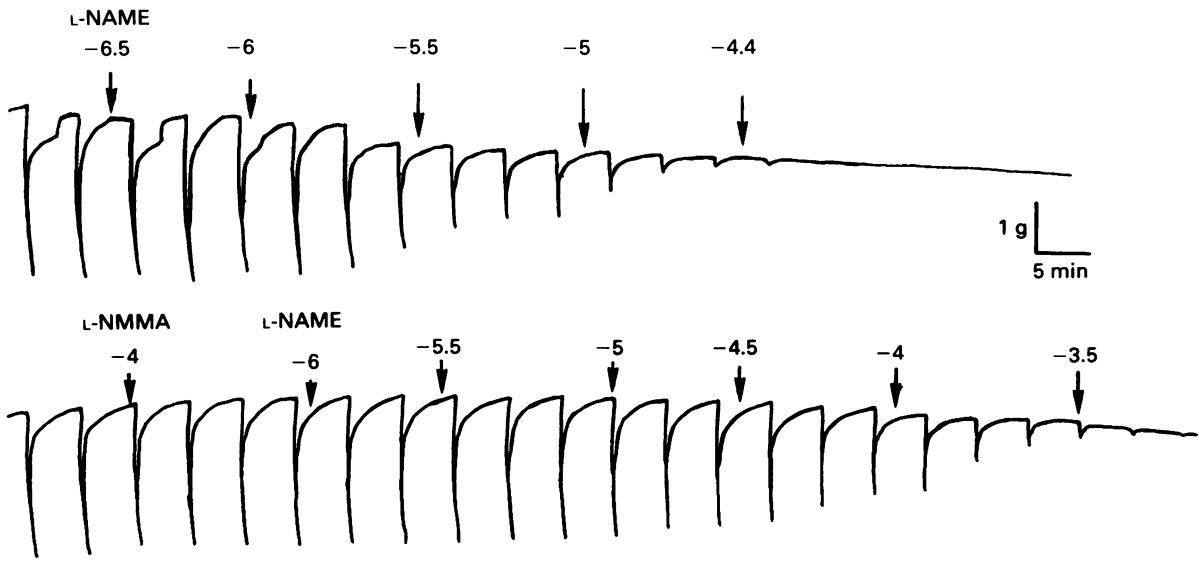


Figure 2 The non-adrenergic, non-cholinergic (NANC) relaxant response of strips of bovine retractor penis muscle obtained following electrical field stimulation at 10 Hz (100 pulses, 0.5 ms, supramaximal voltage) in the presence of guanethidine (30 μM)-induced tone was blocked in a concentration-dependent manner by N^G-nitro L-arginine methyl ester (L-NAME, 0.3–30 μM). Pretreatment with N^G-monomethyl L-arginine (L-NMMA, 100 μM) had no effect by itself on NANC relaxation, but inhibited the ability of L-NAME to block relaxation. All final molar concentrations are given in log units.

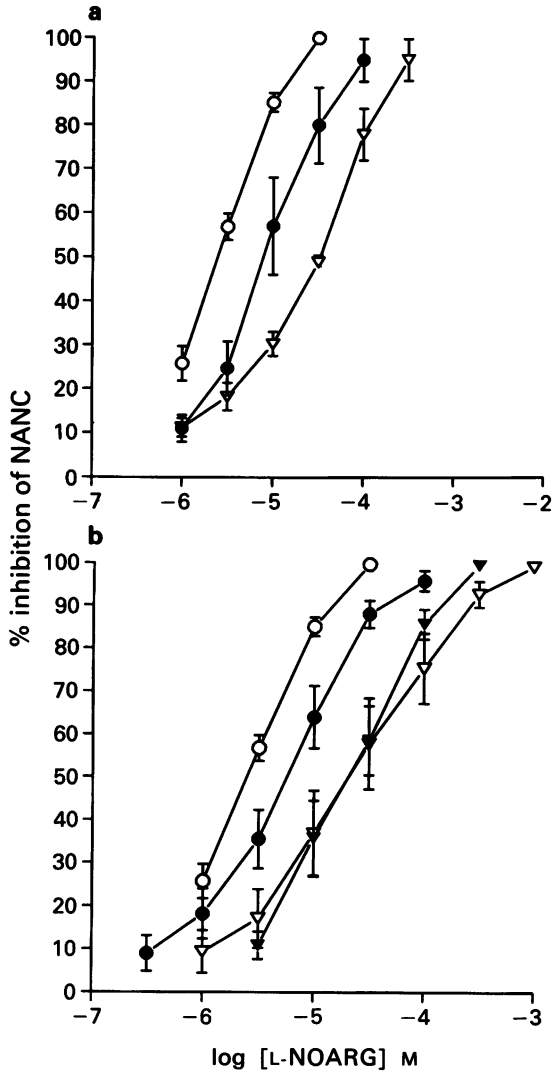


Figure 3 N^G-nitro L-arginine (L-NOARG, 1–30 μM, ○) produced a concentration-dependent blockade of the non-adrenergic, non-cholinergic (NANC) relaxant response of strips of bovine retractor penis muscle obtained upon electrical field stimulation at 10 Hz (100 pulses, 0.5 ms, supramaximal voltage). Pretreatment with L-arginine (a) at 1 mM (●) and 10 mM (▽) or with N^G-monomethyl L-arginine (b) at 100 μM (●), 300 μM (▽) and 1 mM (▼) inhibited the ability of L-NOARG to block NANC relaxation. Points are the mean, and vertical bars indicate the s.e.mean of 6–12 observations.

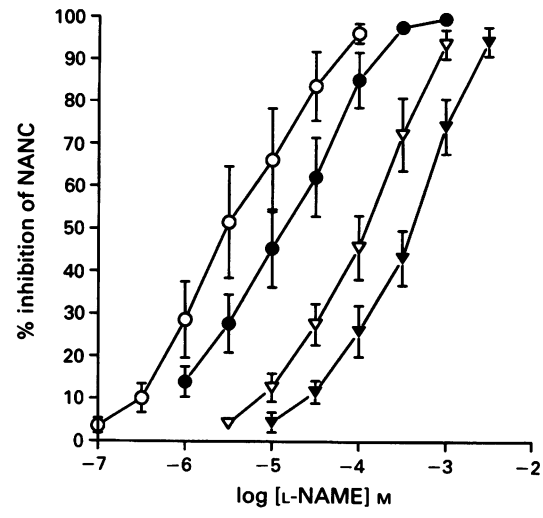


Figure 4 N^G-nitro L-arginine methyl ester (L-NAME, 0.1–100 μM, ○) produced a concentration-dependent blockade of the non-adrenergic, non-cholinergic (NANC) relaxant response of strips of bovine retractor penis muscle obtained upon electrical field stimulation at 10 Hz (100 pulses, 0.5 ms, supramaximal voltage). Furthermore, pretreatment with N^G-monomethyl L-arginine at 100 μM (●), 300 μM (▽) and 1 mM (▼) inhibited the ability of L-NAME to block NANC relaxation. Points are the mean, and vertical bars indicate the s.e.mean, of 6–12 observations.

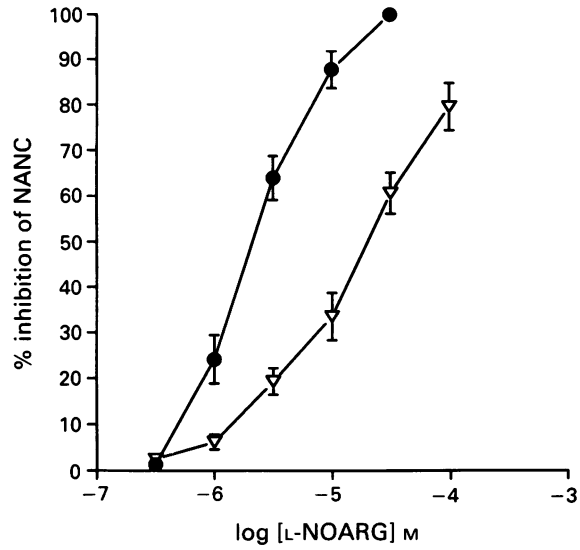


Figure 5 N^G-nitro L-arginine (L-NOARG, 0.1–30 μM, ●) produced a concentration-dependent blockade of the non-adrenergic, non-cholinergic (NANC) relaxant response of rat anococcygeus muscle obtained upon electrical field stimulation at 10 Hz (100 pulses, 0.5 ms, supramaximal voltage). Furthermore, pretreatment with N^G-monomethyl L-arginine at 100 μM (▽) inhibited the ability of L-NOARG to block NANC relaxation. Points are the mean, and vertical bars indicate the s.e.mean, of 6 observations.

penile artery (Liu *et al.*, 1991) are also certain to be mediated by the L-arginine-nitric oxide pathway since they are abolished by L-NOARG. Surprisingly, however, we have found that L-NMMA does not inhibit NANC relaxation in these tissues. This result is all the more surprising since nitric oxide synthase activity isolated from the BRP is reported to be sensitive to inhibition by L-NMMA (Sheng *et al.*, 1991; 1992). Furthermore, in the rat and mouse anococcygeus, where L-NOARG and L-NAME each abolish NANC relaxation, L-NMMA is only partially effective, blocking relaxation by no more than 50% (Gillespie *et al.*, 1989; Gibson *et al.*, 1990).

The major new observation made in this study is that, in the BRP, L-NMMA behaves similarly to the endogenous precursor of nitric oxide synthase, L-arginine, and protects NANC relaxation against the blocking actions of both L-NOARG and L-NAME, but is considerably more potent. The protection afforded by L-NMMA or L-arginine was concentration-dependent; this was seen with all concentrations of L-NMMA (0.1–1 mM) tested against L-NAME, but against L-NOARG the concentration-dependence of blockade was seen only with L-NMMA over the range 100–300 μM, with higher concentrations producing no further effect. Why L-NMMA exhibits only a partial concentration-dependent inhibition of the action of L-NOARG is not clear, but could result from a limitation on access or metabolism of L-NMMA. Nevertheless, the data with both L-NMMA and L-arginine suggest the interactions with L-NOARG and L-NAME to be competitive in nature. Despite this, we found

that neither L-NMMA nor L-arginine was able to reverse convincingly blockade induced by L-NOARG or L-NAME, unless the degree of blockade was substantially sub-maximal. Others working on the vasculature and central nervous system have reported that the blocking actions of L-NOARG cannot be reversed by L-arginine (Unmans, 1990; Dwyer *et al.*, 1991) or are only partially reversible (Steinberg *et al.*, 1990). Randall & Griffith (1991) have shown that the ability of L-NAME to inhibit basal release of EDRF in the rabbit isolated perfused ear is reversed by L-arginine, but inhibition of agonist-induced release is not. When taken together, these findings from the different laboratories suggest that competition does occur, but the rate of dissociation of L-NOARG or L-NAME from nitric oxide synthase is so slow as to give the impression of irreversibility in the normal time course of experiments.

The mechanism by which L-NMMA protects against the blocking actions of L-NOARG and L-NAME on NANC relaxation of BRP is not clear, but certain potential explanations can be excluded. One of these is that the protective effect of L-NMMA results from conversion to L-arginine. It has been shown previously that a deiminase enzyme in bovine aortic endothelial cells can convert L-NMMA, but not L-NOARG, to L-citrulline, which can be metabolized further to L-arginine (Hecker *et al.*, 1990). Alternatively, L-NMMA could potentially by metabolized by a demethylase enzyme directly to L-arginine. Neither of these is possible, however, since L-NMMA is more potent than L-arginine in protecting against the blockade by L-NOARG. It is equally unlikely that L-NMMA acts as an alternative substrate to L-arginine in the formation of nitric oxide by the neuronal nitric oxide synthase, since it partially blocks NANC transmission in the rat anococcygeus. Another possible explanation is that L-NMMA blocks the entry of L-NOARG into the NANC nerves. Again, this is unlikely, since previous work on bovine aortic endothelial cells showing the ability of L-NMMA, but not L-NOARG, to block competitively uptake of L-arginine by the y^+ cationic amino acid transporter (Bogle *et al.*, 1992), suggests separate uptake mechanisms for the two N^G-substituted analogues. Furthermore, L-NMMA also inhibits the blocking action of L-NAME, a lipophilic ester, which is likely to enter cells by simple diffusion rather than by a carrier-mediated process. Our favoured, but speculative explanation is that there is a complex interaction beween L-NMMA, L-NOARG and the endogenous precursor, L-arginine, at the level of the neuronal nitric oxide synthase. In this scheme, L-NMMA behaves as a partial inhibitor of nitric oxide synthse, in a manner similar to the actions of a partial agonist at a drug receptor, and thereby produces a variable degree of blockade by itself (low in BRP but around 50% in rat anococcygeus), but inhibits subsequent blockade by full inhibitors such as L-NOARG and L-NAME. For this to occur, L-NMMA would be required to have a higher affinity as well as a faster rate of dissociation from nitric oxide synthase than L-NOARG or L-NAME and so its presence would provide greater potential for the substrate, L-arginine, to interact with the enzyme than would be possible in the presence of L-NOARG or L-NAME alone. A definitive test of this hypothesis will require a detailed study of the interactions between L-NMMA, L-arginine and L-NOARG or L-NAME on nitric oxide synthase isolated from the NANC nerves, and this is beyond the scope of the present investigation.

In summary, on the BRP where NANC inhibitory neurotransmission is mediated by the L-arginine-nitric oxide pathway, we have found that L-NMMA, a putative inhibitor of nitric oxide synthase, does not block relaxation. Surprisingly, L-NMMA behaves in a similar manner to the endogenous precursor, L-arginine, and blocks competitively the ability of two other inhibitors of nitric oxide synthase, namely L-NOARG and L-NAME, to block NANC relaxation. On the basis of these findings it would seem unwise to use L-NMMA as a sole tool for investigating the role of the L-arginine-nitric oxide pathway in physiological or pathological processes.

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A factor released by monocytes in the presence of dexamethasone stimulates neutrophil locomotion

[1]S. Chettibi, A.J. Lawrence & *R.D. Stevenson

Department of Cell Biology, University of Glasgow, Glasgow G12 8QQ and *Department of Respiratory Medicine, Glasgow Royal Infirmary, Alexandra Parade, Glasgow G31 2ES

1 Steroid-treated monocyte supernatants cause a dramatic increase in the speed of locomotion of human neutrophils and a significant decrease in their adhesion to protein-coated glass. In contrast, control monocyte supernatants have a smaller effect on the speed of locomotion, but cause a large increase in their adhesiveness.

2 This supernatant activity was produced equally well in the presence or absence of serum after 24 h culture at 37°C with 10^{-6} M dexamethasone.

3 The effect of the steroid-treated monocyte supernatants on the speed of locomotion of human peripheral blood neutrophils was not altered by rabbit polyclonal antisera against lipocortins 1–6.

4 Rabbit anti-interleukin-8 antibody which blocked the effect of IL-8 on the speed of locomotion of neutrophils did not antagonize the locomotion stimulating action of steroid-treated monocyte supernatants.

5 The exocellular release of this factor(s) by human mononuclear leucocytes suggests that it may be an *in vivo* mediator of the anti-inflammatory effect of glucocorticoids.

Keywords: Dexamethasone; monocytes; neutrophils; locomotion; adhesion

Introduction

Mononuclear leucocytes are important immune effector cells that play a fundamental role in cellular immunity. In addition to their antigen-presenting and phagocytic activities at the site of inflammation, peripheral blood mononuclear cells are also involved in the synthesis and release of a variety of pro-inflammatory enzymes (Werb, 1978) and polypeptide cytokines (Newton, 1990; Donnelly *et al.*, 1991; Hogquist *et al.*, 1991; Pruzanski & Vadas, 1991) which modulate neutrophil responses (Seitz *et al.*, 1991). The production of these components by monocytes can be suppressed by glucocorticoids (Cupps & Fauci, 1982; Lew *et al.*, 1988; Goulding *et al.*, 1990; Almawi *et al.*, 1991; Standiford *et al.*, 1992) and this has been suggested as the basis for their anti-inflammatory action (Bochner *et al.*, 1987).

Flower (1990) and Wong *et al.* (1991) have attributed many of the anti-inflammatory actions of steroids to the production of lipocortins. This family of proteins has been characterized as inhibitors of phospholipase A_2 and are now known to be members of the wider family of calcium and phospholipid binding proteins (Davidson & Dennis, 1989). Inhibition of phospholipase A_2 activity decreases the production of pro-inflammatory mediators such as prostaglandins and leukotrienes (Blackwell *et al.*, 1982).

Further evidence that anti-inflammatory products are secreted by monocytes under the influence of glucocorticoids was reported when capillary tube polymorph migration studies were carried out. Stevenson (1973) showed that steroid-treated monocytes secreted a protease-sensitive factor(s) that greatly stimulated migration of human neutrophils *in vitro*. The production of this factor(s) by the monocytes was dependent on steroid concentration and was blocked in the presence of cyclohexamide (Stevenson, 1974). The phenomenon of stimulated neutrophil migration was also observed with leucocytes from patients receiving steroid therapy (Stevenson, 1976a). Studies of physico-chemical characteristics showed that this factor(s) was soluble, highly stable over a range of temperatures (− 20°C to + 60°C) and had a molecular weight of 12 to 15 kDa. (Stevenson, 1976c). The migration stimulatory activity of this factor(s) was estimated by measuring the area occupied by polymorphs migrating from the open end of a short capillary tube in a 24 h period in the presence of a steroid-treated mixed mononuclear leukocyte culture supernatant. This assay was simple, but required a high concentration of neutrophils. In addition it was relatively slow and has been considered ambiguous in terms of the parameter actually measured as it may be influenced by many factors including locomotion (chemotaxis, chemokinesis), cell aggregation and cell adhesion to substrata. Frank & Roth (1986) using different assay methods have also shown that culture supernatants from monocytes incubated with 0.5 or 5 $\mu g\,ml^{-1}$ hydrocortisone enhanced random neutrophil migration under agarose.

In this paper we have investigated the steroid-induced stimulatory effect on neutrophil migration using an automated cell tracking assay, which enabled us to study the behaviour of cells migrating on protein-coated glass coverslips and to unravel the various aspects of speed of locomotion and adhesion. In addition, we investigated whether the effect on neutrophils observed with steroid-treated monocyte supernatants could be attributed to lipocortin production by monocytes.

Methods

Preparation of human monocyte culture supernatants

Heparinized venous blood from healthy volunteers was mixed with 5% dextran in a ratio of 1:10 (v/v) and left for 60 min at room temperature for erythrocyte sedimentation to occur. The leukocyte-rich plasma was aspirated and layered over an equal volume of Histopaque-1077 (Sigma Chemical Co., Dorset) and the gradients were centrifuged for 15 min at 750 *g*. The interphase layer containing lymphocytes, monocytes and platelets was aspirated and mixed in a polys-

[1] Author for correspondence.

tyrene Universal bottle with phosphate buffered saline (PBS) pH 7.4, free of calcium and magnesium, washed three times and resuspended in culture medium. Cell viability was tested by trypan blue exclusion (95–99% viable cells).

Culture conditions

Lymphocytes and monocytes were resuspended in 10% Ham's F-10 medium (Gibco, Life Technologies, Paisley) supplemented with 10% (v/v) foetal calf serum (FCS) and 2.85 mM L-glutamine, 47.6 u ml^{-1} penicillin, 47.6 u ml^{-1} streptomycin and 0.3 $\mu g\ ml^{-1}$ Fungizone (amphotericin B). Cells were cultured at a concentration of 5×10^7 cells ml^{-1} in plastic tissue culture flasks. After incubation for 60 min at 37°C non-adherent cells were removed by three gentle washes with PBS. The adherent cells were cultured in culture medium with or without 10% FCS in the presence and absence of 10^{-6} M dexamethasone. After 24 h cell-free conditioned medium was obtained by centrifugation at 1200 *g* for 25 min and stored at 4°C. Viability of the monocytes was tested by trypan blue exclusion (80–90% viable cells).

Dexamethasone preparation

Dexamethasone (Sigma, Chemical Co., Dorset) was freshly made up at a concentration of 10^{-2} M in ethanol and added to the culture medium at a final concentration of 10^{-6} M dexamethasone and 0.001% ethanol. The control culture medium also contained the same concentration of ethanol.

Preparation of human neutrophils

Human neutrophils were purified by a modification of the method of Haslett *et al.* (1985). In brief, whole blood from healthy volunteers was allowed to sediment on 5% dextran after which the polymorphs were isolated on Histopaque-1077 (Sigma, Chemical Co., Dorset). The gradients were centrifuged for 10 min at room temperature at 750 *g*. The pellet consisted of polymorphonuclear leucocytes and some contaminating erythrocytes. Erythrocytes were eliminated by a 15–20-s hypotonic lysis, and pure polymorphs were washed twice with PBS and resuspended in the same buffer at a concentration of 10^6 cells ml^{-1}. Cells prepared by this method were >95% neutrophils. Viability was tested by trypan blue exclusion (98–99% viable cells).

Neutrophil adhesion assay

Human neutrophils prepared as described above were resuspended in 1 ml of 0.5% (w/v) bovine serum albumin (BSA) in the presence of 500 μCi of sodium chromate solution (^{51}Cr) (Radiochemical Centre, Amersham). After 45 min of incubation at 37°C the cells were washed three times with PBS, resuspended in 0.5% BSA to the desired concentration and added to a multiwell dish containing 13 mm diameter coverslips coated with 0.5% BSA. After 1 h incubation at 37°C, the coverslips were gently washed three times with PBS to remove non-adherent cells and applied to a Wilj 2001 Gamma counter.

Automated cell tracking

Human neutrophils were contained in a filming chamber; a stainless steel slide 70 × 40 × 1 mm with a 15 mm-diameter circular aperture. The slide was sterilized under flame and a 22 × 22 square glass coverslip was dipped in ethanol, dried under flame and fixed over the aperture with hot paraffin wax/vaseline, the slide turned over and 1 ml of 0.5% BSA was pipetted into the shallow well thus formed. After 1 h the solution of BSA was discarded and 0.2 ml of cell suspension at 10^6 cells ml^{-1} was added to the well and left for 10–15 min. The non-adherent cells were removed by washing the well with PBS and the adherent cells were then treated with 0.3 ml of culture medium containing the supernatant of interest. A second coverslip was sealed over with hot paraffin which was brushed on over the coverslip margins.

Cell tracking was automated by the method of Dow *et al.* (1987). In brief, a phase contrast inverted microscope (Zeiss, West Germany) was attached to a Panasonic CCTV Camera connected to a time lapse recorder (Panasonic video system) with a video digitiser unit (Watford Electronic, UK) and a BBC Master microcomputer. An air curtain incubator (a modified domestic fan-heater) was used to maintain the cells at 37°C.

Successive frames were stored at <20 s intervals for a maximum of 25 min sequences; each frame was acquired by the computer as a 750 × 450 pixel array. The system was capable of randomly selecting and tracking up to 50 cells, with a print-out of the population and of the individual cell motility parameters after the end of a sequence. The statistical analysis of film sequences and population speed were compared using the non-parametic Mann-Whitney U-test (Campbell, 1974).

Lipocortin antibodies

Rabbit polyclonal antisera against lipocortins 1–6 were a gift from Dr R.B. Pepinsky, (Biogen Inc USA.). Antibodies were reconstituted with 50 μl distilled water and incubated with the steroid-treated monocytes supernatant at a dilution of 1:1000 for 4 h at room temperature.

Interleukin-8 and anti-interleukin-8 antibody

Human interleukin-8 was obtained from Professor A.S.G. Curtis (Cell Biology, Glasgow). Rabbit anti-interleukin-8 antibody was a gift from Dr S. Kelly (Yamanouchi Research Institute, Oxford). This was used at a dilution of 1:1000.

Statistical analysis

Student's *t* test was used to carry out statistical analysis of results.

Results

Neutrophil migration in the capillary tube assay described by Stevenson (1978) was repeated and the results obtained confirmed that steroid-treated monocyte supernatants did indeed contain a migration stimulatory activity. The effects of these supernatants on the purely chemokinetic aspects of neutrophil migration were further examined by the automated tracking assay method described in Methods. The assay conditions were first established by choosing an appropriate protein to coat the glass coverslips and 0.5% bovine serum albumin was found to be suitable for human neutrophils to attach and remain rounded (not spread). Therefore all assays were carried out on albumin-treated glass coverslips. Results obtained by this migration assay confirmed that the addition of culture medium from steroid-treated monocytes dramatically stimulated the speed of locomotion of human neutrophils compared with both control monocyte supernatants and control culture medium with or without steroid (Figure 1). The increase in the speed of locomotion of neutrophils in the presence of steroid-treated monocyte supernatants was associated with striking changes in cell shape. In comparison with neutrophils treated with culture medium in the presence of steroid (Figure 2a,b), those incubated with steroid-treated monocyte supernatants were highly motile (Figure 3a) and had active projections, but remained phase bright (Figure 3b), whilst those treated with control monocyte supernatants were of intermediate motility (Figure 4a) but flattened in appearance (Figure 4b).

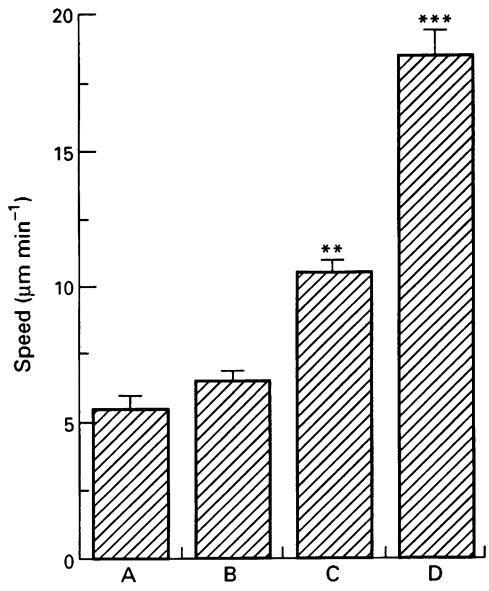


Figure 1 The effect of supernatants from human monocytes cultured for 24 h at 37°C with and without 10^{-6} M dexamethasone on the speed of locomotion of human neutrophils. (A) Culture medium without steroid; (B) culture medium with steroid; (C) control monocyte supernatant; (D) steroid-treated monocyte supernatant. Mean ± s.e.mean (vertical bars), $n = 5$.
$P \leqslant 0.05$, 0.01, 0.001, given by *, **, *** respectively.

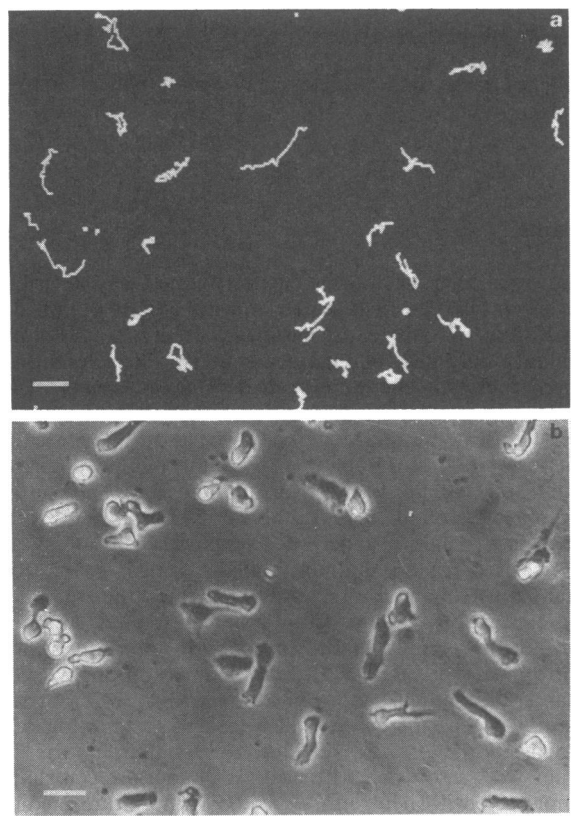


Figure 2 The effect of culture medium with 10^{-6} M dexamethasone on the speed of locomotion of human neutrophils. (a) Automated cell tracking of human neutrophil treated with culture medium in the presence of 10^{-6} M dexamethasone. (b) Cell appearance under phase contrast microscopy using × 32 objective. Bars, (a) 30 μm, (b) 20 μm.

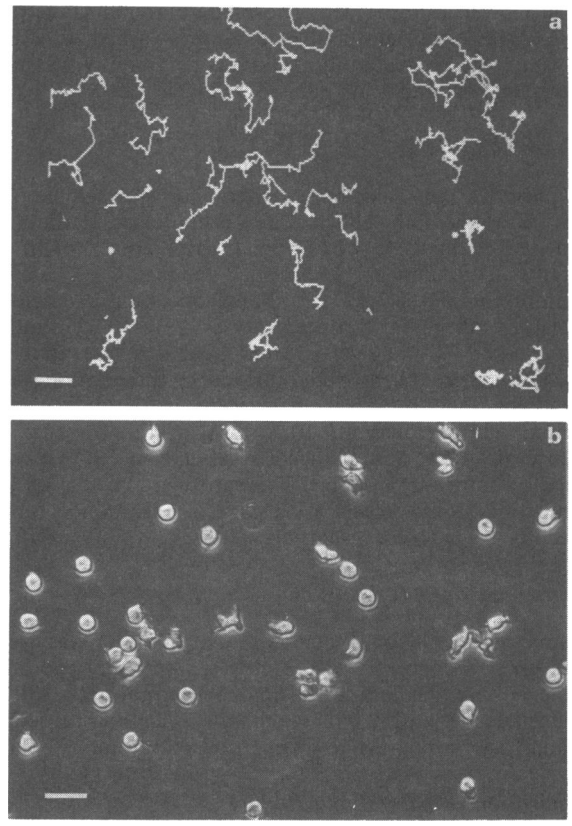


Figure 3 The effect of supernatants from human monocytes cultured for 24 h at 37°C with 10^{-6} M dexamethasone on the speed of locomotion of human neutrophils. (a) Automated cell tracking of human neutrophil tracks after treatment with supernatants from human monocytes cultured for 24 h at 37°C in the presence of 10^{-6} M dexamethasone. (b) Cell appearance under phase contrast microscope using × 32 objective. Bars, (a) 30 μm, (b) 20 μm.

Adhesion experiments were carried out to confirm the observations obtained in the tracking assay with radiolabelled neutrophils. Figure 5 shows that the adhesion of human neutrophils to 0.5% BSA-coated glass coverslips in the presence of steroid-treated monocyte supernatants was significantly reduced compared to both culture medium with and without steroid and control monocyte supernatants. In contrast the addition of control monocyte supernatants showed a large increase in neutrophil adhesion compared to both steroid-treated monocyte supernatants and culture medium.

Stevenson (1978) also demonstrated that a neutrophil migration stimulator was produced by monocytes from patients receiving steroid therapy. We therefore cultured monocytes from peripheral blood of healthy volunteers and from patients 7 h after a dose of 50 mg prednisolone. Figure 6 shows that monocyte culture supernatants from patients receiving steroid therapy significantly stimulated the speed of locomotion of human neutrophils in the tracking assay compared with culture supernatants from healthy volunteers, but the stimulation here was less than that produced by monocytes cultured *in vitro* in the presence of dexamethasone.

In view of the biological and biochemical advantages of reducing the serum concentration of the cultures, we investigated the possibility of reducing the serum dependence, working on the hypothesis that serum was necessary to establish a healthy monocyte culture, but was not required for its continued maintenance. Serum-free supernatants were obtained by the following procedure: mixed mononuclear leucocytes were incubated with culture medium supplemented with

10% foetal calf serum at 37°C for 1 h then discarding the medium. Adherent cells were washed three times with PBS and cultured with serum-free culture medium in the presence and absence of 10^{-6} M dexamethasone for 24 h. This was

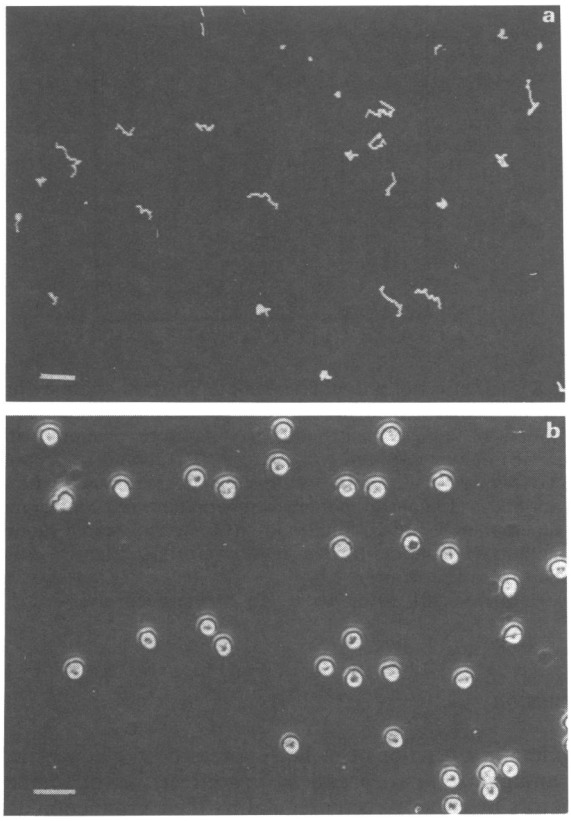


Figure 4 The effect of supernatants from human monocytes cultured for 24 h at 37°C in the absence of 10^{-6} M dexamethasone on the speed of locomotion of human neutrophils. (a) Automated cell tracking of human neutrophil tracks after treatment with supernatants from human monocytes cultured for 24 h at 37°C. (b) Cell appearance under phase contrast microscopy using × 32 objective. Bars, (a) 30 μm, (b) 20 μm.

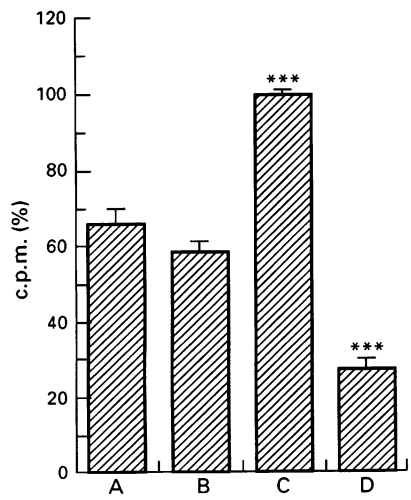


Figure 5 The effect of supernatants from cultured human monocytes for 24 h at 37°C with and without 10^{-6} M dexamethasone on human neutrophil adhesion to 0.5% bovine serum albumin coated-glass coverslips. (A) Culture medium without steroid; (B) culture medium with steroid; (C) control monocyte supernatant; (D) steroid-treated monocyte supernatant. Mean ± s.e.mean (vertical bars) $n = 3$. $P \leqslant 0.05$, 0.01, 0.001, given by *, **, *** respectively.

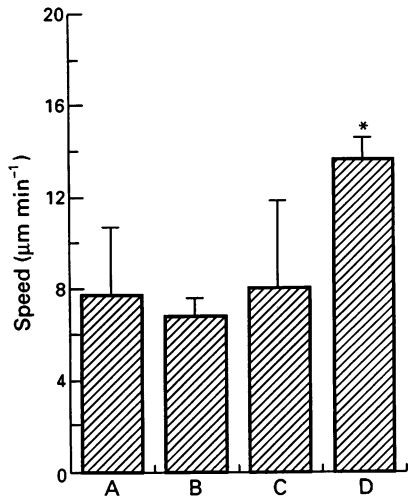


Figure 6 The speed of locomotion of human neutrophils after incubation with culture supernatants of monocytes from peripheral blood of healthy volunteers and of patients receiving steroid therapy. (A) Culture medium without steroid; (B) culture medium with steroid; (C) monocyte supernatant from healthy volunteers; (D) monocyte supernatant from patient receiving steroid treatment. Mean ± s.e.mean (vertical bars), $n = 5$.
$P \leqslant 0.05$, 0.01, 0.001, given by *, **, *** respectively.

found by experiment to be the optimum concentration for the production of the motility stimulating activity. Furthermore higher concentrations of the drug tended to reduce the speed of neutrophil locomotion in control assays. The effect produced by serum free monocyte supernatants on the speed of neutrophil locomotion using the tracking assay system was not significantly different from that produced by supernatants from monocytes cultured in the presence of 10% foetal calf serum (Figure 7). The ability to prepare the neutrophil migration stimulator from cells cultured in the absence of serum had clear advantages for further studies, not only be reducing the biological effects of serum in the tracking assays, but also by obviating the need to remove serum components during subsequent purification steps. The physico-chemical characteristics of this factor(s), which include solubility, stability and sensitivity to proteases were reinvestigated in this study and are found to be very similar to those described by Stevenson (1973; 1974; 1976b) (results not shown).

Glucocorticoids were reported to induce the synthesis and release of phospholipase A_2 inhibitory proteins known as lipocortins from human monocytes (Goudling *et al.*, 1990), and since the production of the neutrophil migration stimulator was induced by glucocorticoids from cultured monocytes it was possible that this stimulator could be related to the lipocortin family. To test this possibility, polyclonal antibodies against lipocortins 1-6 were added to steroid-treated monocyte supernatants. Table 1 shows that none of the antibodies inhibited the effect of steroid-treated monocyte supernatants on the speed of locomotion of the polymorphs in the tracking assay. In addition, a phospholipase A_2 inhibitory fraction was prepared from pig lung by the procedure described by Khana *et al.* (1987) and tested in the tracking assay and no stimulatory activity on the speed of locomotion of human neutrophils was observed (result not shown).

The increase in the speed of neutrophil locomotion could also be attributed to the release of interleukin-8 (IL-8) which is a potent neutrophil-activating and chemotactic peptide. Although the production of IL-8 by monocytes is almost totally inhibited in the presence of glucocorticoids (Seitz *et al.*, 1991) its possible involvement could not be ruled out.

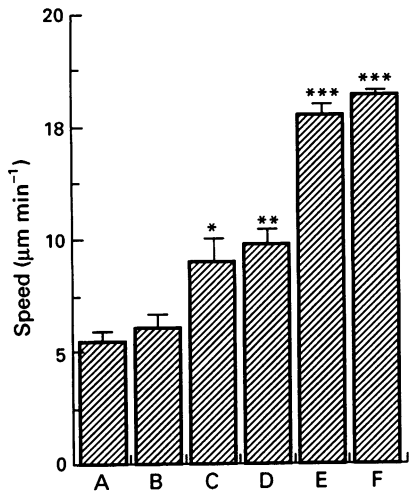


Figure 7 The effect of supernatants from monocytes cultured in the presence and absence of 10% foetal calf serum for 24 h at 37°C with and without 10^{-6} M dexamethasone on the speed of locomotion of human neutrophils. (A) Culture medium with serum; (B) culture medium without serum; (C) control monocyte supernatant with serum; (D) control monocyte supernatant without serum; (E) steroid-treated monocyte supernatant with serum; (F) steroid-treated monocyte supernatant without serum. Mean ± s.e.mean (vertical bars), $n = 3$,
$P \leqslant 0.05$, 0.01, 0.001, given by *, **, *** respectively.

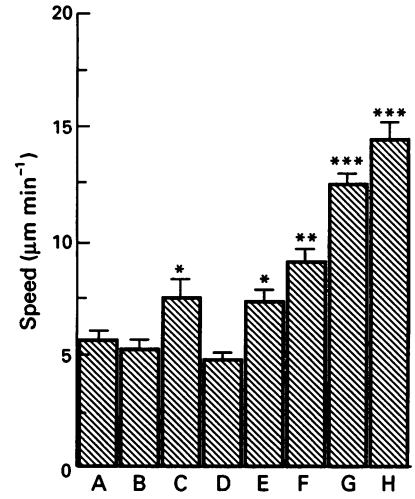


Figure 8 The effect of anti-interleukin-8 (anti-IL-8) antibody on the activity of steroid-treated monocyte supernatants on the speed of locomotion of human neutrophils. (A) Culture medium without steroid; (B) culture medium with steroid; (C) human neutrophils + IL-8; (D) human neutrophils + IL-8 preincubated for 4 h with anti-IL-8 antibody; (E) control monocyte supernatant; (F) control monocyte supernatant + anti-IL-8 antibody; (G) steroid-treated monocyte supernatant; (H) steroid-treated monocyte supernatant + anti-IL-8 antibody. Mean ± s.e.mean $n = 3$.
$P \leqslant 0.05$, 0.01, 0.001, given by *, **, *** respectively.

Figure 8 shows that the addition of 80 ng ml^{-1} recombinant human interleukin-8 (monocyte-derived) to human peripheral blood neutrophils caused an increase in their speed of locomotion in the tracking assay similar to that produced by control monocyte supernatants. This increase in the speed of locomotion of human neutrophils was completely abolished when IL-8 was incubated for 4 h with rabbit and anti-IL-8 antibody. In contrast, the effect of a steroid-treated monocyte supernatant on the speed of locomotion of human neutrophils was not altered when incubated for the same period of time with anti-IL-8 antibody.

Most significantly, pretreatment with anti-IL-8 abolished the phase darkening (flattening) produced by treatment of neutrophils with control monocyte supernatants, but did not enhance their motility. Therefore enhanced locomotion produced by steroid-treated monocyte supernatants cannot be attributed to the absence of IL-8 but to the presence of other factor(s).

Discussion

Inflammatory reactions can be suppressed by glucocorticoids that antagonize *in vitro* and *in vivo* effects of inflammatory mediators by either directly regulating their production at the gene level (Mukaida *et al.*, 1992) or by inducing molecules such as lipocortins (Errasfa & Russo-Marie, 1989) and protease inhibitor (Seifert & Gelehrter, 1978). Administration of glucocorticoids was reported to cause an increase in the number of circulating neutrophils and inhibit their accumulation at sites of inflammation (Bourcheir & Weston, 1991; Davis *et al.*, 1991). It has been suggested that some of the effects of glucocorticoids on neutrophil behaviour *in vivo* could be due to direct interaction between these cells and steroid drugs (Watanabe *et al.*, 1991), but pharmacological concentrations of these agents did not alter the behaviour of human isolated peripheral blood neutrophils in our assays. In

Table 1 The effect of different lipocortin antibodies on the activity of steroid-treated monocyte supernatants on the speed of locomotion of human neutrophils

Treatments	*Speed of locomotion of human neutrophils* (μm min^{-1})
Culture medium without steroid	5.77 ± 0.74
Culture medium with steroid	4.90 ± 0.44
Control monocyte supernatant	8.94 ± 0.69
Steroid-treated monocyte culture supernatant	14.19 ± 1.22
Steroid-treated monocyte culture supernatant + anti-lipocortin-1 antibody	13.06 ± 0.71
Steroid-treated monocyte culture supernatant + anti-lipocortin-2 antibody	14.43 ± 0.86
Steroid-treated monocyte culture supernatant + anti-lipocortin-3 antibody	12.48 ± 0.70
Steroid-treated monocyte culture supernatant + anti-lipocortin-4 antibody	13.58 ± 0.53
Steroid-treated monocyte culture supernatant + anti-lipocortin-5 antibody	12.38 ± 0.67
Steroid-treated monocyte culture supernatant + anti-lipocortin-6 antibody	13.62 ± 1.03

The results represent the mean ± s.e.mean of five measurements.

contrast, we showed that addition of monocyte supernatants with or without steroid to human peripheral blood neutrophils caused significant changes in their speed of locomotion and adhesiveness. It is therefore very likely that the observed effect of monocyte supernatants on neutrophils in this study and the effect shown by Stevenson, (1978) using the capillary tube assay are mediated by an indirect, but unknown mechanism.

Frank & Roth (1986) suggested that glucocorticoids alter neutrophil function by two separate mechanisms; one involving inhibition of arachidonic acid metabolism which results in decreased neutrophil oxidative metabolism, and the other involving release of a peptide(s) from steroid-treated monocytes which alters neutrophil random migration, and the results presented in this paper strongly support the second hypothesis. We have used an *in vitro* assay of cell locomotion, based on computer aided image analysis (Dow *et al.*, 1987) to demonstrate that incubation of human monocytes with dexamethasone promotes the release of a neutrophil migration stimulating factor(s) which is not present in control monocyte supernatants. The computer randomly selects 50 cells from a field containing 80–100 cells, isolates the images of these cells by binary filtering and tracks the positions of the cell centres in successive frames by searching the area around the cell centroid in the previous frame using a box-search algorithm. This is highly successful at low cell densities given that the x,y co-ordinates of the cell in the previous frame are known, and the cell stands out clearly from its background.

Neutrophils incubated with steroid-treated monocyte supernatants not only showed an increase in the speed of locomotion, but also a significant decrease in adhesion. The decrease in adhesiveness in this case could be attributed to the suppression of polypeptide cytokines which are known to be released by monocytes, and which increase the adhesion of neutrophils to endothelial cells (Gimbrone, 1985, Pohlman *et al.*, 1986), and are known to be suppressed in the presence of glucocorticoids (Mukaida *et al.*, 1992). This suggestion is supported by the experiment using human monocyte-derived IL-8, a major cytokine which enhances neutrophil chemotactic activity. In this study, IL-8 had a similar effect on the speed of neutrophil locomotion and adhesiveness as the control monocyte supernatants. It follows that the increase in the speed of locomotion found with steroid-treated monocyte supernatants is due to a factor(s) released by the monocytes in the presence of dexamethasone. The dramatic decrease in adhesiveness brought about by steroid-treated monocyte supernatants cannot be attributed to the absence of pro-adhesive cytokines simply because such factors are not present in control medium. Similarly the increased locomotion produced by steroid-treated monocyte supernatants cannot be attributed to the absence of the pro-adhesive IL-8 because the addition of anti-IL-8 to control monocyte supernatant does not change the speed of neutrophil locomotion. The factor(s), therefore, appears to have anti-adhesive activity together with locomotion stimulating activity and further studies will be required to determine whether or not the effect on locomotion is a direct consequence of the changes of adhesiveness.

Lipocortin proteins are known to be produced by monocytes in the presence of steroid and may serve as anti-inflammatory mediators, raising the possibility that the factor(s) described here could be one of this family of proteins. The speed of locomotion of human neutrophils in the presence of steroid-treated monocyte supernatants was not reduced by antibodies to lipocortins 1–6 suggesting that this factor(s) is not a known lipocortin. This finding is also supported by the fact that the anti-inflammatory effect of lipocortin is reported to be due to the inhibition of phospholipase A_2 and is not known to have any *in vitro* effect on neutrophil behaviour.

The release of the motility stimulating factor(s) by cultured monocytes obtained from patients on steroid treatment indicates that it can also be produced *in vivo* and could be partially responsible for the anti-inflammatory effect of glucocorticoids.

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Effects of hyperkalaemia on the depression of maximum rate of depolarization by class I antiarrhythmic agents in guinea-pig myocardium

Kenneth R. Wyse, Victor Ye & [1]Terence J. Campbell

Departments of Clinical Pharmacology and Cardiology, St. Vincent's Hospital, Darlinghurst, NSW 2010, Sydney, Australia

1 Standard microeletrode methods were used to record intracellular action potentials from strips of guinea-pig right ventricular myocardium superfused with either standard physiological saline ($[K^+] = 5.6$ mM) or the same solution modified to contain $[K^+] = 11.2$ mM.

2 The effects on action potential parameters of three therapeutic concentrations of mexiletine, quinidine and disopyramide were studied under both conditions at four different drive rates (interstimulus intervals = 2400, 1200, 600 and 300 ms).

3 Hyperkalaemia in the absence of drugs produced reductions in resting potential (-86.7 ± 2.5 mV to -71.8 ± 3.7 mV; $n = 30$; $P < 0.001$), maximum rate of depolarization (300 ± 46.5 V s^{-1} to 205.6 ± 37.6 V s^{-1}; $P < 0.0001$), and action potential duration (205 ± 26 ms to 188 ± 32 ms; $P < 0.05$).

4 All three drugs produced increased depression of maximum rate of depolarization in hyperkalaemia compared to control conditions, but at all three concentrations this enhancement of effect was greater for mexiletine than for quinidine, with disopyramide exhibiting intermediate behaviour.

5 Mexiletine behaved very similarly to therapeutic concentrations of lignocaine as described in previous reports from this laboratory.

6 Quinidine behaved very similarly to Class Ic agents.

7 It is concluded that mexiletine demonstrated significantly greater selectivity for depolarized myocardium than quinidine and that this may have implications in terms of proarrhythmic potential.

8 Disopyramide exhibited intermediate selectivity for depolarized myocardium between mexiletine and quinidine.

Keywords: Quinidine; disopyramide; flecainide; mexiletine; lignocaine; hyperkalaemia; depolarization; myocardium; sodium channel; Class I antiarrhythmic agents; antiarrhythmic drugs; cardiac action potential; ischaemia

Introduction

In a recent series of publications we have demonstrated that the Class Ic antiarrhythmic drugs, encainide and flecainide, exhibit significantly less enhancement of their blockade of the fast inward sodium channel in simulated ischaemic conditions than lignocaine or amiodarone (Campbell & Hemsworth, 1990; Campbell *et al.*, 1991). The greater selectivity for cells subjected to acidosis and hyperkalaemia demonstrated for lignocaine and amiodarone over flecainide and encainide implies that the first two agents may be given in concentrations that markedly depress ischaemic areas of myocardium but have little or no effect on normal tissue. This desirable behaviour seems less likely to be achieved with Class Ic agents such as flecainide and encainide. We hypothesised that this may cast further light on the clinically observed proarrhythmic properties of these latter agents. It was noted during the course of these experiments, in which three of the main components seen in ischaemic myocardium (hyperkalaemia, acidosis and hypoxia), were studied individually, that hypoxia had no effect on drug binding, acidosis had only modest effects and by far the largest contributing factor to the enhanced blockade of the sodium channel by Class I drugs in our model of simulated ischaemia was depolarization subsequent to hyperkalaemia. For this reason, in the experiments to be described below we have studied the effects of this intervention only.

In 1987, Hondeghem suggested that Class I drugs which appear to bind with high affinity to inactivated sodium channels, should be more likely to exhibit such ischaemia-selective behaviour than drugs which bind preferentially to activated (open) channels. It was this suggestion that led us to compare Class I agents known to bind selectively to the inactivated state of the sodium channel (such as lignocaine and amiodarone), with those which behave predominantly as open channel blockers (flecainide, encainide and other Class Ic agents). Earlier work from this laboratory had already shown that the relative degree of inactivated versus activated sodium channel block of various Class I antiarrhythmic drugs correlated with the kinetics of their interaction with the sodium channel (Campbell, 1983). Thus compounds with fast kinetics (Class Ib agents and amiodarone) have a high affinity for the inactivated state of the sodium channel and appear to demonstrate a great degree of selective depression of ischaemic myocardium, whereas Class Ic agents with slow kinetics demonstrate significantly less selectivity for ischaemic myocardium. The Class Ia antiarrhythmic agents such as quinidine, disopyramide and procainamide have intermediate kinetics of interaction with the sodium channel, and we suggested that they may demonstrate an intermediate affinity for ischaemic myocardium. We therefore set out in the series of experiments to be described below, firstly to confirm that the Class Ib agent, mexiletine, shared the high affinity for ischaemic myocardium we had previously demonstrated for lignocaine and amiodarone, and then to test the hypothesis that Class Ia antiarrhythmic agents would show intermediate degrees of selectivity for ischaemic tissue in our model.

Lignocaine has the practical disadvantage of being useful only as an intravenous infusion. Mexiletine on the other hand is active when given orally and has virtually identical cellular electrophysiological properties to lignocaine. For this reason it is often used by clinicians to treat arrhythmias for

[1] Author for correspondence.

which intravenous lignocaine has already been successful. Ischaemia-selective behaviour would only be of clinical importance if demonstrated at therapeutically relevant concentrations. The therapeutic range for mexiletine is approximately 3.5 to 10 μM (Campbell *et al.*, 1978) and we therefore studied this agent at concentrations of 5, 10 and 20 μM, in a preparation identical to that used in the previous publications from this laboratory.

The accepted therapeutic ranges of plasma concentration for quinidine and disopyramide are approximately 5 to 15 μM (Hoffmann *et al.*, 1975) and 8 to 22 μM (Niarchos, 1976) respectively. To provide adequate coverage of this range, we studied both agents at the same concentrations as used for mexiletine, namely 5, 10 and 20 μM.

Methods

Guinea-pigs of either sex weighing 300 to 700 g were decapitated and their hearts quickly removed. Strips (approximately 5 mm × 3 mm × 1 mm) were cut from the right ventricular free wall and pinned to the base of a bath (volume 0.5 ml). The tissue was superfused at 3 ml min^{-1} with 'control' solution, gassed with 95% O_2 and 5% CO_2 and maintained at 36 ± 0.2°C by a Peltier element. The composition of this standard superfusate was as follows (mM): NaCl 125, KCl 5.6, $CaCl_2$ 2.16, $NaHCO_3$ 25, $MgCl_2$ 1.0, NaH_2PO_4 0.44 and glucose 11. The 'hyperkalaemic' superfusate was identical to this except that the KCl concentration was doubled to 11.2 mM.

Preparations were allowed to equilibrate in control superfusate for at least 1 h. When required by the protocol, they were driven by bipolar platinum electrodes providing rectangular pulses of 2 ms duration and sufficient amplitude to limit variations in latency between stimulus and action potential upstroke to less than 2 ms.

Action potentials were recorded from cells on the surface of the preparation with conventional glass microelectrodes filled with 3 M KCl coupled to a high input impedance d.c. amplifier with negative capacitance compensation (either WPI 750 or Neurolog NL 102). To study rate-dependent effects, the preparations were driven at four different interstimulus intervals (ISI) ranging from 300 to 2400 ms, for periods long enough to achieve a stable level of $\dot{V}_{max}$. In the absence of drug, stimulation led to minor, rate-dependent falls in $\dot{V}_{max}$ with rapid equilibration at a new, reduced value of $\dot{V}_{max}$ which then remained constant during continued stimulation at the same rate. In the presence of all three drugs, there were more marked, rate-dependent reductions in $\dot{V}_{max}$. All such reductions have been expressed as percentage change from the value of $\dot{V}_{max}$ recorded for the first action potential after prolonged quiescence in drug-free superfusate. All experiments involved continuous impalements of single cells.

Action potentials were monitored on an oscilloscope, and sampled at 20 kHz with a DT 2801-A analog-digital converter, fitted to a IBM personal computer. Analysis was performed by use of software developed in the department (the maximum rate of depolarization being calculated by simple differencing of adjacent samples).

Lignocaine hydrochloride and quinidine sulphate were purchased commercially (Sigma, St. Louis, MO, U.S.A.). Mexiletine was kindly donated by Boehringer Ingelheim and disopyramide by Roussel. All drugs were made as stock solutions in distilled water and then diluted freshly in the relevant superfusate. During repetitions of experiments, the sequence of exposure to the various solutions under study was randomized where possible to minimize any order effects.

All data are expressed as mean ± standard deviation. One way analysis of variance (ANOVA), was used to test for the significance of drug effect on $\dot{V}_{max}$, followed in some cases by Student's *t* tests with the Bonferroni correction for multiple comparisons, to define differences between specific pairs of samples. Two-way ANOVA was used to compare the effects of different drugs across a range of stimulation rates.

All the experiments described have been approved by the Committee for the Use of Animals in Research and Teaching, University of New South Wales, and the Animal Ethics Committee, St. Vincent's Hospital.

Results

Effects of hyperkalaemia in the absence of drugs

The effects on action potential parameters of superfusion for 20 min with high K^+ solution (K^+ = 11.2 mM versus 5.6 mM in control), were very similar to those previously reported by us for this preparation (Campbell *et al.*, 1991). The resting membrane potential fell from −86.7 ± 2.5 mM to −71.8 ± 3.7 mM ($n = 30$; $P < 0.001$). There was a corresponding reduction in action potential amplitude from 123.2 ± 4.3 mV to 106.4 ± 4.4 mV ($P < 0.001$), and action potential duration at 90% of repolarization (APD), fell from 205 ± 26 ms to 188 ± 32 ms ($P < 0.05$). The maximum rate of depolarization ($\dot{V}_{max}$) fell from 300 ± 46.5 V s^{-1} to 205.6 ± 37.6 V s^{-1} ($P < 0.0001$). These data are those recorded at an interstimulus interval of 1200 ms. Qualitatively similar results were obtained at other rates. All of these effects were fully reversible after 15 to 20 min washing in control solution.

Modulation of drug effects by hyperkalaemia

No drug in any of the three concentrations used has a statistically significant effect on resting potential or action potential amplitude in control superfusate. This was also true in the hyperkalaemic superfusate. There was a non-significant trend for mexiletine to shorten action potential duration (APD_{90}) in a dose-dependent manner. At ISI = 1200 for example, APD_{90} in drug-free solution was 223.9 ± 13.8 ms and this fell in a concentration-dependent manner to 194.3 ± 18.3 ms at mexiletine 20 μM. Disopyramide and quinidine on the other hand showed a trend to slightly increase action potential duration, which was more evident at slower stimulation rates and lower concentration-ranges. This trend reached significance only for 5 μM of disopyramide at 1200 and 2400 ms ISI and represented a prolongation of less than 10% in APD.

All drugs produced highly significant, partially or completely reversible reductions in $\dot{V}_{max}$ in some or all of the four ISIs investigated (2400, 1200, 600 and 300 ms). This was true in both superfusates. Because of the wide between-cell variations in control $\dot{V}_{max}$ values, this effect has been expressed as total percentage depression of $\dot{V}_{max}$ below control values, recorded in previously quiescent tissue (the same parameter used in our previous reports). At each ISI, stimulation was continued until $\dot{V}_{max}$ achieved a stable level. These data are presented in Table 1.

Mexiletine As noted above, superfusion with high K^+, drug-free solution produced large rate-dependent reductions in $\dot{V}_{max}$. The dose- and rate-dependent reductions of $\dot{V}_{max}$ produced by each of the drugs was also enhanced by changing from normal K^+ to high K^+ superfusate (Table 1). In this regard, as can be seen from Figure 1, mexiletine behaved very similarly to lignocaine at each of the three concentrations used. It can be seen that in the lower two concentrations for each drug, suppression of $\dot{V}_{max}$ at each ISI in both normal and high K^+ superfusate was virtually identical. At the highest concentration (20 μM mexiletine and 30 μM lignocaine), mexiletine produced greater depression of $\dot{V}_{max}$ than did lignocaine ($P < 0.001$), but this difference between the drugs was maintained on changing to high K^+ solution.

Table 1 Percentage reduction of $\dot{V}_{max}$ by four drugs at four different stimulation rates in normal and high K^+ solutions

		Normal K^+ superfusate				*High K^+ superfusate*			
Drug	*ISI =*	300	600	1200	2400	300	600	1200	2400
Drug-free ($n = 30$)		7.2 ± 2.6	4.0 ± 2.1	3.5 ± 1.6	2.3 ± 1.5	44.6 ± 14.0	37.3 ± 12.1	34.4 ± 11.9	31.9 ± 11.9
Lignocaine 7.5 μM ($n = 13$)		27.2 ± 7.9***	13.0 ± 6.0**	9.7 ± 4.9*	8.5 ± 4.8*	69.0 ± 15.9***	53.6 ± 9.5**	46.6 ± 9.2*	43.0 ± 9.3
Lignocaine 15 μM ($n = 12$)		32.3 ± 8.3***	16.8 ± 5.1***	11.9 ± 6.7**	9.1 ± 6.1*	7.39 ± 11.8***	61.4 ± 15.2***	52.0 ± 10.9***	47.0 ± 11.2**
Lignocaine 30 μM ($n = 11$)		42.5 ± 9.6***	20.9 ± 6.5***	14.9 ± 6.6***	12.5 ± 6.5***	86.9 ± 13.1***	72.0 ± 16.2***	63.6 ± 15.0***	55.4 ± 8.1***
Mexiletine 5 μM ($n = 5$)		29.9 ± 4.9***	18.6 ± 4.3***	13.4 ± 5.1*	11.9 ± 6.3*	66.7 ± 11.0*	56.1 ± 13.0*	50.0 ± 14.3	45.9 ± 14.2
Mexiletine 10 μM ($n = 6$)		36.8 ± 6.9***	22.8 ± 4.7***	16.5 ± 5.8***	13.5 ± 5.5*	84.1 ± 13.0***	67.1 ± 18.0***	52.9 ± 9.9*	48.1 ± 10.4*
Mexiletine 20 μM ($n = 5$)		55.6 ± 8.6***	33.9 ± 7.5***	23.2 ± 8.3***	20.0 ± 9.1***	92.4 ± 11.0***	79.0 ± 13.8***	72.9 ± 17.0***	67.8 ± 20.7***
Quinidine 5 μM ($n = 6$)		27.9 ± 15.9***	20.3 ± 13.9***	14.3 ± 11.2**	10.1 ± 7.6	50.2 ± 9.3	46.4 ± 11.5	37.6 ± 13.4	33.3 ± 14.5
Quinidine 10 μM ($n = 5$)		44.9 ± 10.8***	35.8 ± 11.2***	28.2 ± 7.7***	21.2 ± 7.2***	72.8 ± 18.3**	56.0 ± 13.0	52.4 ± 13.1*	45.9 ± 13.2
Quinidine 20 μM ($n = 5$)		52.3 ± 16.4***	46.9 ± 16.5***	40.6 ± 17.0***	31.7 ± 17.0***	82.3 ± 18.4***	67.5 ± 8.7***	59.3 ± 9.3***	54.0 ± 11.3**
Disopyramide 5 μM ($n = 6$)		25.5 ± 8.3***	16.3 ± 3.5**	13.8 ± 3.2**	10.0 ± 3.8	61.5 ± 10.3	53.9 ± 10.6	48.4 ± 10.2	42.1 ± 8.8
Disopyramide 10 μM ($n = 5$)		36.1 ± 9.6***	26.6 ± 9.0***	21.5 ± 3.2***	16.8 ± 4.5***	67.1 ± 11.8*	59.6 ± 8.0*	50.8 ± 7.2	44.6 ± 8.1
Disopyramide 20 μM ($n = 5$)		38.5 ± 4.8***	28.7 ± 4.9***	24.2 ± 3.3***	22.4 ± 6.7***	90.5 ± 9.3***	67.8 ± 12.2***	59.7 ± 12.2**	51.1 ± 13.4*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, significance of difference from drug-free superfusate (one-way ANOVA with Bonferroni correction). Data points are percentage reductions in $\dot{V}_{max}$ during prolonged stimulation compared to maximum $\dot{V}_{max}$ recorded following prolonged quiescence in drug-free superfusate.
ISI = interstimulus interval.

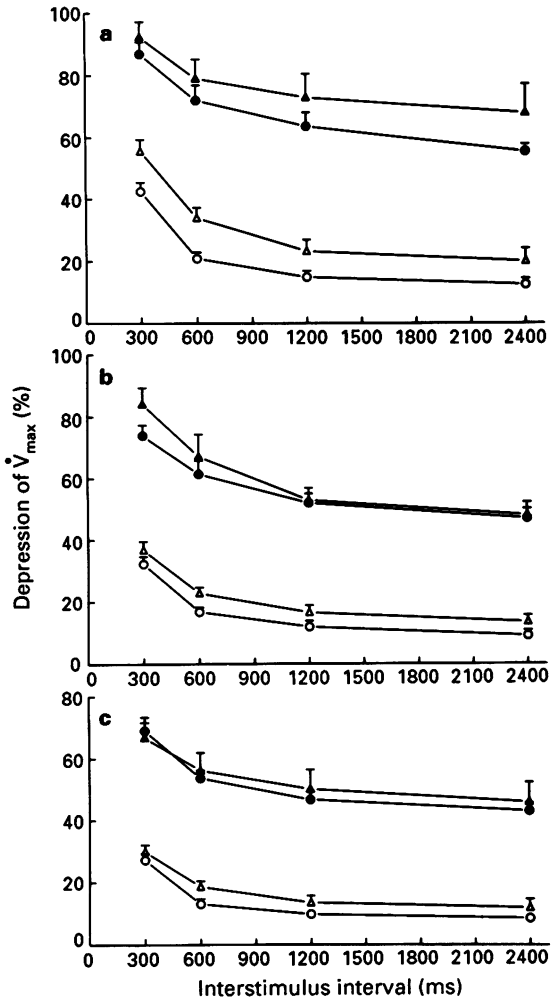


Figure 1 Relationship between interstimulus interval and percentage reduction in $\dot{V}_{max}$ under normal (K^+ = 5.6 mM; open symbols) and hyperkalaemic (K^+ = 11.2 mM; closed symbols) conditions in the presence of lignocaine (○, ●) and mexiletine (△, ▲). Concentrations of each agent used are: (a) lignocaine 30 μM and mexiletine 20 μM; (b) lignocaine 15 μM and mexiletine 10 μM; (c) lignocaine 7.5 μM and mexiletine 5 μM. At each concentration range in the normal K^+ superfusate, lignocaine tends to produce slightly less depression of $\dot{V}_{max}$ than does mexiletine. The main point of the figure is that the depression of $\dot{V}_{max}$ by both agents is markedly and approximately equally enhanced in the presence of high K^+ superfusate.

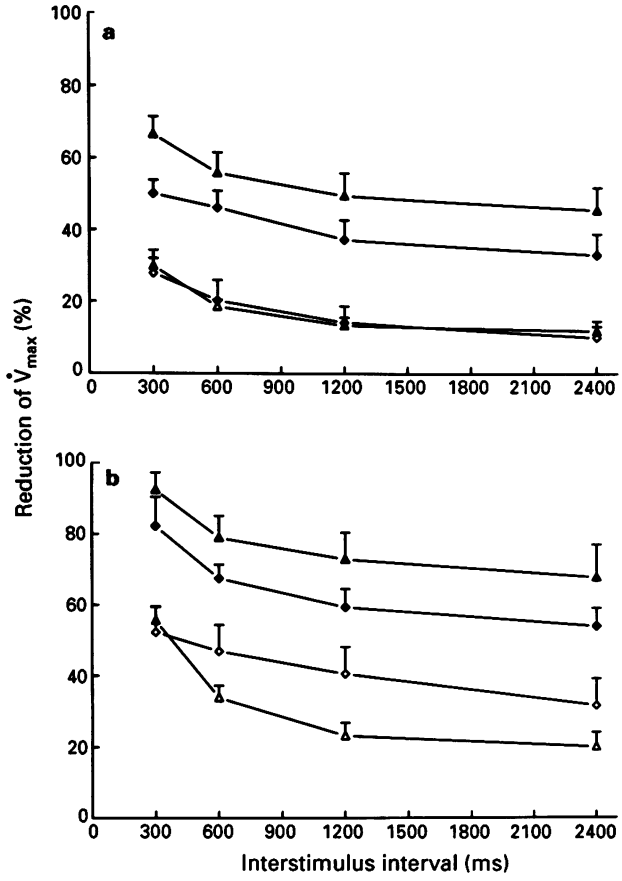


Figure 2 Format as for Figure 1, comparing mexiletine (△, ▲) and quinidine (◆, ◇) at equimolar concentrations of 5 μM (a) and 20 μM (b). At 5 μM in control superfusate, quinidine and mexiletine are approximately equipotent. In hyperkalaemic superfusate, the depression of $\dot{V}_{max}$ by mexiletine is significantly greater ($P = 0.002$; two-way ANOVA) than that of quinidine. In high concentrations the disparity is more marked. In control superfusate, mexiletine has significantly less effect on $\dot{V}_{max}$ than does quinidine ($P = 0.02$). In hyperkalaemic superfusate, the situation is reversed with mexiletine having significantly more effect than does quinidine ($P = 0.008$).

Quinidine and disopyramide

Quinidine contrasts with mexiletine and lignocaine in that its depression of $\dot{V}_{max}$ shows significantly less enhancement in the presence of hyperkalaemia than was seen with the Class Ib agents (Table 1). Thus in normal K^+ superfusate, at each of the three concentrations used (5 μM, 10 μM and 20 μM), quinidine produced the same or more depression of $\dot{V}_{max}$ than did the corresponding dose of mexiletine at any given ISI. The situation is quite the reverse when high K superfusate is substituted. Figure 2 illustrates this for the low and high concentrations of these agents.

Similar though less striking contrasts were seen between disopyramide and mexiletine, particularly in the higher concentration-ranges (Figure 3). Thus disopyramide 5 μM and mexiletine 5 μM behaved rather similarly, both in normal K^+ and high K^+ superfusate, but a lower selectivity of disopyramide for depolarized tissue was evident at higher concentrations, so that at 20 μM, disopyramide and mexiletine had similar effects in low K superfusate ($P = 0.06$), but except at the faster rate, mexiletine 20 μM exhibited significantly more depression of $\dot{V}_{max}$ than did disopyramide 20 μM in high K superfusate ($P = 0.02$ overall; Figure 3).

The fact that the behaviour of disopyramide in this preparation is intermediate between quinidine and mexiletine is further illustrated by Figure 4. It can be seen that at both the low and high ends of the concentration-ranges, disopyramide exhibited greater enhancement of its depression of $\dot{V}_{max}$ in the presence of high K superfusate than did quindine. Figure 5 provides further information on this point, using previously published data from this laboratory studying the effects of 1 μM flecainide in the same preparation ($n = 5$; Campbell *et al.*, 1991). It can be seen that while 5 μM disopyramide exhibited significantly more selectivity for depolarized myocardium than did 1 μM flecainide, 5 μM quinidine behaved in a very similar fashion to the Class Ic agent, flecainide. Thus disopyramide behaved in an intermediate fashion in terms of its selectivity for depolarized myocardium, being significantly less selective than the Class Ib agent, mexiletine and significantly more selective than the Class Ic agent, flecainde. Quinidine however, behaves in a manner essentially similar to that previously described for Class Ic agents.

Discussion

The potential for antiarrhythmic agents to worsen or actually provoke arrhythmias has recently been brought into sharp

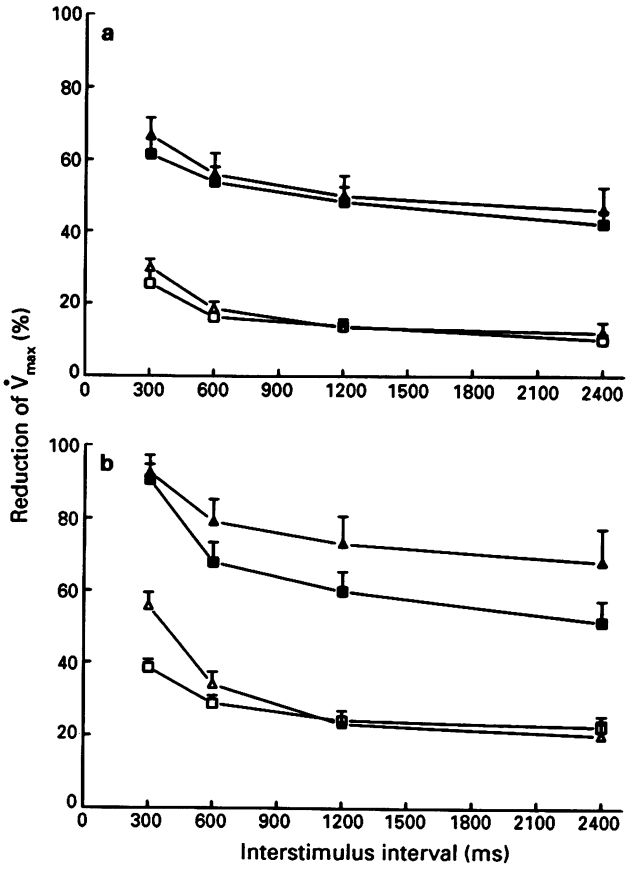


Figure 3 Format as for Figure 2, comparing mexiletine (Δ, ▲) and disopyramide (□, ■) at concentrations of 5 μM (a) and 20 μM (b). At 5 μM, disopyramide and mexiletine behave essentially identically in both control superfusate and hyperkalaemic superfusate ($P = 0.2$ and 0.4 respectively). At 20 μM however, while the two drugs remain essentially equipotent in terms of their depression of $\dot{V}_{max}$ at K^+ = 5.6 mM ($P = 0.07$), mexiletine demonstrates significantly greater depression of $\dot{V}_{max}$ in hyperkalaemic superfusate than does disopyramide ($P = 0.02$).

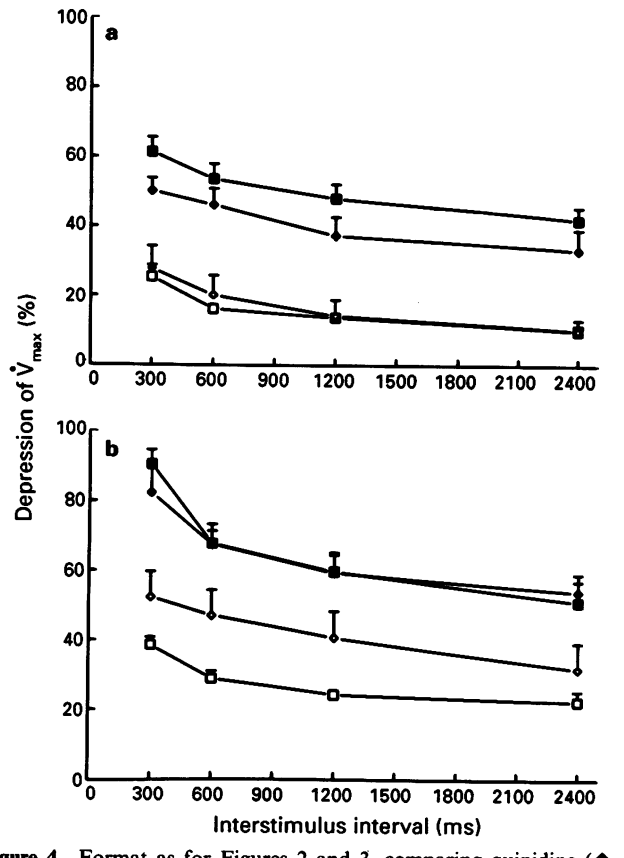


Figure 4 Format as for Figures 2 and 3, comparing quinidine (◆, ◇) and disopyramide (□, ■) at 5 μM (a) and 20 μM (b). The depression of $\dot{V}_{max}$ by disopyramide is enhanced to a significantly greater extent than that seen for quinidine in both concentrations. At 5 μM, the two drugs are equipotent in control solution ($P = 0.5$) and disopyramide is significantly more depressant than quinidine in hyperkalaemic superfusate ($P = 0.004$). At 20 μM, quinidine depresses $\dot{V}_{max}$ significantly more than does disopyramide in the control superfusate ($P < 0.001$), but the two agents produce equal depression in the hyperkalaemic superfusate ($P = 0.7$).

focus by the results of the Cardiac Arrhythmia Suppression Trial (CAST; Echt *et al.*, 1991), and by meta-analyses of trials of the use of quinidine in atrial fibrillation and ventricular arrhythmias (Coplen *et al.*, 1990; Morganroth & Goin, 1991). It seems likely that one of the major mechanisms of proarrhythmia is facilitation by Class I antiarrhyhmic drugs of actual or potential re-entrant circuits due to a critical reduction of conduction velocity within these areas of myocardium (Campbell, 1990). It seems intuititively likely that the greater the degree of myocardial damage, either permanent (due to ischaemic heart disease, cardiomyopathy etc.) or transient (due to ischaemia), the greater the likelihood that the electrophysiological actions of an antiarrhythmic agent might 'uncover' or facilitate a potential substrate for re-entry. It is certainly well documented that agents such as flecainide produce a higher incidence of proarrhythmic effects in patients with more severe ventricular dysfunction (Morganroth & Horowitz, 1984; Morganroth *et al.*, 1986a; Morganroth, 1987). Similar, though less striking trends have been reported for encainide and quinidine (Morganroth, 1986b; 1987; Denes *et al.*, 1981).

The most likely explanation for this is the well known interaction between Class I antiarrhythmic drugs and depolarized myocardium (Hondeghem, 1987). An almost universal response of myocardium to an acute or chronic insult such as ischaemia, is partial depolarization. This in turn will tend to enhance the conduction slowing effects of any antiarrhythmic agents to which the myocardium may happen to be exposed at the time. One of the working hypotheses behind the present experiments was the proposition that Class I drugs which possess more marked selectivity for partially depolarized cells could be given in concentrations that markedly depress ischaemic tissues (thus maximizing the likelihood of abolishing the focus of the index arrthythmia) but which have little or no effect on normal myocardium (thus minimizing the propensity for producing new arrhythmogenic sites within the heart). Widespread, non-selective depression of conduction velocity is well known to enhance the likelihood of re-entrant arrhythmias (Hondeghem, 1987; Campbell, 1990), and indeed very recent laboratory studies have confirmed the ability of flecainide to produce 'fibrillation' in normal, non-ischaemic myocardium *in vitro* (Brugada *et al.*, 1991).

It follows from this argument therefore, that the proarrhythmic propensity of a Class I agent might be expected to correlate with the degree of selectivity it possesses for depressing ischaemic or depolarized myocardium, relative to normal fully polarized cells (which in turn has been shown to correlate with affinity for the inactivated state of the sodium channel: Hondeghem, 1987; Campbell, 1983). The experiments previously reported by us (Campbell & Hemsworth, 1990; Campbell *et al.*, 1991), have alrady confirmed significantly greater selectivity for ischaemic tissue for amiodarone and lignocaine over the Class Ic agents, flecainide and encainide. The data presented above confirm that the Class Ib drugs, mexiletine and lignocaine, exhibit similar degrees of

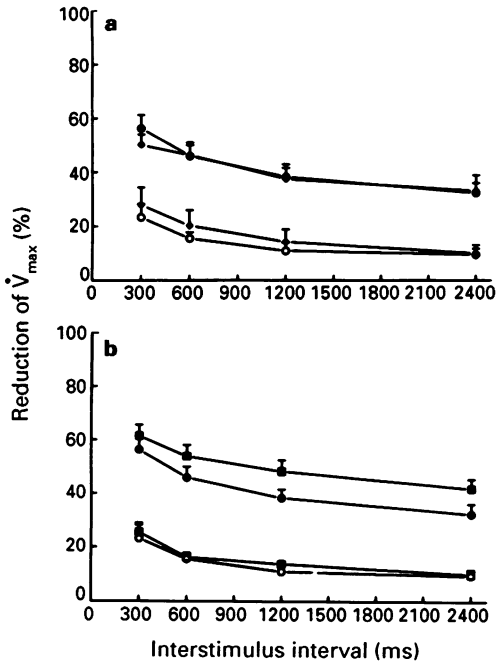


Figure 5 Format as for Figures 2 to 4, comparing quinidine 5 μM (◆, ◇) with flecainide 1 μM (○, ●; a) and disopyramide 5 μM (□, ■) with flecainide 1 μM (○, ●; b). Quinidine and flecainide exhibit very similar degrees of depression of $\dot{V}_{max}$ in both control and high K^+ solution ($P = 0.3$ and 0.7 respectively), whereas disopyramide shows significantly greater selectivity for depolarized myocardium than does flecainide ($P = 0.4$ in control and 0.006 in high K^+).

selectivity for depolarized cells, and that the Class Ia agents quinidine and disopyramide possess significantly less selectivity. Furthermore, comparison of the present data with experiments on the Class Ic agent, flecainide, previously published from this laboratory, demonstrate that the behaviour of disopyramide is truly intermediate between that of Ib and Ic agents, whereas quinidine behaves essentially the same as the Class Ic agents, demonstrating minimal selectivity for ischaemic myocardium. In the original study comparing nine drugs from each of the three sub-classes of sodium channel blockers in terms of their kinetics of interaction with the sodium channel, the kinetics of the Class I agents were (as noted above), intermediate between those of Class Ib and Class Ic. In addition to this, it was noted that disopyramide had the fastest onset kinetics of all the Class Ia agents and demonstrated the greatest ability to prolong the effective refractory period relative to the action potential duration of the sub-group. This would be consistent with its possessing greater selectivity for depolarized myocardium than quinidine, as was the case in the present study (Campbell, 1983; Vaughan Williams, 1984).

Clinical relevance

Does this correlate with clinically observed incidences of proarrhythmia with the various agents? Hard data on this issue are difficult to find in the literature, largely due to the scarcity of large-scale studies which compare two or more Class I antiarrhythmic agents 'head-to-head' in terms of their proarrhythmic propensity. There is certainly a widespread perception among cardiologists that the newer Class Ic agents such as flecainide and encainide appear more likely to produce proarrhythmia than Class Ia drugs, and that mexiletine is less prone to proarrhythmic problems than many other Class I compounds (Morganroth, 1992). In support of this, the best data relevant to the present study which compare mexiletine to other agents, come from the Mexiletine-Quinidine Research Group (Morganroth and Mexiletine-Quinidine Research Group, 1987), who studied approximately 500 patients with ventricular ectopic beats and/or ventricular tachycardia, in which the incidence of proarrhythmia was 9% for those patients on quinidine, compared to 5% for patients on mexiletine. The 95% confidence intervals for the mortality risk difference between mexiletine and quinidine ranged from 0 to 40%. In other words, this study fell just short of showing a statistically significant benefit for mexiletine over quinidine in terms of mortality.

The only direct comparison of disopyramide and quinidine available had only 62 patients in each group and focused on efficacy (Arif *et al.*, 1983). The authors reported no mortality from either drug. Certainly the two compounds behave very similarly in the Electrophysiology Laboratory both *in vitro* and *in vivo*, thus adding further support to the hypothesis that their proarrhythmic profiles are not markedly different from each other.

It is more difficult to be certain whether the current perception that Ic agents are more dangerous than quinidine or disopyramide is truly justified. Indeed, if the data presented here are of major relevance to proarrhythmia, one might expect quinidine at least to be comparable to flecainide in this regard, with disopyramide possibly a little safer. Certainly, data from a recent meta-analysis of the use of quinidine for ventricular arrhythmias do not suggest that quinidine is significantly safer than flecainide (Morganroth & Goin, 1991), but the issue here is clouded because quinidine and disopyramide are far more likely than Ib or Ic antiarrhythmic agents to produce early afterdepolarizations and 'torsades de pointes' (Jackman *et al.*, 1988; Campbell, 1990). It thus becomes very difficult to ascertain whether quinidine and disopyramide are less likely than flecainide or encainide to provoke arrhythmias specifically via their effects on conduction, the only mechanism of relevance to the present study. Neither compound was included in the Cardiac Arrhythmia Suppression Trial, and the prospects of a future large-scale trial bieng organised to answer this question seem rather low. Suffice it to say however, that the data presented above are quite consistent with the hypotheses that proarrhythmia due to facilitation of re-entrant tachycardia is less likely to occur with antiarrhytmic agents that show a high degree of selectivity for partially depolarized myocardium (mexiletine and similar drugs) and more likely to occur with drugs demonstrating intermediate or low degrees of selectivity for such tissue (disopyramide, quinidine and Ic agents respectively).

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α_2-Autoreceptor subclassification in rat isolated kidney by use of short trains of electrical stimulation

C. Bohmann, P. Schollmeyer & [1]L.C. Rump

Medizinische Universitätsklinik Freiburg, Innere Medizin IV, Hugstetter Str. 55, 7800 Freiburg, Germany

1 Rat kidneys were perfused with Krebs-Henseleit solution and incubated with [^{3}H]-noradrenaline. The renal nerves were electrically stimulated at either 1 Hz for 30 s or 100 Hz for 0.06 s. The stimulation induced (S-I) outflow of radioactivity was taken as an index of endogenous noradrenaline release.

2 At a frequency of 1 Hz for 30 s the α-adrenoceptor antagonists BRL 44408 (0.01, 0.1 μM) and imiloxan (0.1, 1.0 μM) enhanced S-I outflow of radioactivity. However, at a frequency of 100 Hz for 0.06 s the α-adrenoceptor antagonists, idazoxan (0.1, 1.0 μM), imiloxan (0.1, 1.0 μM), BRL 44408 (0.1, 1.0 μM), BRL 41992 (0.1, 1.0 μM) and prazosin (0.01 μM) failed to enhance S-I outflow of radioactivity.

3 Thus, the rat isolated kidney stimulated at 100 Hz for 0.06 s, avoids autoinhibition by endogenous noradrenaline and α-adrenoceptor antagonist affinities (pK_B) at the prejunctional α-autoreceptor were estimated without disturbance by the endogenous activator.

4 The α_2-adrenoceptor agonist, clonidine, inhibited the S-I outflow of radioactivity with a maximum of 90% and an EC_{50} of 7.2 nM.

5 All α-adrenoceptor antagonists used caused parallel shifts of the concentration-response curve for clonidine to the right. The rank order of potencies was: rauwolscine ($\alpha_{2A/B}$) > idazoxan ($\alpha_{2A/B}$) > phentolamine ($\alpha_{2A/B}$) > WB 4101 (α_{2A}) > BRL 44408 (α_{2A}) > BRL 41992 (α_{2B}) > prazosin (α_{2B}) = imiloxan (α_{2B}).

6 These data, when compared with binding, molecular and functional data of various other tissues and cell lines, indicate that prejunctional α_2-autoreceptors in rat kidney do not belong to the α_{2B}- or α_{2C}-subtype. The α_2-autoreceptor of rat kidney seems to be of the α_{2A}-subtype. However, α_2-autoreceptor affinities of the present study correlate also well with binding affinities of the recently described α_{2D}-ligand binding site in bovine pineal gland.

Keywords: Noradrenaline release; prejunctional α_2-adrenoceptors; rat kidney; α-adrenoceptor subclassification

Introduction

On the basis of radioligand binding and some molecular cloning studies, it has been suggested that α_2-adrenoceptors can be divided in four subtypes: $\alpha_{2A\text{-}D}$-adrenoceptors (Bylund, 1988; Bylund *et al.*, 1991; Harrison *et al.*, 1991; Lanier *et al.*, 1991; Lomasney *et al.*, 1991). α_2-Adrenoceptors are located pre- and postjunctionally. Binding studies cannot easily differentiate between pre- and postjunctional α_2-adrenoceptors and only a few studies have systematically investigated α_2-adrenoceptor heterogeneity in functional experiments. Thus, it seems that inhibition of adenosine 3':5'-cyclic monophosphate (cyclic AMP) production is mediated via activation of the α_{2A}-subtype in the HT29 cell line and via activation of the α_{2B}-subtype in the NG 108 cell line (Bylund & Ray-Prenger, 1989). More recently, prostaglandin production in rabbit aorta has been linked to activation of the α_{2C}-subtype (Nebigil & Malik, 1992). It is well known that postganglionic sympathetic nerve terminals possess α_2-adrenoceptors which when activated inhibit the amount of noradrenaline released per nerve impulse (Starke, 1987; Rand *et al.*, 1990). Moreover, α-adrenoceptor antagonists enhance noradrenaline release by interrupting the negative feedback loop of endogenous noradrenaline by blocking inhibitory α_2-autoreceptors (Starke, 1987). This has also been shown for the rat isolated kidney (Rump & Majewski, 1987). The available data so far suggest that prejunctional α_2-autoreceptors either belong to the α_{2A}-subtype: rat vas deferens (Connaughton & Docherty, 1990; Smith *et al.*, 1992), rat submandibular gland (Smith *et al.*, 1992), rabbit brain cortex (Limberger *et al.*, 1991), rat brain synaptosomes (Gobbi *et al.*, 1990), guinea-pig submucosal plexus (Shen *et al.*, 1990), guinea-pig urethra (Alberts, 1992) or the α_{2B}-subtype: cat and bovine cerebral arteries (Arribas *et al.*, 1991) and rat atrium (Connaughton & Docherty, 1990; Smith *et al.*, 1992).

It has been suggested that the rat kidney possesses only α_{2B}-binding sites (Neylon & Summers, 1985; Cheung *et al.*, 1986; Michel *et al.*, 1989). The aim of the present study was to investigate whether or not the prejunctional α_2-autoreceptor of rat kidney resembles the α_{2B}-binding site. This was done by determining antagonist potencies for a selection of α-adrenoceptor antagonists against clonidine at the α_2-autoreceptor in rat kidney. Since endogenous noradrenaline competes with clonidine for the prejunctional α-autoreceptor, the pseudo-one-pulse technique (Singer, 1988) was used. This technique, which is free from autoinhibition by endogenous noradrenaline in central nervous tissue (Singer, 1988), was established in the rat isolated kidney. Some of these results have been published in abstract form (Rump & Bohmann, 1992).

Methods

Kidneys of male Wistar rats weighing 250–340 g were isolated according to a method described by Rump *et al.* (1992). The kidneys were transferred into jacketed glass chambers, maintained at a temperature of 37°C and perfused with Krebs-Henseleit solution at a constant rate of 6 ml min^{-1}. The solution was continuously gassed with carbogen (95% O_2 and 5% CO_2) and passed through a 0.8 μm filter before it reached the kidneys. Bipolar platinum electrodes were placed around the renal arteries to stimulate the renal nerves. Perfusion pressure was measured continuously with a Statham P23 Db pressure transducer coupled to a Watanabe pen recorder.

[1] Author for correspondence.

Experimental protocol

The kidneys were incubated with (−)-[ring-2,5,6-^{3}H]-noradrenaline (specific activity 43.7 mCi mmol^{-1}, 0.02 μl) for 15 min. During this perfusion the effluent was continuously gassed with carbogen, filtered through glass wool and a 0.8 μm filter before it was recirculated into the kidneys. After the incubation the kidneys were perfused with drug-free Krebs-Henseleit solution for 90 min to remove loosely-bound radioactivity. After 70 min of this washing procedure a priming stimulation at 5 Hz for 30 s was given; 24 consecutive 3-min samples were collected. There were 6 short trains of electrical stimulation (S_1-S_6) each at 100 Hz for 0.06 s, applied 6, 18, 30, 42, 54 and 66 min after the start of collection. Pressor responses to electrical stimulation were calculated as the maximum increase of perfusion pressure above basal perfusion pressure. Cumulative concentration-response curves for the α_2-adrenoceptor agonist, clonidine, were constructed. Clonidine was infused directly into the perfusion line at a rate of 0.15 ml min^{-1} by a perfusion apparatus (Braun, Melsungen, Germany) in increasing concentrations 6 min before S_2, S_3, S_4, S_5, and S_6. When an α-adrenoceptor antagonist was present for all stimulation periods (throughout), it was added to the perfusion solution directly after the priming stimulation. In some experiments there were only three electrical stimulations (S_1-S_3) at either 100 Hz for 0.06 s or 1 Hz for 30 s to test the effects of the α-adrenoceptor antagonists by themselves. In this case the α-adrenoceptor antagonists were added in two increasing concentrations just 6 min before S_2 and S_3.

Phenoxybenzamine pretreatment

After incubation with [^{3}H]-noradrenaline (see above) there was a washing period of 165 min. After 10 min of washing the kidneys were exposed to the irreversible α-adrenoceptor antagonist, phenoxybenzamine (1.0 μM) for 40 min. A priming stimulation was given at 5 Hz for 30 s after 145 min of the washing procedure and the experimental protocol was then similar to that described above. In some experiments α-adrenoceptors were protected against phenoxybenzamine by either prazosin (0.1 and 1.0 μM) or BRL 44408 (0.3 μM). Prazosin or BRL 44408 were added prior to the phenoxybenzamine exposure (immediately after the incubation period) and the exposure to phenoxybenzamine was always stopped 20 min before washout of the protecting α-adrenoceptor antagonists was started. A priming stimulation was given at 5 Hz for 30 s after 145 min of the washing procedure and the experimental protocol was then similar to that described above. The complete washout of prazosin was confirmed in separate experiments by comparison of a pressor response induced by the α_1-adrenoceptor agonist, methoxamine (10 μM) prior to the protection procedure with a pressor response induced by methoxamine just before the priming stimulation.

Estimation of radioactivity

Aliquots (4 ml) of the effluent were mixed with 10 ml scintillation fluid (Picofluor 40, Packard Instruments) to measure the amount of radioactivity by liquid scintillation counting.

Calculation of data

The spontaneous (resting) outflow of radioactivity was determined as the mean of the amount of radioactivity in the effluent collected during the 3 min before and 3 min after the start of stimulations (S_1-S_6). The S-I outflow of radioactivity was calculated by subtracting the spontaneous outflow of radioactivity from the radioactivity present in the 3-min sample collected immediately after the beginning of stimulation. S_1 served as a reference stimulation and the S-I outflow of radioactivity in S_n (S_2-S_6) is expressed as a percentage of that in S_1 (S_n as % of S_1). The spontaneous outflow of radioactivity during S_n is expressed as a percentage of that during S_1 (R_n as % of R_1). For further evaluation of drug effects the S_n/S_1 ratios were calculated as a percentage of the values determined in the corresponding control experiments (S_n/S_1 as % of control) and in the case of clonidine further expressed as percentage inhibition. All data are arithmetic means ± s.e.mean and were analyzed by unpaired Student's t test where appropriate. Sigmoid curves were fitted to all percentage inhibition values (except phenoxybenzamine experiments) according to Waud (1975) to yield EC_{50} values of clonidine in the presence and absence of antagonists. The pK_B values for the α-adrenoceptor antagonists were determined according to equation 4 of Furchgott (1972).

Drugs and vehicles

The Krebs-Henseleit solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.45, $NaHCO_3$ 25, KH_2PO_4 1.03, D-(+)-glucose 11.1, disodium edetate 0.067, ascorbic acid 0.07. The following drugs were purchased: (−)-[ring-2,5,6-^{3}H]-noradrenaline (NEN, Dreieich, Germany); phentolamine mesylate (Ciba-Geigy, Basel, Switzerland); methoxamine HCl (Sigma, St. Louis, Missouri, U.S.A.); yohimbine HCl and rauwolscine HCl (Roth, Karlsruhe, Germany); N-[2-(2,6-dimethoxyphenoxy) ethyl]-2,3-dihydro-1,4-benzodioxin-2-methanamine-HCl (WB 4101) and phenoxybenzamine HCl (Research Biochemicals Inc., Massachusetts, U.S.A.). The following drugs were generous gifts: clonidine HCl (Boehringer Ingelheim, Ingelheim, Germany); prazosin HCl (Pfizer, Karlsruhe, Germany); idazoxan HCl (Reckitt & Colman, Kingston-upon-Hull, UK); imiloxan HCl (Syntex Research, Palo Alto, California, U.S.A.); 1,2-dimethyl-2,3,9,13b-tetrahydro-1H-dibenzo[c,f]) imidazo[1,5-a]azepine (BRL 41992) and (2-[2H-(1-methyl-1,3-dihydroiso-indole)methyl]-4,5-dihydroimidazole (BRL 44408) (Smith Kline Beecham, Surrey, UK).

All drugs were dissolved in distilled water before being diluted with Krebs-Henseleit solution except prazosin, BRL 41992, BRL 44408, WB 4101 and phenoxybenzamine. Prazosin was diluted in a solution with the following composition: 0.5 ml glycerol, 3.5 ml 5% dextrose and 6 ml distilled water before being diluted with Krebs-Henseleit solution. BRL 41992, BRL 44408 and WB 4101 were dissolved in 10 mM HCl and phenoxybenzamine was dissolved in a solution of the following composition: 2.0 ml ethanol, 1.5 ml 0.2 M acetic acid and 10.0 ml distilled water before being diluted with Krebs-Henseleit solution. The respective vehicles had no effect on the release of noradrenaline and control experiments with the vehicles and without were combined.

Results

Kidneys were perfused with Krebs-Henseleit solution and labelled with [^{3}H]-noradrenaline. After a washing procedure the renal sympathetic nerves were electrically stimulated at 100 Hz for 0.06 s (S_1-S_6). The S-I outflow of radioactivity and S-I pressor responses in S_1 in the presence and absence of drugs are given in Table 1.

Effect of α-adrenoceptor antagonists on renal nerve stimulation at either 1 Hz for 30 s or 100 Hz for 0.06 s

When the renal nerves were stimulated at 1 Hz for 30 s, the α-adrenoceptor antagonists imiloxan (0.1, 1.0 μM) and BRL 44408 (0.01, 0.1 μM) significantly enhanced S-I outflow of radioactivity (Figure 1). However, at 100 Hz for 0.06 s imiloxan (0.1, 1.0 μM) had no effect on the S-I outflow of radioactivity and BRL 44408 (0.1, 1.0 μM) even slightly inhibited S-I outflow of radioactivity (Figure 1). Moreover, the α-adrenoceptor antagonists, idazoxan (0.1, 1.0 μM), prazosin (0.01 μM) and BRL 41992 (0.1, 1.0 μM) did not significantly alter S-I outflow of radioactivity at 100 Hz for 0.06 s. Pra-

Table 1 Absolute S-I outflow of radioactivity and S-I pressor responses in the first stimulation period (S_1) in the presence and absence of drugs and after phenoxybenzamine pretreatment in rat isolated kidney preincubated with [^{3}H]-noradrenaline

Treatment	*Frequency of stimulation*	n	*S-I outflow of radioactivity in S_1* (d.p.m.)	*S-I pressor response in S_1* (mmHg)
No drug	100 Hz, 0.06 s	57	11767 ± 587	15 ± 0.7
Prazosin (0.3 μM)	100 Hz, 0.06 s	7	9214 ± 1135	4 ± 0.6*
Idazoxan (0.1 μM)	100 Hz, 0.06 s	20	8930 ± 661*	13 ± 1.3
Imiloxan (0.3 μM)	100 Hz, 0.06 s	9	9775 ± 1104	15 ± 1.9
BRL 44408 (0.3 μM)	100 Hz, 0.06 s	14	9238 ± 528*	9 ± 0.9*
BRL 41992 (0.3 μM)	100 Hz, 0.06 s	19	10712 ± 756	5 ± 0.5*
Phentolamine (0.1 μM)	100 Hz, 0.06 s	8	7498 ± 898*	5 ± 0.8*
Rauwolscine (0.1 μM)	100 Hz, 0.06 s	18	8439 ± 578*	13 ± 1.6
WB 4101 (0.3 μM)	100 Hz, 0.06 s	7	6526 ± 538*	6 ± 1.0*
Phenoxybenzamine (1 μM)	100 Hz, 0.06 s	8	12681 ± 368	5 ± 0.9*

Values shown are mean ± s.e.mean.
*Indicates significant difference from no drug experiments, Student's unpaired t test, $P<0.05$.

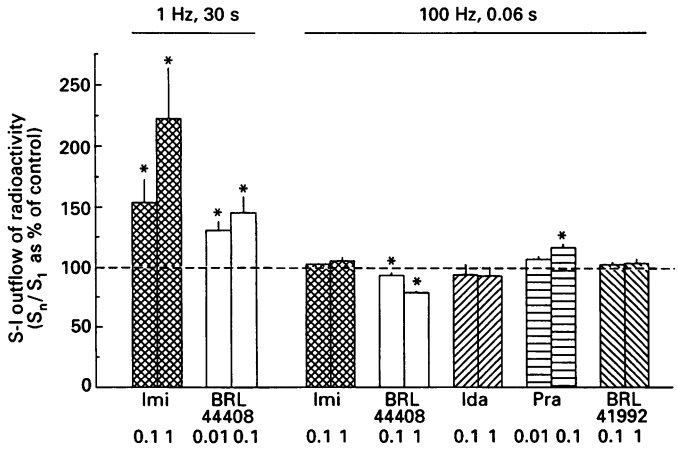


Figure 1 Effect of imiloxan (Imi), BRL 44408, idazoxan (Ida), prazosin (Pra) and BRL 41992 on S-I outflow of radioactivity from rat kidneys preincubated with [^{3}H]-noradrenaline. There were three stimulation periods (S_1–S_3) at either 1 Hz for 30 s or 100 Hz for 0.06 s and drugs were added in increasing concentrations before S_2 and S_3. Results are expressed as the ratios S_n/S_1 (S_2/S_1, S_3/S_1) as a percentage of the ratios of the corresponding controls (1 Hz for 30 s, $n=4$; 100 Hz for 0.06 s; $n=7$). All data are mean ± s.e.mean (vertical lines). There were two sets of experiments: (1) At 1 Hz, 30 s: BRL 44408 (0.01, 0.1 μM, $n=3$) and Imi (0.1, 1.0 μM, $n=3$). (2) At 100 Hz, 0.06 s: Imi (0.1, 1.0 μM, $n=4$), BRL 44408 (0.1, 1.0 μM, $n=4$), Ida (0.1, 1.0 μM, $n=5$), Pra (0.01, 0.1 μM, $n=4$) and BRL 41992 (0.1, 1.0 μM, $n=4$–5). *Indicates significant difference from corresponding control, Student's t test, $P<0.05$.

zosin (0.1 μM) slightly enhanced S-I outflow of radioactivity (Figure 1), however, at the same time it increased the spontaneous outflow of radioactivity to 126.5 ± 4.0% ($n=4$) as compared to control of 83.6 ± 3.0% ($n=6$). BRL 44408 (0.1, 1.0 μM), BRL 41992 (0.1, 1.0 μM), imiloxan (0.1, 1.0 μM), prazosin (0.01 μM) and idazoxan (0.1, 1.0 μM) had no effect on the spontaneous outflow of radioactivity (data not shown).

Influence of α-adrenoceptor antagonists on the inhibitory effect of clonidine in rat kidney at 100 Hz for 0.06 s

Clonidine concentration-dependently inhibited S-I outflow of radioactivity with an EC_{50} of 7.2 nM and a maximum of 90% (Figure 2). All α-adrenoceptor antagonists used caused parallel shifts of the concentration-response curve of clonidine with no suppression of the maximum response (except phenoxybenzamine). The interaction of clonidine with the α_{2B}-selective antagonist prazosin and the α_{2A}-selective antagonist WB 4101 is shown as an example (Figure 2). The pK_B values for all α-adrenoceptor antagonists are shown in Table 2. Clonidine had no effect on the spontaneous outflow of radioactivity (data not shown).

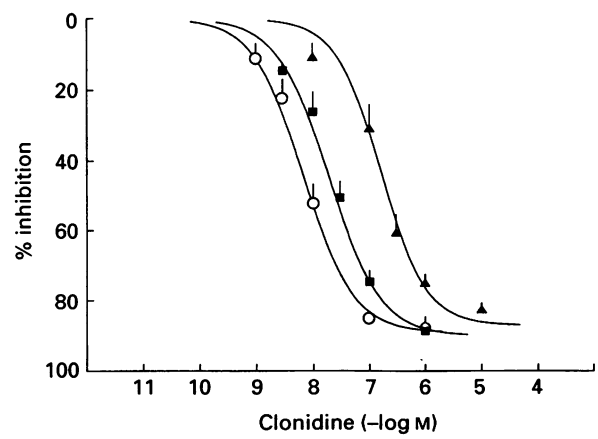


Figure 2 Effect of clonidine on the S-I overflow of radioactivity from rat isolated kidney preincubated with [^{3}H]-noradrenaline and its interaction with prazosin and WB 4101. There were six stimulation periods (S_1-S_6, each 100 Hz, 0.06 s). Clonidine was added in increasing concentrations before S_2-S_6. Results are expressed as the ratios S_n/S_1 (S_2/S_1, S_3/S_1, S_4/S_1, S_5/S_1, S_6/S_1) as a percentage of the ratios of the corresponding controls. Each point represents the mean of 4–5 experiments. Clonidine alone (○), clonidine in the presence of prazosin (0.3 μM, ■) and in the presence of WB 4101 (0.3 μM, ▲).

Table 2 pK_B values of α-adrenoceptor antagonists against clonidine at the prejunctional α_2-autoreceptor of rat kidney

α-Adrenoceptor antagonist	pK_B	n
Imiloxan (0.3 μM)	6.79 ± 0.08	5
Prazosin (0.3 μM)	6.75 ± 0.14	4
BRL 41992 (0.3 μM)	7.21 ± 0.08	5
BRL 44408 (0.3 μM)	7.75 ± 0.09	5
WB 4101 (0.3 μM)	7.91 ± 0.14	4
Phentolamine (0.1 μM)	8.17 ± 0.11	4
Idazoxan (0.1 μM)	8.29 ± 0.17	5
Rauwolscine (0.1 μM)	8.52 ± 0.08	4

pK_B values were calculated according to Furchgott (1972) as described in the Methods.

Receptor protection experiments with phenoxybenzamine

After pretreatment of the kidneys with the irreversible α-adrenoceptor antagonist, phenoxybenzamine (1.0 μM) for 40 min clonidine failed to inhibit the S-I outflow of radioactivity (Figure 3). However, when α-adrenoceptors were protected by either prazosin (0.1 μM) or prazosin (1.0 μM) during the phenoxybenzamine exposure, clonidine induced a maximal inhibition of the S-I outflow of radioactivity of 40% and 56%, respectively (Figure 3). Protection of α-adrenoceptors by BRL 44408 (0.3 μM) was most effective, so that clonidine induced a maximal inhibition of the S-I outflow of radioactivity of 70% (Figure 3). In these experiments, in which prazosin (1.0 μM) had been used to protect α-adrenoceptors, imiloxan (3.0 μM) and BRL 44408 (0.3 μM) caused parallel rightward shifts of the concentration-response curve of clonidine (Figure 4). The pK_B-values for imiloxan (3.0 μM) and BRL 44408 (0.3 μM) were 6.5 and 7.3, respectively.

Correlation of α-autoreceptor affinities in rat kidney and binding affinities of various cell lines and tissues

Correlation between functional α_2-autoreceptor affinities of rat kidney and α_2-binding affinities of other tissues and cell lines is shown in Table 3. A significant correlation was found neither for the α_{2B}-binding site of rat neonatal lung and kidney, the NG108-15 cell line nor for the α_{2C}-binding site of the OK cell line and human C4 clone. The prejunctional α_2-autoreceptor of rat kidney correlated better with the α_{2A}-binding site of human platelet, human C10 clone, HT29 cell line and rabbit spleen than with the α_{2D}-binding site of rat

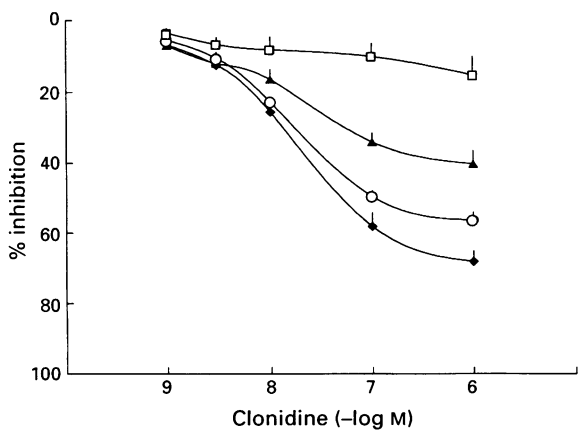


Figure 3 Effect of clonidine on the S-I outflow of radioactivity from rat isolated kidney pretreated with phenoxybenzamine (1.0 μM, □). There were six stimulation periods (S_1-S_6, each 100 Hz, 0.06 s). Clonidine was added in increasing concentrations before S_2-S_6. Results are expressed as the ratios S_n/S_1 (S_2/S_1, S_3/S_1, S_4/S_1, S_5/S_1, S_6/S_1) as a percentage of the ratios of the corresponding controls. Each point represents the mean of 3–4 experiments. Clonidine alone (□), clonidine when α-adrenoceptors were protected by prazosin (0.1 μM, ▲), clonidine when α-adrenoceptors were protected by prazosin (1.0 μM, ○), clonidine when α-adrenoceptors were protected by BRL 44408 (0.3 μM, ◆).

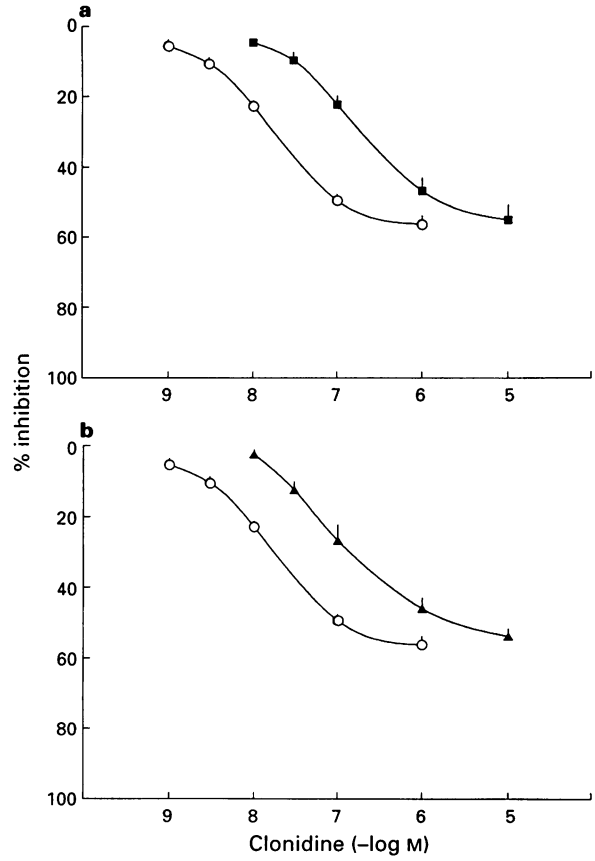


Figure 4 Effect of clonidine on the S-I outflow of radioactivity from rat isolated kidney pretreated with phenoxybenzamine (1.0 μM) and its interaction with (a) imiloxan and (b) BRL 44408. During phenoxybenzamine (1.0 μM) pretreatment α-adrenoceptors had been protected by prazosin (1.0 μM). There were six stimulation periods (S_1-S_6, each 100 Hz, 0.06 s). Clonidine was added in increasing concentrations before S_2-S_6. Results are expressed as the ratios S_n/S_1 (S_2/S_1, S_3/S_1, S_4/S_1, S_5/S_1, S_6/S_1) as a percentage of the ratios of the corresponding controls. Each point represents the mean of 3–4 experiments. Clonidine alone (○); clonidine in the presence of imiloxan (3.0 μM, ■), clonidine in the presence of BRL 44408 (0.3 μM, ▲).

Table 3 Correlation between α-adrenoceptor antagonist potencies (pK_B) of rat kidney and pk_i values of various tissues and cell lines

	r	n
HT29 cell (α_{2A})[a]	0.92*	5
Human C10 clone (α_{2A})[b]	0.97*	4
Human platelet (α_{2A})[c]	0.90*	5
Rabbit spleen (α_{2A})[d]	0.90*	6
Rat kidney (α_{2B})[d]	0.69	6
Rat lung (α_{2B})[a]	0.50	5
NG108-15 cell (α_{2B})[c]	0.42	5
OK cell (α_{2C})[a]	0.66	5
Human C4 clone (α_{2C})[b]	0.62	4
Bovine pineal gland (α_{2D})[b]	0.98*	4
Rat submaxillary gland (α_{2D})[d]	0.83*	6

Correlation coefficient is *r*, the number of pairs of data points in the analysis is *n*.
*Significant differences from 0, $P<0.05$.
Data are taken from: [a]Blaxall *et al.*, 1991; [b]Simonneaux *et al.*, 1991; [c]Bylund *et al.*, 1988; [d]Michel *et al.*, 1989.

submaxillary gland. However, correlation between the prejunctional α_2-adrenoceptor of rat kidney and the α_{2D}-binding site of bovine pineal gland was as good as that for the α_{2A}-binding site of the human C10 clone.

Discussion

In the present study, rat kidneys were isolated and perfused with Krebs-Henseleit solution. After incubation with [^{3}H]-noradrenaline the renal nerves were electrically stimulated and the stimulation induced (S-I) outflow of radioactivity was taken as an index of endogenous noradrenaline release (Starke, 1977; Rump *et al.*, 1990). When the renal sympathetic nerves were stimulated at a frequency of 1 Hz for 30 s, the α_2-adrenoceptor antagonists, imiloxan (Michel *et al.*, 1990) and BRL 44408 (Young *et al.*, 1989) enhanced the S-I outflow of radioactivity. This suggests, that under these conditions neuronally released noradrenaline activates prejunctional α_2-autoreceptors to inhibit its own release. Imiloxan and BRL 44408 interrupt this negative feedback loop to enhance transmitter release. Similar results have been obtained in a previous study with other α-adrenoceptor antagonists in rat isolated kidney stimulated at 1 and 5 Hz for 30 s (Rump & Majewski, 1987). It has been suggested in the literature that the prejunctional α_2-autoreceptor may generally be of the α_{2B}-subtype (Bylund *et al.*, 1988). Therefore, the aim of the present study was to characterize the α_2-autoreceptor of rat kidney in respect of the $\alpha_{2A\text{-}D}$-adrenoceptor subclassification (Bylund *et al.*, 1991). This was done by determining antagonist affinities (pK_B) of various α-adrenoceptor antagonists especially prazosin (high affinity for α_{2B}-subtype) at the α_2-autoreceptor in rat isolated kidney. Clonidine, which is not selective for α_{2A}- or α_{2B}-binding sites (Bylund, 1988; Michel *et al.*, 1989), served as an α_2-adrenoceptor agonist. However, clonidine has to compete with endogenous noradrenaline for the α-autoreceptor and this disturbs exact pK_B determinations for α-adrenoceptor antagonists (Starke, 1987; Singer, 1988; Limberger *et al.*, 1989). Therefore experimental conditions free of autoinhibition by endogenous noradrenaline were established in the present study in rat isolated kidney. According to the so-called 'pseudo-one-pulse technique' described for brain slices (Singer, 1988), the renal nerves were stimulated at 100 Hz for 0.06 s only. Using this stimulation protocol in rat isolated kidney the α-adrenoceptor antagonists imiloxan and BRL 44408, in contrast to the stimulation at 1 Hz for 30 s, failed to enhance S-I outflow of radioactivity. Furthermore, idazoxan, BRL 41992 and prazosin (0.01 μM) also failed to enhance S-I outflow of radioactivity, although there was a substantial and measurable S-I outflow of radioactivity at 100 Hz for 0.06 s. This suggests, that the rat isolated kidney stimulated at 100 Hz for 0.06 s is free of autoinhibition. It is feasible, that there is not enough time for endogenous noradrenaline to activate prejunctional α_2-autoreceptors during this short train of electrical impulses. A similar time-dependence for autoinhibition by neuronally released noradrenaline has been described earlier (Story *et al.*, 1981; Singer, 1988; Limberger *et al.*, 1989). BRL 44408 even slightly inhibited S-I outflow of radioactivity at 100 Hz for 0.06 s, which may be due to a partial agonist action of this compound. A similar inhibitory effect of BRL 44408 has been observed in rat atrium (Limberger *et al.*, 1992). Prazosin (0.1 μM) slightly enhanced S-I outflow of radioactivity, but at the same time also enhanced the spontaneous outflow of radioactivity from the tissue. Thus it seems likely, that the slight facilitatory effect of prazosin (1.0 μM) on S-I outflow of radioactivity was due to an unspecific, possibly reserpine-like (Commarato *et al.*, 1978) effect, reported recently for the rabbit brain cortex in the absence of autoinhibition (Limberger *et al.*, 1991). The increase in perfusion pressure in response to renal nerve stimulation at 100 Hz for 0.06 s was very small. Therefore, the interpretation of the effects of the α-adrenoceptor antagonists on these responses is difficult. It seems, however, that all α-adrenoceptor antagonists with well known α_1-adrenoceptor blocking activity such as prazosin, WB 4101, phentolamine and phenoxybenzamine inhibited pressor responses to renal nerve stimulation whereas the more selective α_2-adrenoceptor antagonists such as imiloxan, idazoxan and rauwolscine had no effect. Postjunctional vasoconstrictor α-adrenoceptors of rat kidney are almost exclusively α_1-adrenoceptors (Schmitz *et al.*, 1981; Rist *et al.*, 1991). Thus, it may be concluded that the inhibition of pressor responses by prazosin, WB 4101, phentolamine, phenoxybenzamine, BRL 44408 and BRL 41992 was due to blockade of postjunctional α_1-adrenoceptors, which are normally activated by neuronally released noradrenaline in rat kidney. At 100 Hz for 0.06 s, clonidine concentration-dependently inhibited S-I outflow of radioactivity to a maximum of 90% with an EC_{50} of 7.2 nM. All α-adrenoceptor antagonists used shifted the concentration-response curve for clonidine to the right in a parallel manner. The effects of the antagonists on the inhibitory effect of clonidine were surmountable in all cases. Thus, the necessary requirements to determine correct antagonist potencies at the α_2-autoreceptor are given. Sigmoid curve fitting (Waud, 1975) was performed to yield EC_{50} values and pK_Bs for the α_2-adrenoceptor antagonists were calculated according to equation 4 of Furchgott (1972). A small population of α_{2A}-binding sites (about 10% of the total number of sites) has been recently described in rat kidney (Uhlen & Wikberg, 1991a,b) but the majority of α_2-binding sites of rat kidney is of the α_{2B}- subtype (Neylon & Summers, 1985; Cheung *et al.*, 1986; Michel *et al.*, 1989). However, our functional autoreceptor affinities in rat kidney did not correlate with affinities obtained in binding studies for the α_{2B}-binding site in rat neonatal lung (Blaxall *et al.*, 1991), NG108-15 cell (Bylund *et al.*, 1988) and rat kidney (Michel *et al.*, 1989). For example, our functional pK_B for prazosin is far lower than the binding pK_Bs for prazosin published earlier (Simonneaux *et al.*, 1991). Moreover, imiloxan, which like prazosin is selective for the α_{2B}-adrenoceptor subtype (Michel *et al.*, 1990), showed the lowest affinity of all α-adrenoceptor antagonists used in the present study in rat kidney. In contrast, idazoxan, a structural analogue of imiloxan (Michel *et al.*, 1990) but non-selective in respect of $\alpha_{2A/B}$-adrenoceptors, was 32 times more potent than imiloxan. The α_{2A}-selective compound BRL 44408 (Young *et al.*, 1989) was about 4 times more potent than the α_{2B}-selective compound BRL 41992 (Young *et al.*, 1989) at the prejunctional α_2-adrenoceptor in the present study, whereas at the α_{2B}-binding site of rat lung BRL 41992 had a 130 times higher affinity than BRL 44408 (Young *et al.*, 1989). Furthermore, WB 4101, selective for α_{2A}-adrenoceptors (Bylund, 1988), was 12 times more potent than prazosin and imiloxan in the present study, which is in contrast to findings of Michel *et al.* (1990) reported for the α_{2B}-ligand binding site of rat kidney. Taking all these data together, it seems very unlikely, that the α_2-autoreceptor of rat kidney is of the α_{2B}-subtype. However, α_{2A}- and α_{2B}-adrenoceptors may coexist not only in one tissue but possibly on the same sympathetic nerve ending. Therefore, receptor protection experiments using phenoxybenzamine in order to eliminate all putative α_{2A}-receptors were performed. During the exposure to phenoxybenzamine α_2-autoreceptors were either protected by the α_{2A}-selective antagonist BRL 44408 or the α_{2B}-selective antagonist prazosin. Phenoxybenzamine pretreatment in the absence of other antagonists indeed totally abolished the inhibitory effect of clonidine, which suggests that all prejunctional α_2-adrenoceptors were irreversibly alkylated. However, the α_{2A}-selective drug, BRL 44408 (0.3 μM) more effectively protected α_2-autoreceptors against alkylation by phenoxybenzamine than did the α_{2B}-selective prazosin (0.1 and 1.0 μM). Moreover, in experiments, in which α_2-autoreceptors had been protected by prazosin (1.0 μM), BRL 44408 again shifted the concentration-response curve more potently to the right than the α_{2B}-selective imiloxan, with a similar ratio as in previous

experiments without phenoxybenzamine pretreatment. Thus, even this approach did not reveal evidence for prejunctional α_{2B}-adrenoceptors in rat isolated kidney. However, in favour of an α_{2A}-adrenoceptor there was an excellent correlation between our functional autoreceptor affinities and binding affinities for the α_{2A}-binding site of the human C10 clone (Zeng *et al.*, 1990), human platelet (Bylund *et al.*, 1988), rabbit spleen (Michel *et al.*, 1989) and HT29 cell (Blaxall *et al.*, 1991). Thus it is more likely, that the prejunctional α_2-autoreceptor of rat kidney is an α_{2A}-adrenoceptor. As far as further α_2-adrenoceptor subtypes are concerned, there is no evidence that prejunctional α_2-autoreceptors of rat kidney are of the α_{2C}-subtype. No significant correlation was found between our autoreceptor antagonist affinities and binding affinities for the α_{2C}-binding site of the OK cell (Blaxall *et al.*, 1991) and the human C4 clone (Regan *et al.*, 1988). However, recently a fourth α_2-binding site, termed α_{2D}, has been described in bovine pineal (Simonneaux *et al.*, 1991) and perhaps rat submaxillary gland (Michel *et al.*, 1989). The correlation between the prejunctional α_2-adrenoceptor of rat kidney and the α_{2D}-binding site of bovine pineal gland was as good as that for the α_{2a}-binding site of the human C10 clone. However, the prejunctional α_2-autoreceptor of rat kidney correlated better with the α_{2A}-binding site of human platelet, human C10 clone, HT29 cell line and rabbit spleen than with the α_{2D}-binding site of rat submaxillary gland. Molecular studies have demonstrated, that α_{2A}- and α_{2D}-adrenoceptors are to a great extent homologous and possibly represent species variations of one and the same receptor (Lanier *et al.*, 1991). Therefore, it may be difficult to separate the pharmacological characteristics of α_{2A}- and α_{2D}-adrenoceptors in functional experiments (Smith *et al.*, 1992).

Note added in proof

Renal prejunctional α_2-adrenoceptors of Sprague-Dawley rats have been suggested to be of the α_{2D}-subtype (Schwartz, D.D. & Malik, K.K. (1992). Characterization of prejunctional alpha-2 adrenergic receptors involved in modulation of adrenergic transmitter release in the isolated perfused rat kidney. *J. Pharmacol. Exp. Ther.*, **261**, 1050–1055).

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Influence of rhein anthrone on peristaltic reflex of guinea-pig isolated ileum: involvement of prostaglandins

[1]Geert Nijs, Peter de Witte, *Karel Geboes, †Ann Meulemans, †Jan Schuurkes & Joseph Lemli

Laboratory of Pharmaceutical Biology and Phytopharmacology, Institute of Pharmaceutical Sciences, K.U. Leuven, E. Van Evenstraat 4, B-3000 Leuven; *Laboratory of Histo- and Cytochemistry, Department of Medical Research, K.U. Leuven, Minderbroedersstraat 12, B-3000 Leuven and †Department of Gastrointestinal Pharmacology, Janssen Research Foundation, Janssen Pharmaceutica, Turnhoutseweg 30, B2340 Beerse, Belgium

1 The influence of rhein anthrone on the peristaltic reflex was studied with a modified Trendelenburg technique in a range from 10^{-8} M to 4×10^{-5} M, on a normal and reversed guinea-pig ileum segment. Rhein anthrone had no significant effects on longitudinal muscle tension, intraluminal pressure or volume displacement when tested on the normal segment in doses up to 10^{-5} M. When applied to the mucosal side (reversed segment), rhein anthrone produced a dose-dependent increase of longitudinal muscle tension (significant from 10^{-7} M), of intraluminal pressure (significant from 3×10^{-6} M) and of volume displacement (significant from 10^{-7} M). The data show that rhein anthrone possesses *in vitro* activity which is dependent on contact with the mucosa.

2 The action of rhein anthrone on the reversed segment was inhibited by BW755C (a dual inhibitor of cyclo-oxygenase and lipoxygenase), by indomethacin and by SC19220 (an antagonist of prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$). The effects remaining on longitudinal muscle tension, intraluminal pressure and volume displacement, calculated as percentage (mean ± s.e.mean) of the initial value, were respectively: 13 ± 8; 23 ± 13; 112 ± 5 for BW755C; 66 ± 19; 51 ± 8; 53 ± 8 for indomethacin and 27 ± 12; 13 ± 7; 50 ± 5 for SC19220. It is concluded that arachidonic acid metabolites, especially PGE_2 and $PGF_{2\alpha}$ are involved in the effects of rhein anthrone on the reversed segment.

Keywords: Rhein anthrone; peristaltic reflex; guinea-pig isolated ileum; prostaglandins; cathartics; anthraquinones; gastrointestinal motility

Introduction

Sennosides are a group of natural compounds found in several laxative preparations used all over the world. Their laxative potency has been recognized for a long time and the bacterial degradation of sennosides into sennidins and the active compound rhein anthrone is well known (Lemli, 1988).

In spite of this, the exact mechanism of the laxative action remains to be elucidated. Both an influence on secretion and motility have been observed in *in vivo* experiments. The former has been explained as the result of an influence on adenosine 5'-triphosphate (ATP) content of mitochondria (Verhaeren, 1980) or on Na^+,K^+-ATPase (Wanitschke & Karbach, 1988). As to the latter, one of the problems encountered was how to reproduce the very clear effects on motility (increase of peristalsis) perceived *in vivo* (Fioramonti *et al.*, 1988; Wienbeck *et al.*, 1988; Leng-Peschlow, 1988; Staumont *et al.*, 1988; Spiessens *et al.*, 1988) by an *in vitro* technique. The absence of these motility stimulating effects *in vitro* (Odenthal & Ziegler, 1988) made it difficult to gain more insight of the mechanism of action. Recently, prostaglandin mediation (Beubler & Kollar, 1988; Yagi *et al.*, 1988; 1990; Nijs *et al.*, 1991; 1992) has been reported which can possibly explain both effects on secretion and motility.

In this paper we describe an *in vitro* system, based on a Trendelenburg set-up by which these effects on motility of rhein anthrone can be demonstrated. The role of prostaglandins in the observed effects was also evaluated.

Methods

Guinea-pigs of either sex (weighing ca. 350 g, fasted for 24 h with free access to tap water) were killed by decapitation. The ileum was isolated, washed three times with Tyrode solution (37°C, gassed with 95% O_2 and 5% CO_2) and freed from adhering tissue. Peristaltic reflex was studied on guinea-pig isolated ileum segments of 7 cm length mounted on a U-shaped glass device used to build up the intraluminal pressure in a modified Trendelenburg set-up as described by Van Nueten *et al.* (1973). A maximum of 4 segments was taken from each animal, *n* being the number of tissue preparations. Tyrode solution at 37°C, gassed with 95% O_2 and 5% CO_2 with the following composition (mM): NaCl 136.8, $NaHCO_3$ 11.9, D-glucose 5.6, $NaH_2PO_4.H_2O$ 0.4, KCl 2.7, $MgCl_2.6H_2O$ 1.0, $CaCl_2$ 1.8 was used as bathing solution. Intraluminal pressure was increased from − 5 to 25 mm hydrostatic pressure over a 50 s time period, and maintained at this level during challenge with the test drug, by means of an automatic lift control unit. Parameters of peristaltic reflex recorded were changes in longitudinal muscle tension measured with an isometric Cannon transducer, development of intraluminal (intraintestinal) pressure detected with a Statham transducer and volume displacement measured by an ultrasonic Janssen transit-time technique (Geivers *et al.*, 1974). All results were recorded on a Graphtec Linearcorder F-WR3701.

The test drugs (rhein anthrone and rhein) were given in the bath fluid (100 ml) at the serosal (normal segment) or mucosal side (reversed segment) after 30 s stabilisation of the peristaltic reflex activity at 25 mm hydrostatic pressure. All values were calculated as peak height relative to control peak height (each segment serving as its own control). Results are expressed as means ± s.e.mean; statistical evaluation was performed by Student's paired *t* test. $P < 0.05$ was considered as significant, $P < 0.01$ as highly significant.

[1] Author for correspondence.

Experiments with rhein anthrone and rhein applied to the serosal side of the ileum segment

In a first set of experiments, the serosa was in contact with the bathing solution (normal segment).

Experiments with rhein anthrone and rhein applied to the mucosal side of the ileum segment

In a second set of experiments, the segment was turned inside out by gently pulling the oral edge through the lumen to the aboral edge, obtaining a segment with the mucosa outside (reversed segment).

Experiments with rhein anthrone applied to the mucosal side after incubation with inhibitors of prostaglandin synthesis or a receptor antagonist

In a third set of experiments, the role of prostaglandins in the effects observed after application of rhein anthrone to the mucosal side (reversed segment) was evaluated. The reversed ileum was first tested for a response to rhein anthrone as described above (pressure 25 mm), washed, incubated for 30 min with inhibitors of prostaglandin synthesis or a receptor antagonist (at pressure − 5 mm) to allow adequate penetration of the compounds in the tissue (Bennett *et al.*, 1976), washed and again tested for response to rhein anthrone.

Three compounds were used: indomethacin, SC19220 and BW755C. Indomethacin was used as an inhibitor of cyclo-oxygenase (Baumann *et al.*, 1980; Rainsford, 1988); BW755C was chosen as an inhibitor of cylco-oxygenase and lipoxygenase (dual inhibition) (Baumann *et al.*, 1980; Rainsford, 1988) and SC19220 was used as a competitive antagonist of PGE_2 and $PGF_{2\alpha}$ on guinea-pig ileum (Sanner, 1988). The effects remaining for each of the three parameters (longitudinal muscle tension, intraluminal pressure and volume displacement) after incubation with each inhibitor or receptor antagonist were determined for 4 different concentrations of rhein anthrone (10^{-6} M, 3×10^{-6} M, 10^{-5} M and 4×10^{-5} M) and expressed as percentage (mean ± s.e.mean) of the initial value ($n = 6-10$ for each concentration of rhein anthrone, consequently each mean was calculated from at least 24 results). Statistical significance was assessed by Student's paired t test (effect of rhein anthrone before incubation serving as control).

Drugs

Rhein (1,8-dihydroxy-3-carboxyanthraquinone) was prepared starting from aloin (Mac Farlan Smith Ltd.) according to the method of Bellaart (1952). Rhein anthrone (1,8-dihydroxy-3-carboxy-9-anthrone) was synthesized according to de Witte & Lemli (1988). The purity was 94%, as was proven with [^{14}C]-rhein anthrone. Solutions of rhein anthrone and rhein were freshly prepared by adding 10 ml of Tyrode solution (37°C, pH = 7.8) to appropriate amounts of rhein anthrone or rhein (yielding solutions of 10^{-3} M or 10^{-2} M) and mixing with an ultrasonic mixer. Before adding aliquots of these solutions to the bathing medium, the solution was vortexed.

BW755C.HCl (2.7 mg) (3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride) (The Wellcome Foundation Ltd.) was dissolved in 200 ml Tyrode solution to yield a concentration of 50 μM. Indomethacin (10 mg) (Merck Sharp & Dohme) was first dissolved in 10 ml Tris buffer pH 8.3 (1 M) and freshly diluted 100 times in Tyrode, yielding a solution of 30 μM. SC19220 (5 mg) (1-acetyl-2-(8-chloro-10, 11-dihydrobenz (b,f) (1,4) oxazepine-10-carbonyl) hydrazine) (G.D. Searle & Co.) was dissolved in 200 ml Tyrode solution to yield a solution of 75 μM. These solutions were used as bathing solutions to incubate the tissue.

Results

Influence of rhein anthrone and rhein applied to the serosal side of the ileum segment

When the bathing solution was in contact with the serosa (normal segment), rhein anthrone ($n = 6$) had no significant effect on volume displacement and only for very high doses (10^{-5} M or higher) a significant effect on longitudinal muscle tension and intraluminal presure was noticed (Figure 1). For rhein ($n = 6$) a significant increase ($P < 0.05$) of volume displacement was observed, accompanied by a small but significant decrease ($P < 0.05$) of intraluminal pressure (Figure 2).

Influence of rhein anthrone and rhein applied to the mucosal side of the ileum segment

With the bathing solution in contact with the mucosa (reversed segment), rhein anthrone ($n = 20-50$) significantly ($P < 0.01$) increased the amplitude of all three parameters of peristaltic reflex for concentrations down to 10^{-7} M. The increase in intraluminal pressure was somewhat less pronounced. The effects on all three parameters were clearly dose-de-

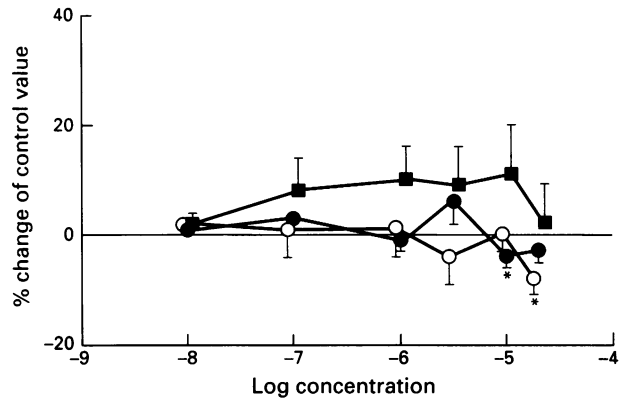


Figure 1 Log concentration-response curves for rhein anthrone on peristaltic reflex of guinea-pig isolated ileum (serosa outside). Changes of longitudinal muscle tension (●), intraluminal pressure (○) and volume displacement (■) are shown. Each point represents the mean of 6 experiments, with s.e.mean shown by vertical bars. *$P < 0.05$; compared to control values for that segment (Student's paired t test).

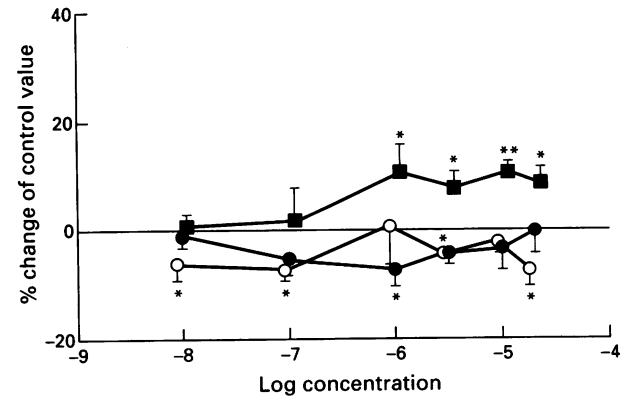


Figure 2 Log concentration-response curves for rhein on peristaltic reflex of guinea-pig isolated ileum (serosa outside). Changes of longitudinal muscle tension (●), intraluminal pressure (○) and volume displacement (■) are shown. Each point represents the mean of 6 experiments, with s.e.mean shown by vertical bars. *$P < 0.05$; **$P < 0.01$; compared to control values for that segment (Student's paired t test).

pendent (Figure 3). Rhein ($n = 6$) had little influence on peristaltic reflex activity except for a significant increase ($P < 0.05$) of volume displacement and a small but significant increase ($P < 0.05$) of longitudinal muscle tension for concentrations exceeding 10^{-7} M (Figure 4).

Influence of rhein anthrone applied to the mucosal side of the ileum segment after incubation with inhibitors of prostaglandin synthesis or a receptor antagonist

After incubation of the reversed segment with BW755C (50 μM) for 30 min, the effects of rhein anthrone on peristaltic reflex were largely prevented ($P < 0.01$ for longitudinal muscle tension and intraluminal pressure) except for the effect on volume displacement ($n = 6-10$ for each concentration of rhein anthrone tested, mean of 36 observations altogether).

After incubation of the reversed segment with indomethacin (30 μM) for 30 min, a considerable decrease of intraluminal pressure and volume displacement ($P < 0.01$ for both) was observed ($n = 6-10$ for each concentration of rhein anthrone tested, mean of 37 observations altogether).

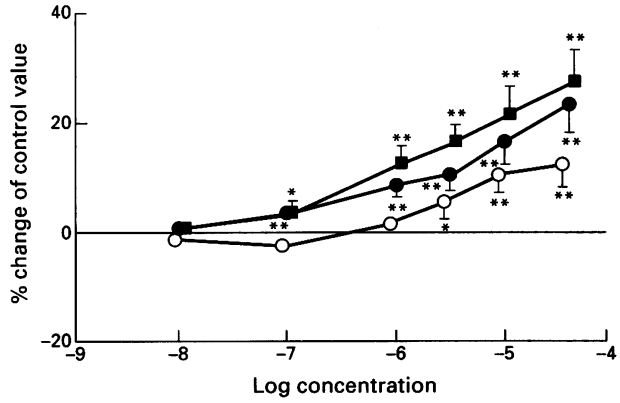


Figure 3 Log concentration-response curves for rhein anthrone on peristaltic reflex of guinea-pig isolated ileum (mucosa outside). Changes of longitudinal muscle tension (●), intraluminal pressure (○) and volume displacement (■) are shown. Each point represents the mean of 20–50 experiments, with s.e.mean shown by vertical bars. $*P < 0.05$; $**P < 0.01$; compared to control values for that segment (Student's paired t test).

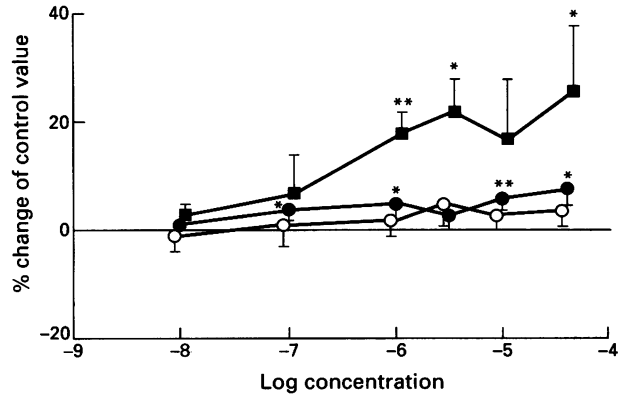


Figure 4 Log concentration-response curves for rhein on peristaltic reflex of guinea-pig isolated ileum (mucosa outside). Changes of longitudinal muscle tension (●), intraluminal pressure (○) and volume displacement (■) are shown. Each point represents the mean of 6 experiments, with s.e.mean shown by vertical bars. $*P < 0.05$; $**P < 0.01$; compared to control values for that segment (Student's paired t test).

Finally after incubation with SC19220 (75 μM) for 30 min, longitudinal muscle tension, intraluminal pressure and volume displacement were strongly reduced ($P < 0.01$ for all three parameters) ($n = 6-10$ for each concentration of rhein anthrone tested, mean of 28 observations altogether).

All the results are summarized in Figure 5, showing the percentage of the effect remaining for each of the three parameters of peristaltic reflex in response to rhein anthrone after incubation with BW755C, indomethacin or SC19220. All effects were significantly reduced by all three compounds (except volume displacement for BW755C and longitudinal muscle tension for indomethacin). In particular SC19220 can inhibit a very large part of the effects of rhein anthrone on peristaltic reflex of the reversed segment.

It was shown in control experiments that incubation at − 5 mm pressure for 30 min with Tyrode solution had no influence on rhein anthrone (10^{-5} M) stimulation of peristaltic activity (values were calculated as percentage, mean ± s.e.mean, compared to rhein anthrone (10^{-5} M) stimulated values before incubation). Longitudinal muscle tension was 98 ± 2; intraluminal pressure 107 ± 4; volume displacement 107 ± 7. Other control experiments showed that incubation with the inhibitor itself did not change basal peristaltic activity of the test segment (values in percentage, mean ± s.e. mean, compared to control before incubation). Longitudinal muscle tension was 101 ± 7 in the case of BW755C, 93 ± 18 for indomethacin, 98 ± 8 for SC19220; intraluminal pressure 97 ± 12 for BW755C, 94 ± 8 for indomethacin, 102 ± 13 for SC19220; volume displacement was 89 ± 8 for BW755C, 114 ± 12 for indomethacin and 92 ± 12 for SC19220 ($n = 6$ for all control determinations).

Discussion

For a long period it was unclear why the distinct *in vivo* effects of sennosides on peristaltis (Fioramonti *et al.*, 1988; Wienbeck *et al.*, 1988; Staumont *et al.*, 1988; Spiessens *et al.*, 1988) could not be reproduced in any way by an *in vitro* technique. Indeed, except for some studies with rhein on rat intestine showing decreased contractility (Odenthal & Ziegler, 1988) no reports on *in vitro* activity on motility of rhein anthrone or rhein have been published. The reason why it is so difficult to demonstrate an *in vitro* effect on motility of rhein anthrone is most likely because drugs are usually ap-

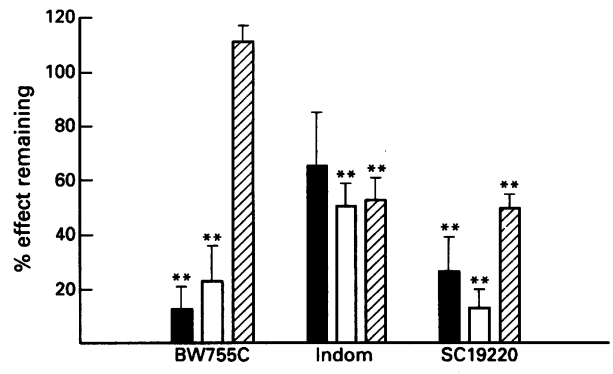


Figure 5 Percentage of the initial effect of rhein anthrone on peristaltic reflex of guinea-pig isolated ileum (mucosa outside) remaining after incubation with BW755C (50 μM), indomethacin (30 μM) or SC19220 (75 μM). Each column represents the mean of the 4 concentrations of rhein anthrone tested (10^{-6}, 3×10^{-6}, 10^{-5}, 4×10^{-5} M; $n = 6-10$ for each concentration of rhein anthrone tested; each mean calculated from at least 24 observations) expressed as percentage, with s.e.mean shown by vertical bars. Changes of effects on longitudinal muscle tension (solid column), intraluminal pressure (open column) and volume displacement (hatched column) are shown. $**P < 0.01$, compared to control values obtained with rhein anthrone before incubation of that segment (Student's paired t test).

plied to the serosal side of the segment in the classical Trendelenburg set-up (Trendelenburg, 1917), whereas rhein anthrone seems to need contact with the mucosa to be active (Van Hoestenberghe *et al.*, 1991). How the drug induces motility by this mucosal contact is however not yet clear.

The test model chosen was a reversed guinea-pig ileum segment, allowing direct contact of the bathing solution with the mucosa. With this model, a strong discrepancy was observed between rhein and rhein anthrone. Rhein significantly increased the volume displacement and caused a small but significant increase in longitudinal muscle tension. The main effect was on the volume displacement parameter, showing some similarity with the results seen with the normal segment. Rhein anthrone, the active metabolite *in vivo*, however, dose-dependently and positively affected all three parameters of peristaltic reflex (longitudinal muscle tension, intraluminal pressure and volume displacement), being active for concentrations down to 10^{-7} M. Longitudinal muscle tension and volume displacement were more affected than intraluminal pressure. Therefore it is inferred that stronger combined contractions of longitudinal muscle and circular muscle are responsible for a larger volume displacement (increased peristalsis). To our knowledge, it is the first time that obvious motility stimulating effects *in vitro* have been observed with rhein anthrone or any other metabolite of sennosides.

Our results also show that rhein anthrone and rhein have only marginal effects on peristaltic reflex of the guinea-pig ileum when applied in the bathing solution and in contact with the serosal side. In these experimental conditions, rhein anthrone has no significant effects, except for very high doses (10^{-5} M or higher). For rhein a significant increase of volume displacement and a small but significant decrease of intraluminal pressure was noticed. This means that more volume is displaced by each peristaltic reflex wave although this is not caused by stronger contractions of longitudinal or circular muscle layers, but on the contrary is associated with small relaxations of circular (and longitudinal) muscle layers. This can be explained by a decrease of the resistance to fluid displacement, causing an increase of the filling stage of the segment, leading to a larger volume displacement. It has been observed earlier that the volume displacement can be increased in two different ways: (1) stronger combined contractions of longitudinal and circular muscle layer, resulting in an improved expulsion of fluid (increased peristalsis) and (2) decrease of circular muscle tension leading to a decreased resistance to filling (Trendelenburg, 1917). Such reduction in contractility after serosal addition of rhein has previously been described (Odenthal & Ziegler, 1988). This reduced motility has also been observed in *ex vivo* preparations (Garcia-Villar *et al.*, 1980).

Rhein is only a prodrug of rhein anthrone (de Witte, 1989) which needs reduction to the latter compound before gaining activity on motility in *in vivo* experiments. Nevertheless, both compounds seem to have similar but limited activity on peristaltic reflex *in vitro* on the normal segment. Therefore, the effect on volume displacement present for rhein (not significant for rhein anthrone) can probably be regarded as not directy linked to the motility enhancing effects observed *in vivo* for rhein anthrone. It should also be noticed that these laxatives normally affect only the mucosa and not the serosa as they are poorly absorbed from the gastrointestinal tract (Lemli, 1988).

In vivo it has been demonstrated that prostaglandins are important in motility effects of rhein anthrone (Yagi *et al.*, 1988; Nijs *et al.*, 1991; 1992). We therefore wanted to verify whether such prostaglandin mediation could also be demonstrated by this *in vitro* technique. It is obvious from Figure 5 that prostaglandins are very important mediators of the effects of rhein anthrone on motility *in vitro*. BW755C is able to block most of the effects on longitudinal muscle tension and intraluminal pressure, but is completely inactive as to the volume displacement parameter. Therefore arachidonic acid metabolites seem to be involved in the effects of rhein anthrone on longitudinal muscle and intraluminal pressure of the reversed segment. The results with indomethacin, which can block effects on intraluminal pressure and volume displacement, again point in the direction of an involvement of prostaglandins. Because even SC19220 is very active (blocking largely the effects on all three parameters), one can say that probably PGE_2 and $PGF_{2\alpha}$ are involved in the observed effects. This hypothesis is supported by earlier findings *in vivo* that the amount of PGE_2 is drastically increased in the small intestine after intraduodenal administration of rhein anthrone (Nijs *et al.*, 1992).

Our experiments demonstrate that the *in vitro* effects on motility of rhein anthrone are prostaglandin-mediated and are only seen if contact between this drug and the mucosa is possible, again stressing the importance of the mucosa in the induction of the laxative effect of sennosides.

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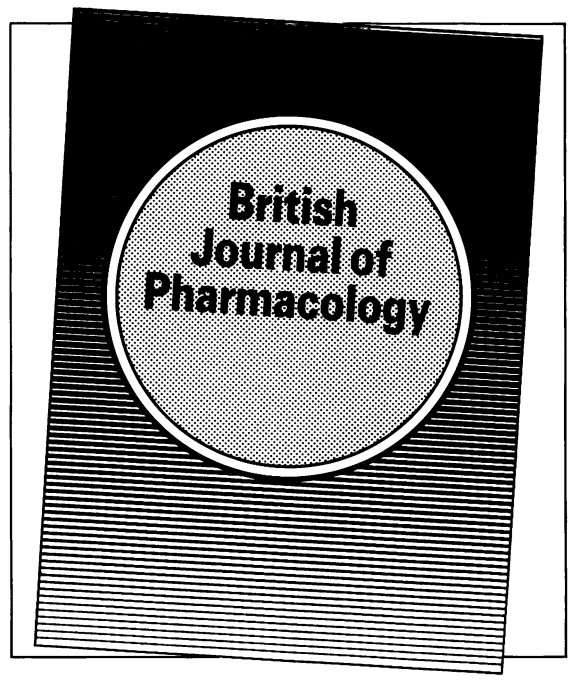
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Special Reports should normally occupy no more than two printed pages of the Journal; two illustrations (Figures or Tables, with legends) are permitted. As a guideline, with type face of 12 pitch and double-line spacing, a page of A4 paper could contain 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 keywords (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.

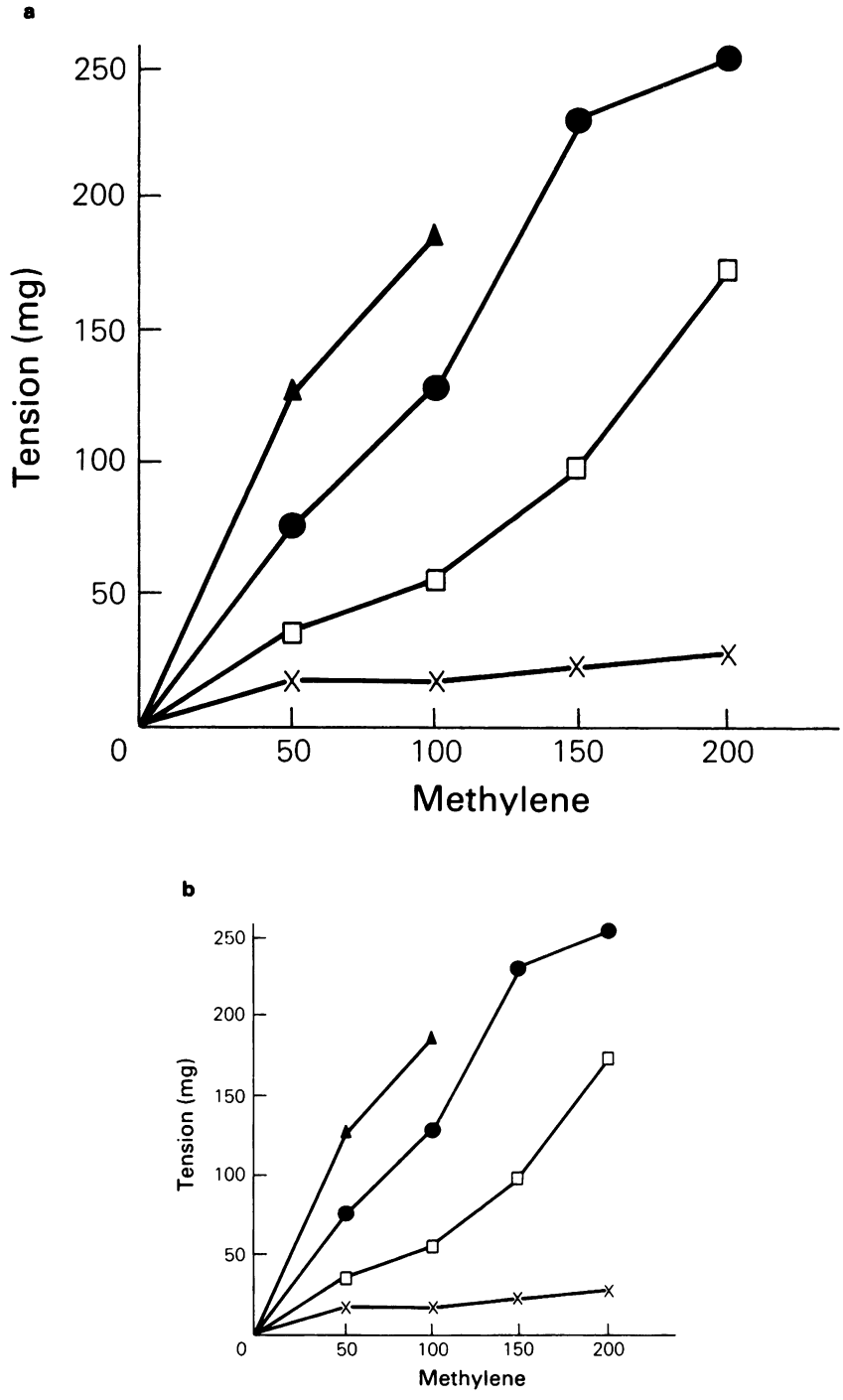


Figure 1 (a) Artwork as drawn. (b) Artwork reduced to 60 per cent of its original size for publication in the Journal.

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ABBREVIATIONS AND SYMBOLS

Physico-chemical quantities

The *British Journal of Pharmacology* uses the SI symbols for units. The following prefixed 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 m μg 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	$t_{\frac{1}{2}}$
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 millon	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 1993

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 have been chosen by international bodies or specialist groups specially convened for the purpose.

For receptor nomenclature, with few exceptions, the Journal generally follows the guidelines laid down in the current *Trends Pharmacol. Sci.* (TIPS) Receptor Nomenclature Supplement.

1 *Definition of receptors and subtypes*

Receptors and their subtypes are defined in relation to structural information where this is available and on the basis of functional studies. With the latter, they are defined in terms of the relative potencies of agonists and selectivities of antagonists and 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 are 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 Keywords, unless qualified by the adjective putative (e.g. . . . mediated by the putative α_{2A}-adrenoceptor).

(b) Only well-established and universally accepted subtype names (e.g. muscarinic and nicotinic acetylcholine receptors; 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. numerical plus letter) are acceptable.

3 *Types of receptor*

The NWP accepts that there are additional receptors to those described below which can be considered as well established. In many cases, however, their existence has been confirmed only in cloning studies and it is as yet unclear how they relate to similar subdivisions proposed on the grounds of differences in agonist and antagonist potencies in various tissues.

(a) *Acetylcholine receptors* The two principal subfamilies are muscarinic and nicotinic acetylcholine receptors (the abbreviations muscarinic AChRs and nicotinic AChRs are acceptable, but not mAChR or nAChR, which may be confused with muscle and neuronal subtypes).

Muscarinic acetylcholine receptors Nomenclature for receptors expressed from an unaltered genome in their native tissue should be confined to only three subtypes, namely M_1, M_2 and M_3; where M_2 refers to the cardiac subtype, and M_3 includes both smooth muscle and glandular subtypes.

When referring to receptors with similar pharmacology to those described above but which have been expressed from DNA/RNA which has been introduced into cells, the conventional terms m1, m2, m3 etc. should be used.

Nicotinic acetylcholine receptors The principal subgroups are muscle and neuronal nicotinic acetylcholine receptors. For receptors in their native cells, the stage of development and source of the tissue should be described fully initially.

When dealing with receptors which have been expressed from cloned genes or cDNAs, the terms muscle and neuronal are not sufficient. The subunit DNAs being expressed should be clearly defined and their origin referenced. Nomenclature for the subunits should follow current usage, (α1, β1, γ, ε and δ for muscle subunits; and a higher number of either α or β for neuronal subunits).

(b) *Adrenoceptors* The principal subtypes are α_1-, α_2-, β_1-, β_2- and β_3-adrenoceptors. Discussion of additional subtypes must be fully referenced.

(c) *Angiotensin receptors* At present only the AT_1 receptor is recognised. The AT_2 binding site should be fully referenced.

(d) *Bombesin receptors* No subtypes are recognised. Proposed subtypes may be used as discussion points, but a full explanation is required.

(e) *Bradykinin receptors* B_1 and B_2 receptors are recognised. Possible additional types and subtypes of bradykinin receptors should not be designated, except as discussion points and should be fully referenced.

(f) *Calcitonin gene-related peptide (CGRP) receptors* No subtypes are recognised. Proposed subtypes may be used as discussion points, but a full explanation is required.

(g) *Cholecystokinin (CCK) receptors* The principal subtypes are CCK_A and CCK_B receptors, CCK_B receptors being known also as gastrin receptors.

(h) *Dopamine receptors* D_1 and D_2 dopamine receptors are currently recognised. Other subtypes must be very clearly defined and fully referenced. DA_1 and DA_2 should not be used for peripheral dopamine receptors.

(i) *Endothelin receptors* Three subtypes are recognised: ET_1, ET_2 and ET_3.

(j) *Excitatory amino acid receptors* At present, no subdivisions of the receptor for N-methyl-D-aspartate (NMDA; see 6(c) below) are permitted except as discussion points.

Four non-NMDA receptors have been established and are named:

(i) AMPA receptors.
(ii) Kainate receptors.
(iii) 2-Amino-4-phosphobutyrate receptors (also sometimes known as ABP or AP4 receptors) to be abbreviated L-AP4 receptors.
(iv) Metabotropic receptors.

Additional proposed receptor subtypes may be discussed but a full explanation is required.

(k) *γ-Aminobutyric acid (GABA) receptors* The principal subtypes are $GABA_A$ and $GABA_B$ receptors. Any other is to be used only as a discussion point and to be fully referenced.

(l) *Histamine receptors* 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 last case a definition and references are desirable.

(m) *Receptors for 5-hydroxytryptamine* The name 5-hydroxytryptamine (5-HT) is preferred to serotonin (see 4(b) below). The principal subtypes recognised are $5\text{-}HT_{1A, 1B, 1C, 1D}$, $5\text{-}HT_2$, $5\text{-}HT_3$ and $5\text{-}HT_4$. $5\text{-}HT_1$-like may be used when the exact $5\text{-}HT_1$ subtype is not known or does not conform to existing nomenclature.

Other types may be debated in the Discussion section but, until there is international agreement on the nomenclature, these subtypes must be referenced.

(n) *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. All leukotriene receptor subtypes should be fully referenced.

(o) *Neuropeptide Y receptors* No subtypes are recognised. Proposed subtypes may be used as discussion points, but a full explanation is required.

(p) *Opioid receptors* The principal subtypes are μ-, δ- and κ-opioid receptors. Other possible subtypes (e.g. ε) are acceptable only as discussion points and must be referenced.

(q) *Oxytocin receptors* (see Vasopressin and oxytocin receptors).

(r) *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.

(s) *Purinoceptors* The principal 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. Proposed subtypes may be used as discussion points, but a full explanation is required.

(t) *Somatostatin receptors* No subtypes are recognised. Proposed receptor subtypes may be used as discussion points, but a full explanation is required.

(u) *Tachykinin receptors* The term tachykinin is preferred to neurokinin. Except as discussion points, only the following tachykinin receptor subtypes are acceptable at present: NK_1, NK_2 and NK_3.

(v) *Vasoactive intestinal peptide (VIP) receptors* No subtypes are recognised. Proposed subtypes may be used as discussion points, but a full explanation is required.

(w) *Vasopressin and oxytocin receptors* The principal subtypes are designated V_1, V_2, V_3 and OT receptors; V_3 has sometimes been known as V_{1B}.

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 acetylcholine receptor) is an inappropriate use of the term. Transmission events involving nitric oxide may be referred to as *nitrergic*. However, *nitrergic* may be used to describe axons only when there is sufficient evidence that nitric oxide is released from them as a neurotransmitter.

(a) *Catecholamine releasing nerve fibres* The adjective to be applied to nerve fibres that release dopamine as a transmitter is dopaminergic (not DAergic).

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 serotoninin and serotoninergic. The term 5-HTergic is not acceptable, except to avoid frequent repetition of 5-hydroxytryptamine.

Likewise, the terms purinergic (ATP) and purinergic (adenosine) are preferred.

5 *Enzymes*

The IUB Enzyme Commission (EC) number and full name (Enzyme Nomenclature 1984, Academic Press, New York

and London) must be quoted when first mentioned in text. Subsequently the accepted trivial name is used. Trivial names may be used in the title.

6 *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 stereoisomers. 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). The alkyl chain should be specified for synthetic PAF e.g. C_{16}-PAF.

(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.

(e) *Eicosanoids* The system of nomenclature to be used for eicosanoids is that published in *Methods in Enzymology*, (1990), **187**, 1–9. This scheme incorporates recent changes in the style of abbreviation of hydroperoxy-, epoxy- and oxo-unsaturated fatty acids e.g. 12(S)-hyroperoxyeicosatetraenoic acid which was formerly abbreviated as 12(S)-HPETE now becomes 12(S)-HpETE. In manuscripts, the first use of the full chemical name of any eicosanoid should indicate double bond geometry when this is known.

(f) *Peptide nomenclature* The preferred style is capital letters to designate the first letter of the word. Otherwise, upper and lower case letters should be used (e.g. Enk-IR, enkephalin-like immunoreactivity). When numbers are used these should be placed after a hyphen on the same line as the abbreviation, e.g. ET-1.

(g) *Cell lines* Cell type, sources and originating species need to be defined.

(h) *Molecular biology* Abbreviations pertaining to molecular biological techniques need to be defined or presented in such a way that they can be recognised by the non-specialist e.g. the oligonucleotide sequence, TAGC.

(i) *Tension* Tension is force and should be calibrated in newtons (1 newton = 1kg ms^{-1}) or in kg weight, g weight, or mg weight etc. It should not be calibrated in units of mass (e.g. kg). (See Miller D.J. in *Trends Pharmacol. Sci.* 1988, 9, 124–5).

(j) *Ions* When referring to ions, the charge should be indicated. e.g. Na^+, Ca^{2+}, $2Na^+/Ca^{2+}$ exchange, etc.

(k) *Inhibitors of nitric oxide synthase* The most commonly used and currently accepted abbreviation for N^G-nitro-L-arginine and N^G-nitro-L-arginine methyl ester are L-NOARG and L-NAME. Unless alternative international agreement is reached, these will be the abbreviations accepted by the journal.

PREPARATION OF MANUSCRIPTS

Authors are strongly recommended to read the full *Instructions to Authors* and *Nomenclature Guidelines for Authors* (*Br. J. Pharmacol.* 1993, **108,** 275–284) 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.
   —to include Key words (10 maximum) at end of summary.

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.

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NOMENCLATURE

Authors are reminded that accepted receptor and associated terminology is laid out in *Nomenclature Guidelines for Authors*, as published in the *British Journal of Pharmacology*, *Br. J. Pharmacol.*, 1993, **108,** 282–284.

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